



Nutritional Status of Micropropagated Plantlets of Two Varieties of *Stevia rebaudiana* Bert. under Different Culture Conditions

Vilariño Susana¹, Florido María del Carmen², García José Luis³ and Cantos Manuel^{3*}

¹Vitrosur Lab SLU. Algodonera del Sur, Spain

²Department of Cristalografía, Mineralogía y Química Agrícola, Universidad de Sevilla, Spain

³IRNAS (CSIC), Spain

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***Corresponding author:** Manuel Cantos, Institute of Natural Resources and Agrobiology of Seville (IRNAS), State Agency Spanish National Research Council (CSIC), Av. Reina Mercedes 10, 41012 Sevilla, Spain

Abstract

Extracts obtained from plants of *Stevia rebaudiana* Bert have high sweetening capacity and without risks for health. The sexual reproduction of this species is characterized by the low germination efficiency of its seeds and the high heterogeneity of the resulting populations. Thus, asexual propagation, particularly micropropagation, is the preferred tool for obtaining a large number of healthy and genetically homogeneous plants in a short time. To study the effects of the substrate composition and culture system on the nutrient contents of resulting plantlets, two stevia varieties (Criolla and Morita), three culture media (Murashige and Skoog and two modifications of this medium) and two culture systems (Temporal Immersion System-TIS- and solid medium) were used. After 30 days of culture the stem length and leaf nutritional status were tested. The nutrient contents of the in vitro well-developed plants are clearly related with the substrate compositions. Except for manganese no differences were observed between both varieties in terms of macro or micronutrients levels. In most cases the nutrient levels were higher in the microplants grown in solid medium. The macro and micronutrient levels of the resulting plantlets reported and discussed here, for the first time, may be useful for future studies in order to establish normal nutritional status of reference for researchers working in stevia micropropagation.

Keywords: Criolla; Morita; Solid medium; Temporal immersion system; PLANTFORM

Abbreviations: TIS: Temporary Immersion System; MS: Murashige Skoog; MSM: Modified MS; G: Grapevine Medium; BA: 6-benzylaminopurine; NAA: Naphthaleneacetic Acid

Introduction

The extract obtained from stevia (*Stevia rebaudiana*, Bert) leaves is remarkable due to its high sweetening capacity and very low-calorie content. Since no side effects from its consumption have been described [1], *S. rebaudiana* has become a real alternative sweetener on the market.

Stevia sexual reproduction is characterized by low germination and a high heterogeneity in the resultant populations from its seeds. For these reasons, asexual propagation methods, mainly micropropagation, have been preferred [2,3]. For this purpose Murashige and Skoog medium (MS) [4] has been routinely applied as basal medium for the micropropagation from buds [5,6]. However, the high levels of nitrogen, chlorine and molybdenum in MS at full strength suggest, according to some authors as Chee &

Pool [7], the possibility of reducing the concentration of MS salts to a half or a third of its usual concentration. Consequently, this substrate could be improved for certain species as stevia.

In micropropagation, mainly for commercial purposes, solid culture media tend to be more costly due to the presence of agar. Accordingly, culture in liquid substrate performed by temporary immersion system (TIS) may be a suitable alternative [8] avoiding continuous contact with the liquid substrate. This system is considered to be highly automatable with important reductions in both costs and contamination [9]. Since the purpose of this study was to answer the question: How the cultured system and nutritional conditions assayed affect the nutritional status of the obtained microplant?

Material and Methods

Plantlets grown previously under *in vitro* conditions of two *Stevia rebaudiana* (Bert) varieties, Criolla and Morita III, were cut into uninodal explants (1-2cm in length with two axillary buds) and subcultivated in two different culture systems: medium

solidified by agar and 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 [10]. MSM and G substrates were modified in relation to MS at macronutrient level, consisting mainly of a gradual reduction in nitrogen content (Table 1 & 2).

Table 1: Basal composition of the substrates used.

