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Effects of Culture System and Substrate Composition on Micropropagated Plantlets of Two Varieties of *Stevia rebaudiana* Bert

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Abstract: Background: Secondary metabolites in stevia leaves offer important therapeutic benefits. The germination problems of stevia seeds and the high heterogeneity of the resulting populations make micropropagation the preferred tool for obtaining a large number of plants in a short time. Until now, studies have focused on optimizing the action of growth regulators for the improvement of stevia micropropagated plantlets. In this project, we study alternative mineral nutrients in the substrate and two culture systems on micropropagated stevia plantlets to obtain proper amounts of enhanced plantlets. Methods: Criolla and Morita varieties; MS, MSM, and G substrates, and temporary immersion (TIS) and agar-medium systems were used. Biometric variables and damage to the cell membrane of the resulting plantlets were tested. Results: More productivity in the Criolla plantlets growing in MS solid medium and TIS. The Morita plantlets presented higher productivity in the solid MS, MSM, and G substrates and TIS. The explants growing in MS and MSM presented the highest productivity. The solid substrate was generally better than TIS. No damage to the cell membrane was found. Conclusions: The micropropagation efficiency of stevia plantlets of the two varieties has been enhanced without negative effects on their physiological condition. Consequently, any of the culture systems used, including bioreactors, can be of industrial application on a large scale.

Keywords: temporal immersion system; productivity; Criolla; Morita; MDA

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1. Introduction

Lately, the bioactive capacity of certain plant compounds that can be used as nutrient-rich food in human health has aroused great interest in different scientific fields, such as biochemistry, medicine, and pharmacy [1]. Of these, glucosides extracted from stevia leaves (*Stevia rebaudiana*, Bert) are notable because they are low-caloric sweeteners and have an elevated sweetening capacity. Since no side effects of human consumption have been described [2,3], *S. rebaudiana* has become an alternative sweetener in the food industry, and in general, for consumers. Its sweetening capacity can be attributed to the steviol glycosides found in leaves, with stevioside and rebaudioside A being the most important glycosides responsible for the sweet taste. Stevioside accounts for the most sweetener (60–70% of the total glucoside content) and is estimated to be 110–120 times sweeter than sugar. However, stevioside is also responsible for the lingering bitter taste similar to that of licorice. In contrast, rebaudioside A is particularly interesting [4] because despite only accounting for 30–40% of glucoside content in leaf extract, it has a sweeter taste considered to be 180 to 400 times sweeter than sugar without bitter taste. Thus, the rebaudioside-A/stevioside ratio is the accepted measure of sweetness quality; hence, the higher this ratio, the greater the quality of the product [5]. Additionally, a number of therapeutic benefits of compounds present in stevia leaves have been reported: antimicrobial (e.g.,

against Streptococcus strains) [6], antifungal (inhibition against *Candida albicans*, *Penicillium chrysogenum* and *Aspergillus niger*) [7], hypotensive, anti-inflammatory, anti-tumor, anti-diarrheal, diuretic and immunomodulatory [8,9]. Studies [3,8] have described the highly effective insulinotropic and anti-hyperglycemic properties of stevia extracts, for example, against type 2 diabetic animal models. The relationship between the high antioxidant activity of these plant extracts has been reported by several authors [10].

Stevia is a perennial plant originally from Paraguay [11]. It is a semi-bush plant belonging to the Asteraceae family. Stevia seeds have high heterogeneity and low germination capacity due to a high percentage of sterile seed and a very difficult seed harvesting caused by low uniformity of flowering and seed ripeness; that is why asexual methods such as tissue culture plants, have been widely used to propagate it [12–14].

The ionic balance of the culture medium is highly dependent on ammonium and nitrate ions, as they are the major components of any substrate for plant in vitro culture [15,16]. The widely used Murashige and Skoog substrate (MS), basal salt medium [17], is considered to be moderately saline despite its high ionic content [18]. As a result, it is used extensively as a suitable culture medium due to its balanced macro and micronutrient composition and also because it can be used at different dilutions. In the case of stevia, MS medium with different combinations and concentrations of growth regulators have been widely applied as a medium for the in vitro culture of many species of plants [19–22]. However, the high total nitrogen with a proportion of NO_3^- 1.9 greater than NH_4^+, the high levels of Cl and Mo, and the relatively low levels of Ca, P, Mg, and Cu in MS at full strength are evident compared to the average concentrations of these elements in plant shoots (dry weight basis) considered to be sufficient for adequate growth reported by [23]. According to Magangana et al., plants grown in low-salt medium have a greater ion uptake capacity than those grown in normal or high-salt media [24].