Components	MS		MSM		G	
Macronutrients						
	mg/l	mM	mg/l	mM	mg/l	mM
CaCl ₂ 2 H ₂ O	440	2.99	-	-	-	-
Ca (NO ₃) ₂ 4 H ₂ O	-	-	1200	5.08	300	1.27
KH ₂ PO ₄	170	1.25	340	2.5	170	1.25
MgSO ₄	370	1.5	600	2.43	370	1.5
NH ₄ NO ₃	1650	20.6	800	10	800	10
KNO ₃	1900	18.8	1900	18.8	800	7.92
FeSO ₄ 7 H ₂ O	27.8	0.1	36.14	0.13	27.8	0.1
Micronutrients						
	mg/l	μM	mg/l	μM	mg/l	μM
CoCl ₂ 6H ₂ O	0.025	0.105	0.025	0.105	0.025	0.105
CuSO ₄ 5H ₂ O	0.025	0.1	0.025	0.1	0.025	0.1
NaEDTA	37.3	100	48.5	130	37.3	100
KI	0.83	5	0.83	5	-	-
H ₃ BO ₃	6.2	100	6.2	100	6.2	100
MnSO ₄ H ₂ O	16.9	100	16.9	100	0.85 ⁽¹⁾	5
NaMoO ₄ 2H ₂ O	0.25	1.03	0.25	1.03	0.25	1.03
ZnSO ₄ 7H ₂ O	8.6	29.9	8.6	29.9	8.6	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	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 (6-benzylaminopurine)	0,072	0.32	0,072	0.32	0,072	0.32
NAA (naphthalenacetic acid)	0,024	0.13	0,024	0.13	0,024	0.13

(1) Adjusted according to Chée & Pool [7].

Table 2: Macronutrient ion content of the assayed media (in mmol/l).

Ion	MS	MSM	G
N total	60.01	43.88	29.18
NO ₃ ⁻	39.4	38.96	20.46
NH ₄ ⁺	20.61	10	10
H ₂ PO ₄ ⁻	1.25	2.5	1.25
K ⁺	20.04	21.3	9.16
Ca ²⁺	2.99	5.08	1.27
Mg ²⁺	1.5	2.43	1.5
SO ₄ ²⁻	1.6	2.58	1.6
Cl ⁻	5.99	-	-
Total	93.38	82.85	45.24

Six treatments using the three substrates mentioned were assayed: three for solid medium adding agar (7gL⁻¹) (MSS, MSMS and GS) and three for 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 (600mL) (100mL per flask) for solid conditions. In the case of the TIS treatments PLANTFORM bioreactors [9,11] (4000mL) (500mL substrate per bioreactor) were used. After substrate distribution the flasks and bioreactors were closed and autoclaved for 20min (120°C, 1kg/cm² of pressure). Ten explants per flask, in the case of the solid medium and 50 per PLANTFORM bioreactor were established in order to provide 10mL of substrate per explant in both cases. Cultures were incubated in a growth chamber at 26±1 °C, 30µEm⁻²s⁻¹ and for a 16h. photoperiod. In the case of the

Table 3: Comparison of the average of stem length (cm) obtained in the micropropagation of stevia varieties in the substrates assayed.

Variety	Assayed Substrates					
	MSS	MSMS	GS	MSTIS	MSMTIS	GTIS
Criolla	8.43 c	7.11 b	8.48 c	7.10 b	5.50 a	5.50 a
Morita	5.21bc	4.62 abc	5.63 c	4.20 ab	4.80 abc	4.00 a

N=50. In each row, means followed by the same letter were not statistically different according to Tukey's test (P<0.05).

Table 3 summarizes the results of the average of stem length (cm) obtained.

As regards stem length in the Criolla plantlets, three statistically well-differentiated groups were observed (Table 3):

- The two longest stems were grown in MSS and GS;
- Two stems of intermediate length - MSMS and MSTIS - were obtained; and
- The two shortest stems were grown on MSMTIS and GTIS. Less homogeneity in length was found in the Morita plantlets.