Consequently, this substrate may not be optimal for certain species [15,25] and can be improved, as shown for persimmon [26] and grapevine [27]. According to Chée and Pool, KI is a non-essential component for the growth and development of grapevine plants and can subsequently be removed from tissue culture media [28]. The authors mentioned above reported that lower Mn concentrations, specifically 0.85 mg/L (5 μM), improved in vitro grapevine development, and the substitution of calcium nitrate with calcium chloride improved the quality of grapevine shoots produced in culture.

In in-vitro plant cultivation, high productivity and efficiency is mandatory. Using agar in solid culture medium increases the cost of the media. For this reason, culture on a liquid substrate has become an economical alternative for the micropropagation of plants and can be performed by continuous immersion or temporary immersion (temporary immersion system–TIS). In continuous immersion, the explants are continuously in contact with the liquid medium, facilitating nutrient absorption and assimilation [29], albeit with the disadvantage of increased hyperhydricity, which severely affects plant survival in ex vitro conditions. In a bioreactor, the explants are submerged at time intervals, with a frequency that can be controlled. This automatable system improves efficiency in the biofactory and reduces costs and contamination [30]. Since the purpose of this study was to improve the production of stevia stems, the objective is to enhance both the development of the aerial part of stevia and its multiplication coefficient using the temporary immersion System and variations in substrate composition.

2. Materials and Methods

2.1. Plant Material and Treatments

Plantlets grown previously under in vitro conditions of two *Stevia rebaudiana* (Bert) varieties, Criolla and Morita III, were cut into uninodal explants (1–2 cm in length with two axillary buds) and subcultivated in two different culture systems: medium solidified by agar; and temporary immersion system (TIS). Three substrates were assayed in each culture systems: MS; MS modified (MSM); and G, also deriving from MS and adapted for in vitro grapevine culture [31]. All three substrates were modified at the macronutrient

level, consisting mainly of a gradual reduction in nitrogen content (Section 4) and the addition of sucrose, vitamins, and plant growth regulators (Section 4).

Six treatments using the three substrates mentioned were assayed: three for solid medium adding agar (7 gL^{-1}) (MSS, MSMS, and GS) and three for the TIS system (MSTIS, MSMTIS, and GTIS). In all situations, the pH was adjusted to 5.7 prior to autoclaving. The different culture media were distributed in each flask (600 mL) (100 mL per flask) for solid conditions. In the case of the TIS treatments, PLANTFORM bioreactors [30,32] (4000 mL) (500 mL substrate per bioreactor) were used. After substrate distribution, the flasks and bioreactors were closed and autoclaved for 20 min (120 °C, 1 kg/cm^2 of pressure). Ten explants per flask, in the case of the solid medium and 50 per PLANTFORM bioreactor were established in order to provide 10 mL of substrate per explant in both cases. Cultures were incubated in a growth chamber at 26 ± 1 °C, 30 $\mu Em^{-2}s^{-1}$ and for a 16 h. photoperiod. In the case of the PLANTFORM. Bioreactor's gas exchange sterilized by filters was controlled through three inlets/outlets anchored to the side. A basket containing the plant material was placed above the inner chamber designed with special grooves to allow nutrients to rise efficiently when pressure was applied. The immersion frequency was 2 min every 8 h, as described elsewhere [33,34]. Each treatment was repeated 5 times.

At 30 days of culture, the following biometric parameters were measured: aerial plant size (length in cm), shoot number per explant (also considered as "multiplication coefficient" in accordance with [35], productivity (shoot number × average stem length) [36], fresh and dry weights (aerial parts were dried at 80 °C for 48 h and then weighed).

2.2. Relative Growth Rate and Water Content

Relative growth rate (RGR) was calculated using the formula: RGR = (ln Df − ln Di). D-1 ($g\ g^{-1}\ day^{-1}$) where Df = final dry weight, Di = initial dry weight (average of 10 explants from each treatment dried at the beginning of the experiment), and D = duration of the experiment (days).

To control the hyperhydration aspect of the new plants, the hydration level was calculated using the following formula: H (%) = [(Fw − Dw/Fw]100 (Fw = fresh weight; Dw = dry weight).

2.3. Lipid Peroxidation

At the end of the experimental period, cell membrane integrity was estimated according to the degree of lipid peroxidation determined from the concentration of malondialdehyde (MDA) [37]. Three samples (250 mg) per treatment were crushed in a mortar cooled in liquid nitrogen. The powder was then extracted using 5 mL of trichloroacetic acid (TCA) at 0.1% (w/v). The extract was centrifuged at 10,000× *g* for 10 min. The supernatant was collected, and 1 mL mixed with 4 mL of 20% TCA containing 0.5% thiobarbituric acid (TBA) (w/v). The mixture was heated at 95 °C for 30 min. Once cooled on ice, the sample was centrifuged at 10,000× *g* and the supernatant was measured in a spectrophotometer at 532 and 600 nm. MDA concentration in the extracts was calculated using a molar extinction coefficient of 155 $mM^{-1}\ cm^{-1}$ as follows:

$$\text{MDA equivalents (nmol mL}^{-1}\text{)} = (\text{Abs532} - \text{Abs600})/155{,}000 \times 106$$

MDA concentration was expressed as nmol.g fw^{-1}.

2.4. Statistical Analysis

Statistical analysis was carried out using IBM SPSS Statistics v.25. Data were analyzed using principal components analysis (PCA) for dimensionality reduction and General Linear Model (GLM) was used to analyze principal effects and the interactions among the three culture media, the two culture systems and the two varieties. Three- and one-way analyses of variance (F-test) and the Bonferroni test for average comparison were employed. Data were tested for normality using the Kolmogorov–Smirnov test and for homogeneity

of variance using the Levene test. The Tukey tests were applied to significant test results for the identification of important contrasts.

3. Results

3.1. Growth

The absence of visual hyperhydricity symptoms and root presence were observed in all plantlets developing from explants (Figure 1).

Figure 1. Stevia plantlet growing in PLANTFORM (**left**) and solid medium (**right**).

Table 1 summarizes the results of the biometric values obtained from the parameters studied.

Table 1. Comparison of the average values of the biometric parameters obtained in the micropropagation of the stevia varieties in the substrates assayed.

Variety	Parameter	Assayed Substrates					
		Solid System			Temporary Immersion System		
		MSS	MSMS	GS	MSTIS	MSMTIS	GTIS
Criolla	Stem length (cm)	8.43 c	7.11 b	8.48 c	7.10 b	5.50 a	5.50 a
	No. of shoots/explant	8.42 c	6.62 b	5.56 a	8.24 c	8.02 c	6.20 b
	Productivity (mm)	71.91 b	48.68 a	49.47 a	59.86 b	46.07 a	34.70 a
	Fresh weight (g)	0.39 ab	0.33 a	0.46 ab	0.49 b	0.33 a	0.42 ab
	Dry weight (g)	0.065 c	0.037 b	0.046 ab	0.049 abc	0.034 a	0.054 bc
	RGR (gg^{-1} day^{-1})	0.051 c	0.032 ab	0.040 abc	0.041 abc	0.028 a	0.046 bc
	Water content (%)	83.65 a	87.70 ab	88.78 ab	87.47 ab	89.89 b	85.90 ab
Morita	Stem length (cm)	5.21 bc	4.62 abc	5.63 c	4.20 ab	4.80 abc	4.00 a
	No. of shoots/explant	9.98 bc	9.65 bc	9.14 bc	6.95 a	10.72 c	8.42 ab
	Productivity (mm)	53.56 b	47.88 ab	54.38 b	31.37 a	56.73 b	35.60 a
	Fresh weight (g)	0.31 c	0.26 bc	0.27 bc	0.143 a	0.175 a	0.201 ab
	Dry weight (g)	0.028 a	0.029 a	0.032 a	0.026 a	0.027 a	0.027 a
	RGR (gg^{-1} day^{-1})	0.032 a	0.034 a	0.037 a	0.024 a	0.032 a	0.029 a
	Water content (%)	88.98 c	87.04 bc	86.14 abc	78.56 ab	77.22 a	85.06 abc

n = 50. In each row, the means followed by similar lower letters (a, b, or c) indicate differences between substrates were not statistically significant according to Tukey's test ($p < 0.05$).