As with the Criolla plantlets, the longest average stem length was obtained in those grown in GS medium, but similar values were obtained for stem grown in MSS, MSMS and MSMTIS. In

PLANTFORM bioreactors gas exchange sterilized by filters was controlled through three inlets/outlets anchored to the side. The immersion frequency was 2 min every 8h, as described elsewhere [12,13]. Each treatment was repeated 5 times.

At 30 days of culture the aerial plant size (length in cm) was measured and the nutrient status of the obtained material was evaluated. The leaf samples were carefully washed with distilled water and then dried at 80°C for 48h and ground. Individual 500mg samples were then digested by wet oxidation with concentrated HNO₃ under pressure in a microwave oven to obtain the extract. Concentrations of P, K, Ca, Mg S, B, Cu Fe, Mn, Na, Mo and Zn in the extracts were determined by inductively coupled plasma optical spectroscopy (ICP-OES) (MS 3.5). Total N concentration was determined by Kjeldahl digestion using an elemental analyzer (Leco CHNS-932, Spain). Analyses were repeated three times.

Statistical analysis was carried out using IBM SPSS Statistics v.25. 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.

Results

Growth

Root presence and absence of visual hyperhydricity symptoms were observed in all the plantlets developing from explants.

contrast, shorter stems were quantified in MSTIS and GTIS media (Table 3). Consequently, considering the combined results for Criolla and Morita, the stems were longer (P<0.05) in solid substrates (average 6.60cm) than with TIS (average 5.21cm).

When comparing the behavior of both stevia varieties and the three substrates (MS, MSM and G) independently of the culture system used (solid medium or TIS), the best development in length was found in stems grown in MS medium (average of 6.33cm), similar statistically to the length achieved in G (average of 5.9cm) and shorter (P<0.05) than the lengths of stems grown in MSM (average of 5.52cm). In general, considering the different averages for all substrates in both solid and TIS, longer stems were obtained for Criolla (7.02cm) (P<0.05) than Morita (4.77cm), respectively.

Nutritional status

Macronutrients

In general, no differences were observed between both varieties in terms of macro or micronutrients levels. Only manganese levels in leaves were higher (Student's t-test $P < 0.05$) in the Morita plantlets (411.45mg/kg) than in the Criolla variety (389.68mg/kg). However, the macronutrient analysis revealed statistical differences (ANOVA Tuckey test $P < 0.05$) among the assayed substrates. Thus, statistically highest nitrogen percentages were found in plants developed in MSS and MSMS, followed by the same TIS substrates (Table 4). The G substrate presented the lowest (ANOVA, Tuckey test $P < 0.05$) concentrations of this element in both solid and TIS conditions, although concentrations were statistically higher in the former than in the latter. As regards nitrogen content, significant differences (Student's t-test ($P < 0.05$)) between solid and TIS systems were observed: 5.12 %

and 4.33%, respectively.

In terms of phosphorous and potassium levels, plantlets cultured in MSS, MSMS and MSMTIS substrates contained the highest percentages (ANOVA Tuckey test $P < 0.05$). In contrast, the plantlets with the lowest phosphorous and potassium contents were those cultured in GTIS and MSTIS. The phosphorous level registered in leaves of plantlets grown in solid media (0.43%) was significantly higher (Student's t-test ($P < 0.05$)) than those found in plantlets developed under TIS conditions, (0.32%). Foliar analysis revealed the highest calcium and magnesium content in plantlets grown in MSMTIS, although in the Morita plantlets the highest percentage (ANOVA Tuckey test $P < 0.05$) was found in MSMS medium (Table 4). For leaves from the both varieties, the highest (ANOVA Tuckey test $P < 0.05$) iron content was obtained in the plantlets grown in MSMTIS medium. In TIS conditions more iron was accumulated in leaves, (171.25mg/kg) than in solid media (108.21mg/kg) (Student's t-test ($P < 0.05$)).

Table 4: Macronutrient averages in stevia plants cultured *in vitro* in different culture media.