According to a preliminary study of our group [38], three statistically well-differentiated groups could be established in the Criolla plantlets according to the results obtained for stem length (Table 1): (1) the two longest stems were grown in GS and MSS; (2) two stems of intermediate length—MSMS and MSTIS—were obtained; and (3) the two shortest stems

were grown on MSMTIS and GTIS. As with the Criolla plantlets, in the Morita plantlets, the longest average stem length was obtained in those grown in GS medium, but similar values were obtained for stems grown in MSS, MSMS, and MSMTIS. In contrast, shorter stems were quantified in MSTIS and GTIS media (Table 1). Consequently, according to the referred study [39], taking into account the combined results for Criolla and Morita, the stems were longer ($p < 0.05$) in solid substrates (average 6.60 cm) than with TIS (average 5.21 cm) (Table S1). On the other hand, for both stevia varieties and the three substrates (MS, MSM, and G) independently of the culture system used (solid medium or TIS), the best length development was found in stems grown in MS medium (average of 6.33 cm), similar to the length achieved in G (average of 5.9 cm) and shorter ($p < 0.05$) than the lengths of stems grown in MSM (average of 5.52 cm). In general, considering the different averages for all substrates both in solid and in TIS, longer stems were obtained for Criolla (7.02 cm) ($p < 0.05$) than for Morita (4.77 cm), respectively.

Table 1 shows the average number of buds per explant, indicating that the Criolla explants developed in the MSS, MSTIS, and MSMTIS substrates presented the largest ($p < 0.05$) number of buds per explant, 8.42; 8.24 and 8.02, respectively. Those grown in MSMS and GTIS yielded intermediate values, 6.62 and 6.20, respectively, and the shortest lengths were found in G, in solid medium (5.56). For Morita plantlets, as with the results described for length parameters, the results were slightly less homogeneous. All explants grown in solid media and MSMTIS produced a large number ($p < 0.05$) of shoots per explant, always more than 9.1, while those developed in MSTIS and GTIS never exceeded 8.42. Differences were observed among the three substrates assayed, 8.46 and 8.76 for MS and MSM, respectively, representing similar values, and 7.33 for G ($p < 0.05$). However, no differences were detected between solid (8.03) and TIS (8.12) systems (Table S1). Morita plantlets had a larger average number of buds per plant 9.21, ($p < 0.05$), than the Criolla variety plantlets (7.18).

As a consequence of results obtained for both parameters, the product of length per shoot number, i.e., productivity, was higher ($p < 0.05$) in the Criolla plantlets grown in MSS and MSTIS substrates, with values above 59.8 mm, than the other four substrates assayed, which never exceeded 49.5 mm. Morita plantlets grown in the MSS, GS, and MSMTIS substrates presented higher productivity (higher than 54.3 mm), which was nevertheless similar ($p < 0.05$) to the levels obtained for the plantlets grown in MSMS. The plantlets with the lowest productivity (less than 35.6 mm) were those grown in the GTIS and MSTIS substrates. Explants grown in MS substrate developed a high productivity–55.1-similar to that of the explants cultured in MSM (49.8) and lower ($p < 0.05$) than those grown in the G substrate (43.54 mm). In general, the solid substrate was better (average 54.31 mm, $p < 0.05$) for explant productivity than TIS (average 44.40 mm) and differences were observed between Criolla and Morita productivity (averages 51.78 and 47.0 mm, respectively) (Table S1).

According to Table 1, Criolla plantlets reached the highest fresh weight in MSTIS substrate (0.49 g), although this was similar to the weights obtained in the other substrates with the exception of those developed in MSMS and MSMTIS, with 0.33 g in both cases. The highest fresh weight of Morita plantlets was registered in solid media. In contrast, lower values were obtained in MSTIS and MSMTIS: 0.14 and 0.17 g, respectively. Thus, plantlets with the lowest fresh weight were those developed under TIS conditions (average 0.30 g) compared to those grown in solid media (average 0.33 g) ($p < 0.05$). The Criolla plantlets also had a higher average fresh weight (0.40 g) than the Morita plantlets (0.23 g) ($p < 0.05$). These results may explain the differences found when the fresh weights in the six cultures assayed were compared. MSM developed plantlets with the lowest fresh weight (0.28 g) in both varieties, Criolla and Morita ($p < 0.05$), but while Morita plantlets grown in MS and MSM substrates using TIS achieved a low fresh weight, the Criolla plantlets grown in MS and G using both solid and TIS presented the highest fresh weights, resulting in a statistically lower fresh weight in the TIS system (0.30 g) than in solid conditions (0.33 g) ($p < 0.05$).

The dry weight results maintained the same pattern as those for the fresh weight. However, the higher dry weights found in plantlets grown under solid conditions were similar to those of the plantlets grown in TIS media (both with an average dry weight of 0.04 g) (Table S1). Similarly to fresh weight, Criolla plantlets presented the highest dry weight (0.05 g) when compared to Morita plantlets (0.028 g) ($p < 0.05$). MSM also produced plantlets with a lower dry weight ($p < 0.05$) in both varieties (0.032 g) when compared to MS and G (0.041 g and 0.040 g, respectively) (Table S1). The high dry weight found in Criolla plantlets developed in MSS (0.06 g) was similar to that quantified in GTIS (0.05 g). No differences were observed between the plantlets with respect to the dry weights achieved for the assayed media in the Morita variety (Table 1).

Over the 25 days of the experiment, the daily development (RGR) of Criolla plantlets was higher (0.0398 gg^{-1} day^{-1}) ($p < 0.05$) than in the Morita plantlets (0.0317 gg^{-1} day^{-1}) (Table S1). This difference can be explained by the better development of Criolla plantlets in MS media under both solid conditions and TIS, and in GTIS (Table 1), while the RGR of the Morita plantlets was not affected by the culture system used in any of the six substrates assayed. Thus, the best substrates for both varieties were MS and G (0.0379 gg^{-1} day^{-1}) in both cases and the worse ($p < 0.05$) MSM (0.031 gg^{-1} day^{-1}) (Table S1). When all the results were considered, differences ($p < 0.05$) between solid and TIS (0.0377 and 0.0338 gg^{-1} day^{-1}, respectively) were found.

Although no statistical differences were observed in the water content percentage among the three substrates used (84.90, 85.50, and 86.47 for MS, MSM, and G, respectively), the variations in fresh weight together with stable dry weight in Morita plantlets resulted in higher ($p < 0.05$) water content in plantlets of this variety cultured in MSS (88.98%) (Table 1). This percentage was similar to the percentages obtained for MSMS, GS, and GTIS (Table 1). However, the low water content in plantlets of this variety developed in MSMTIS resulted in lower water content ($p < 0.05$) in Morita (83.97%) compared to Criolla (87.23%) and in TIS conditions (84.17%) compared to solid substrates (87.03%) (Table S1). In any case, hyperhydricity signs were not identified in any of the assayed plantlets.

3.2. Lipid Peroxidation (MDA Levels)

Regarding MDA content, no significant differences were observed among the substrates used in either solid conditions (3.41 $nmolg^{-1}$) or TIS media (4.06 $nmolg^{-1}$). However, due to the MDA levels in the Criolla leaves in both MSMTIS and GTIS (Table 2), the average MDA levels found in Criolla (4.28 $nmolg^{-1}$) were higher ($p < 0.05$) than those of Morita (3.21 $nmolg^{-1}$) (Student's *t*-test test $p < 0.05$).

Table 2. Comparison of average MDA values obtained in the micropropagation of stevia varieties in the substrates assayed.

Variety	MDA ($nmolg^{-1}$)					
	Solid System			Temporary Immersion System		
	MSS	**MSMS**	**GS**	**MSTIS**	**MSMTIS**	**GTIS**
Criolla	3.69	3.93	2.77	5.14	4.59	5.53
Morita	2.98	4.12	2.99	3.22	2.64	3.27

3.3. Principal Component Analysis

The principal component analysis score plot (Figure 2) shows the means for each combined treatment of PC1 (39.58% of variance) versus PC2 (25.47% of variance).