Culture media	Macronutrients											
	Nitrogen (%)		Phosphorous (%)		Potassium (%)		Calcium (%)		Magnesium (%)		Iron (mg/kg)	
	Criolla	Morita	Criolla	Morita	Criolla	Morita	Criolla	Morita	Criolla	Morita	Criolla	Morita
MSS	5.61 e	5.68 c	0.46 c	0.36 b	3.42 d	3.13 b	0.48 c	0.49 d	0.16 a	0.14 a	67.13 a	121.30 b
MSMS	5.44 e	5.48 c	0.51d	0.56 d	3.99 f	3.87 d	0.69 d	0.87 e	0.2 b	0.25 e	94.40 b	170.67 c
GS	3.71 b	4.67 b	0.30 b	0.41 bc	2.15 b	2.63 a	0.41 b	0.36 b	0.16 a	0.19 c	63.53 a	82.40 a
MSTIS	5.06 d	4.53 b	0.26 a	0.29 a	3.17 c	3.53 c	0.42 b	0.42 c	0.16 a	0.17 b	120.30 c	120.00 b
MSMTIS	4.21 c	5.13 c	0.46 d	0.42 c	3.73 e	3.47 c	0.87 e	0.88 e	0.25 c	0.22 d	278.00 d	279.33 d
GTIS	3.24 a	3.55 a	0.24 a	0.25 a	1.97 a	2.04 a	0.31 a	0.31 a	0.16 a	0.16 b	74.90 a	134.60 b

The different letters in the columns indicate statistical differences (Tukey test, $P < 0.05$) between each macronutrient considered for the assayed substrates.

Micronutrients

In relation to micronutrients (Table 5), with exception of molybdenum with substrate averages of 12.98 and 149.45mg/kg

in Criolla and Morita leaves, respectively (Student's t-test $P < 0.05$), the other microelements analyzed did not present any differences in contents for either variety.

Table 5: Averages micronutrient levels in stevia plantlets cultured *in vitro* in different culture media.

Culture Media	Micronutrients													
	Boron (mg/kg)		Copper (mg/kg)		Sulphur (%)		Manganese (mg/kg)		Sodium (mg/kg)		Molybdenum(mg/kg)		Zinc(mg/kg)	
	Criolla	Morita	Criolla	Morita	Criolla	Morita	Criolla	Morita	Criolla	Morita	Criolla	Morita	Criolla	Morita
MSS	54.0 a	50,2 a	2,8 d	5.1 d	0.39 b	0.46 cd	254.0 b	269.7 a	0.09 d	0,07 c	9.0 a	114.0 a	84.7 a	114.0 a
MSMS	71.4 b	101,3 b	3,3 e	5.1 d	0.51 c	0.53 e	336.3 c	398.0 b	0.09 d	0,11 d	13.0 d	147.0 b	103.0 c	147.0 b
GS	78.0 b	106,1 b	1,7 a	2.5 a	0.31 a	0.40 ab	653.3 f	620.7 d	0.05 c	0,07 c	9.3 ab	140.0 b	110.0 c	140.0 b
MSTIS	49.9 a	53,8 a	2,5 c	3.0 b	0.4 b	0.43 bc	227.6 a	255.0 a	0.02 a	0,03 a	11.8 c	118.0 a	101.0 b	118.0 a
MSMTIS	101.4 c	101,7 b	4,1 f	4.3 c	0.48 c	0.46 d	381.6 d	408.7 b	0.04 b	0,03 a	24.6 e	172.7 c	173.7 e	172,7 c
GTIS	95.2 c	101.0 b	2,2 b	3.0 b	0.37 b	0.37 a	485.3 e	516.6 c	0.04 b	0,04 b	10.2 b	205.0 d	163.7 d	205.0 d

The different letters in the columns indicate statistical differences (Tukey test, $P < 0.05$) between each micronutrient.