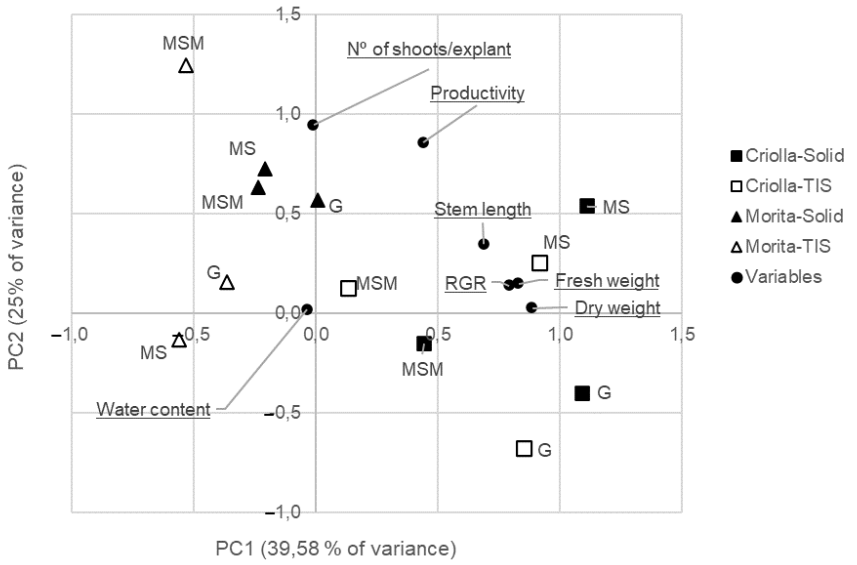

Figure 2. Principal component analysis mean scores plot for each combined treatment (black squares: Criolla with solid system; white squares: Criolla with TIS; black triangles: Morita with solid system; white triangle: Morita with TIS. black circle. The variables are underlined and (Table 3) are in bold. MS, MSM, and G are the culture media.

Table 3. Correlation coefficients between each variable and the three factors resulting from Varimax-rotated principal component analysis, eigenvalues, and percentage variance accounted for.

Variables	Factor 1	Factor 2	Factor 3	Communalities
Dry weight	**0.886**	0.025	−0.302	0.877
Fresh weight	**0.830**	0.148	0.322	0.815
RGR	**0.792**	0.137	−0.400	0.807
Stem length	**0.688**	0.345	0.276	0.669
No. of shoots/plant	−0.009	**0.944**	−0.064	0.895
Productivity	0.440	**0.855**	0.117	0.939
Water content	−0.034	0.018	**0.939**	0.883
Eigenvalue	2.771	1.783	1.331	
Percent variance	39.582	25.472	19.011	
cum.variance	39.582	65.054	84.065	

Extraction method: PCA. Rotation method: Varimax with Kaiser normalization. Communalities show the proportion of each variable accounted for. Values in bold indicate the variables with the highest loadings for each Factor.

The possible association among the different parameters was tested by Varimax-rotated principal component analysis (PCA). The correlation coefficients between each principal component and each variable are shown in Table 3. These show that dry weight, fresh weight, RGR, and stem length were associated with one another within the first component (PC1), whereas the number of shoots/explant was associated with productivity within the second component (PC2). The water content was in a third component (PC3). RGR was negative and weakly associated with the third component. Productivity was weakly associated with the first component. More than 84% of the variance was accounted for by the analysis, and high commonalities were found for all variables. This confirmed the relationship between productivity and the number of shoots/explant, although its relationship with stem length was not clear. Thus, productivity was more dependent on the number of shoots per plantlet than on stem length.

4. Discussion

The success of any in vitro culture procedure is highly dependent on the medium used. Media should ideally be optimized for the species to be cultured, ensuring a correct balance and absorption of nutrients. As indicated above, there exists extensive literature on the improvement of stevia micropropagation that describes the use of a variety of substrates. Nevertheless, those studies have focused mainly on optimizing the dose and quality of growth regulators [39,40]. The present study considered three factors to increase the quality and quantity of micropropagated plants. The first factor was the cultivar used. Two commercial varieties (Criolla and Morita III) were selected for this purpose. The other two factors considered were the culture system (solid and TIS) and medium (MS, MSM, and G), with special emphasis on the mineral composition of the media. Thus, organic components (sucrose, vitamins, and growth regulators) were kept constant (Table 4). To understand the overall influence of the culture medium (M), the culture system (S), and the variety (V) on the production of stevia plantlets and thus distinguish the effects and interactions of these three variables on plantlets, PCA and ANOVA were used. The interactions between the experimental designs were significant for the majority of variables. The Levene test for each individual variety showed that the variances were generally non-homogeneous, and the one-way ANOVA could then be used to test the significant dependence on the treatment of each variable.

Table 4. Basal composition of the substrates used.

COMPONENTS	MS		MSM		G	
			Macronutrients			
	mg/L	mM	mg/L	mM	mg/L	mM
$CaCl_2 2H_2O$	440.00	2.99	-	-	-	-
$Ca(NO_3)_2 4H_2O$	-	-	1200.00	5.08	300.00	1.27
KH_2PO_4	170.00	1.25	340.00	2.50	170.00	1.25
$MgSO_4$	370.00	1.50	600.00	2.43	370.00	1.50
NH_4NO_3	1650.00	20.60	800.00	10.00	800.00	10.00
KNO_3	1900.00	18.80	1900.00	18.80	800.00	7.92
$FeSO_4 7H_2O$	27.80	0.1	36.14	0.13	27.80	0.1
			Micronutrients			
	mg/L	µM	mg/L	µM	mg/L	µM
$CoCl_2 6H_2O$	0.025	0.105	0.025	0.105	0.025	0.105
$CuSO_4 5H_2O$	0.025	0.1	0.025	0.1	0.025	0.1
NaEDTA	37.30	100	48.5	130	37.30	100
KI	0.83	5	0.83	5	-	-
H_3BO_3	6.20	100	6.20	100	6.20	100
$MnSO_4 H_2O$	16.90	100	16.90	100	0.85 [1]	5
$NaMoO_4 2H_2O$	0.25	1.03	0.25	1.03	0.25	1.03
$ZnSO_4 7H_2O$	8.60	29.9	8.60	29.9	8.60	29.9
			Vitamins			
	mg/L	µM	mg/L	µM	mg/L	µM
Myo-Inositol	100	555	100	555	100	555
Thiamine	1.00	2.96	1.00	2.96	1.00	2.96
			Carbohydrates			
	(g/L)	(mM)	(g/L)	(mM)	(g/L)	(mM)
Sucrose	20	58.4	20	58.4	20	58.4
			Growth regulators			
	mg/L	µM	mg/L	µM	mg/L	µM
BA	0.072	0.32	0.072	0.32	0.072	0.32
NAA	0.024	0.13	0.024	0.13	0.024	0.13

[1] Adjusted according to Chée and Pool (1987) [28].

A three-way ANOVA was performed using the general linear model, revealing that the three designed factors influenced ($p < 0.05$) one or more of the parameters studied to different degrees (Table S1).

According to Figure 2, the stem length, weight, and RGR were associated with varietal differences (Criolla > Morita), while the number of shoots and productivity were associated with the culture system or culture media, although differences were observed between the varieties. For Criolla, in both culture systems, the number of shoots was associated with culture media (MS > MSM > G), whereas in Morita, the same variable was associated with the culture system. However, in TIS, the order was (MSM > G > MS), while in solid conditions, no differences were observed.

According to the partial Eta-square values, the experimental factor with the highest incidence was the variety ($\eta p^2 = 0.470$), followed by the culture system ($\eta p^2 = 0.139$) and, with a lower incidence, culture medium ($\eta p^2 = 0.070$).

The variety (Criolla/Morita) influenced all the proposed variables. Criolla plantlets produced the highest values in all parameters, except for the average number of shoots per plant, with much larger levels in the Morita plantlets (Table S1).

The culture system influenced all variables, with the exception of shoot number and dry weight. In the cases influenced by the culture system, the highest values were always found in the solid system (Table S1).

The culture media influenced all variables with the exception of water content. The highest stem length, productivity, number of shoots, fresh weight, and RGR values were obtained in MS. MSM was similar to MS in productivity, and the number of buds and G was similar to MS in stem length, fresh and dry weights, and RGR. Thus, according to the ANOVA analysis, it may be assumed that the best combination of the three variables for suitable micropropagation of the stevia varieties considered was Criolla in solid conditions using MS culture medium.