Boron content was higher in the leaves of Criolla plantlets grown in MSMTIS and GTIS media (ANOVA Tuckey test $P < 0.05$). The pattern was similar for the Morita variety, but in this case analogous levels were obtained for both media and also in solid conditions (Table 5).

The highest copper content quantified in Criolla leaves was obtained in the plantlets cultured in MSMTIS medium (ANOVA Tuckey test $P < 0.05$). However, in the Morita variety these contents were significantly higher ($P < 0.05$) in plantlets cultured in the MSS and MSMS media. The highest concentrations of sulphur (ANOVA Tuckey test $P < 0.05$) were found in the plantlets growing in the MSMS and MSMTIS substrates, while the lowest contents were quantified in plantlets grown in the GS and GTIS substrates (Table 5). The highest levels of manganese were found in plantlets from the two varieties grown in GS and the lowest in MSMTIS (ANOVA Tuckey test $P < 0.05$). Significant differences (ANOVA Tuckey test $P < 0.05$) in sodium content were also observed, with maximum values in plantlets multiplied in MSS and MSMS media (ANOVA Tuckey test $P < 0.05$). In this case, the Na concentration identified in leaves of plantlets developed in solid conditions (0.082mg/kg) was higher than in those quantified in TIS (0.033mg/kg) (Student's t-test ($P < 0.05$)). For molybdenum and zinc, the highest values detected in Criolla plantlets corresponded to those grown in MSMTIS medium. However, for Morita plantlets the highest levels of both elements were found in GTIS medium. Leaves developing in TIS presented higher levels of zinc (158.82mg/kg) than those of plantlets grown in solid substrates (122.34mg/kg) (Student's t-test ($P < 0.05$)).

Discussion

In general, the length ranges obtained are in accordance with the normal length found by other authors micropropagating stevia explants [3,6]. However, it may be suggested for stem length that the best combination consisted of Criolla plantlets growing in solid MS or G media. Many publications on steviosides and rebaudiosides, as main responsible for sweet taste of stevia plantlets cultured *in vitro*, can be found in current literature. On the contrary, no references about macro and micronutrient status of leaves from micropropagated plants have been found. For this reason, the nutrient levels reported here may be useful as references for future studies. In general, no differences were observed between both varieties in terms of macro or micronutrients levels. Only manganese levels in leaves were higher (Student's t-test $P < 0.05$) in the Morita plantlets (411.45mg/kg) than in the Criolla variety (389.68mg/kg). However, the macronutrient analysis revealed statistical differences (ANOVA Tuckey test $P < 0.05$) among the assayed substrates. Thus, statistically highest nitrogen percentages were found in plants developed in MSS and MSMS, followed by the same TIS substrates (Table 4). The G substrate presented the lowest (ANOVA, Tuckey test $P < 0.05$) concentrations of this element in both solid and TIS conditions, although concentrations were statistically higher in the former than in the latter. These N contents are clearly related with the substrate compositions

(Table 1 & 2), $r = 0.7544$ ($P < 0.01$). As regards nitrogen content, significant differences (Student's t-test ($P < 0.05$)) between solid and TIS systems were observed: 5.12% and 4.33%, respectively. A similar result was found by Deng et al. [14] in explants of *Fraxinus mandshurica* that the absorption of nitrogen was favored in agar gelled culture media when compared with plants grown in TIS.

In terms of phosphorous and potassium levels, plantlets cultured in MSS, MSMS and MSMTIS substrates contained the highest percentages (ANOVA Tuckey test $P < 0.05$). In contrast, the plantlets with the lowest phosphorous and potassium contents were those cultured in GTIS and MSTIS. Levels of both macronutrients were directly correlated with its contents in the substrate ($r = 0.7521$ and $r = 0.9649$, $P < 0.01$) respectively. This strongest correlation in the case of potassium indicate that a dose almost three fold added to the MSM substrate may be suitable for good *in vitro* development of stevia plantlets. The phosphorous level registered in leaves of plantlets grown in solid media (0.43%) was significantly higher (Student's t-test ($P < 0.05$)) than those found in plantlets developed under TIS conditions, (0.32%). In a similar way, K content in leaves of both stevia varieties was slightly higher (3.18%) when cultured in solid substrates than in TIS (2.96%). Foliar analysis revealed the highest calcium and magnesium content in plantlets grown in MSMTIS, although in the Morita plantlets the highest percentage (ANOVA Tuckey test $P < 0.05$) was found in MSMS medium (Table 4). Calcium levels showed a very strong correlation with the calcium added to the substrates ($r = 0.9134$, $P < 0.01$). George et al. [15] indicated that calcium concentrations within *in vitro* plant tissue culture tend to be proportional to those in soil. Magnesium levels were also clearly related with the supply of Mg in the substrates ($r = 0.8911$, $P < 0.01$). For leaves from both varieties, the highest (ANOVA Tuckey test $P < 0.05$) iron content was obtained in the plantlets grown in MSMTIS medium. However, the direct correlation with the Fe in the substrates was maintained ($r = 0.7118$, $P < 0.05$). In TIS conditions more iron was accumulated in leaves, (171.25mg/kg) than in solid media (108.21mg/kg) (Student's t-test ($P < 0.05$)).

In relation to micronutrients (Table 5), with exception of molybdenum with substrate averages of 12.98 and 149.45mg/kg in Criolla and Morita leaves, respectively (Student's t-test $P < 0.05$), the other microelements analyzed did not present any differences in contents for either variety.

Boron content was higher in the leaves of Criolla plantlets grown in MSMTIS and GTIS media (ANOVA Tuckey test $P < 0.05$). The pattern was similar for the Morita variety, but in this case analogous levels were obtained for both media and also in solid conditions (Table 5).

The highest copper content quantified in Criolla leaves was obtained in the plantlets cultured in MSMTIS medium (ANOVA Tuckey test $P < 0.05$). However, in the Morita variety these contents were significantly higher ($P < 0.05$) in plantlets cultured in the MSS and MSMS media. The highest concentrations of sulphur (ANOVA Tuckey test $P < 0.05$) were found in the plantlets growing in the

MSMS and MSMTIS substrates, while the lowest contents were quantified in plantlets grown in the GS and GTIS substrates. No differences were observed between the solid and TIS systems (0.43 and 0.42%, respectively). The highest levels of manganese were found in plantlets from the two varieties grown in GS and the lowest in MSMTIS (ANOVA Tuckey test $P < 0.05$). This was the only element significantly inversely correlated with its concentration in the substrates ($r = -0.8653$, $P < 0.01$). This result can be explained by the antagonism between K content in the substrate and Mn in leaves. Tavares et al. [16] reported a significant Mn decrease in leaves of *Aechmea blanchetiana* micropropagated in medium with high K levels. According to this antagonism, our results indicate a similar inverse correlation between K added to the medium and Mn concentration in leaves ($r = -0.8252$, $P < 0.01$). Significant differences in sodium content were also observed, with maximum values in plantlets multiplied in MSS and MSMS media (ANOVA Tuckey test $P < 0.05$). In this case, the Na concentration identified in leaves of plantlets developed in solid conditions (0.082mg/kg) was higher than in those quantified in TIS (0.033mg/kg) (Student's t-test ($P < 0.05$)). For molybdenum and zinc, the highest values detected in Criolla plantlets corresponded to those grown in MSMTIS medium. However, for Morita plantlets the highest levels of both elements were found in GTIS medium. Leaves developing in TIS presented higher levels of zinc (158.82mg/kg) than those of plantlets grown in solid substrates (122.34mg/kg) (Student's t-test ($P < 0.05$)) [17-20].

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Compliance with Ethical Standards

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Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors designed the performed experiments according to the consensual conception of the study. Material preparation and data collection were performed by S.V. Statistical analysis was performed by M.C.F. The first draft of the manuscript was written by M.C. and J.L.G. All authors contributed to the revision of scientific contents and approved the final version of the drafted manuscript.

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