However, certain considerations may be established for each parameter studied (Table S1). For stem length, the optimal combination consisted of Criolla plantlets grown in solid MS or G media. Morita explants developed the largest number of shoots for plantlet in TIS using MSM or MS media. The Criolla plantlets yielded the highest fresh and dry weights; the highest fresh weights were obtained in plantlets of this variety grown in MS or G in solid conditions; the highest dry weights were reached for the Criolla plantlets developed in MSS and GS.

Thus, for both weights, the lowest water contents were found in plantlets of the Morita variety grown in MS and MSM in TIS conditions. No high accumulation of water in leaves was detected, according to the absence of visual symptoms of the hyperhydricity indicated.

Of the biometric indicators evaluated, the number of shoots x the average stem length, defined as productivity in accordance to [40], and the evolution of growth over time expressed as RGR, are of special interest in micropropagation improvement, especially for commercial purposes. According to our results, the best combination from a productivity standpoint was Criolla grown in MS, in both solid and TIS conditions, but Morita plantlets can achieve similar productivity in MSMTIS. TIS is able to foster good development with both varieties, but the culture medium is important; Criolla explants must be micropropagated in MS and Morita in MSM, while solid conditions are suitable for Criolla and MS. For RGR the optimal combination would be Criolla in solid or TIS conditions using MS or G. However, Morita explants achieved similar development in solid G medium.

According to studies, manganese sulphate concentration in the substrate must be reduced for grapevine in vitro culture since manganese is a cofactor in the oxidation of indolacetic acid (IAA) and has also been reported to catalyze the oxidation of auxin protectors, thus accelerating IAA oxidation and deactivation by endogenous peroxidase [41]. Thus, refs. [41,42] reported a clear improvement of callogenesis in grapevine, strongly related to manganese sulphate reduction.

In the present study, the very low manganese sulphate concentration supplied by G medium did not substantially improve shoot length or shoot number per explant, indicating that this modification of culture substrate cannot be applied to all species cultured in vitro. The good development achieved in the solid substrates quantified here contrasts with the finding reported by authors such as [43], who reported a lower growth of potato

plantlets in a solid medium compared with liquid as a result of less nutrient absorption from the substrate.

In our assays, the plantlets are unrooted because the objective was to improve the multiplication factor. Thus, transportation within the plant occurs via the xylem. This effect is favored by the continuously open stomata characteristic of the micropropagation system [44], which provides high relative humidity in the culture vessel. Contrary to what could be expected, despite the easy assimilation of the liquid medium, it was not the plantlets in TIS that had the highest water content in their cells. This explains why no hyperhydricity symptoms were observed in the micropropagated plantlets in TIS.

Taking into account that MDA is the result of the decomposition of unsaturated fatty acids in the cell membrane, its level is directly correlated with oxidative stress. Despite the high levels of MDA detected in the leaves of Criolla plantlets, they were similar to those reported by [45] as normal (4.9 $nmolg^{-1}$) for stevia seedlings cultured in vitro on MS substrate. Thus, the treatments and culture system used did not cause damage to the stevia cells. On the other hand, [46] reported a suitable nutritional status of these plantlets for both varieties assayed.

According to Etienne and Berthouly, [29], immersion time, either duration or frequency, is a decisive parameter for TIS efficiency. Nevertheless, other authors [46,47] did not report significant improvements in culture performance linked to immersion time variations. Although this aspect was not examined here, it needs to be reviewed as a path for further improvement in stevia micropropagation.

Although the outcome of the PCA test established the best combination of factors (variety, substrate, and culture system) for optimum results, it is worthwhile noting that our results show that stevia explants, independently of the variety, possess a remarkable ability for in vitro culture, regardless of the culture system or medium used. Other authors have recently reported the similarity of behavior between PLANTFORM bioreactors and on agar in plants of *Digitalis lutea* × *purpurea*, *Echinacea purpurea*, and *Rubus idaeus* [48].

5. Conclusions

In conclusion the micropropagation efficiency of stevia can be enhanced through the use of alternative substrates and culture systems without any negative effect on the physiological condition of plantlets. Finally, this was true for both varieties tested, although a different behavior of plantlets of each one depending on the media and culture systems tested was found.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/physiologia3010006/s1, Table S1: three-way ANOVA table.

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