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Steviol Glycoside Content Dynamics during the Growth Cycle of *Stevia rebaudiana* Bert

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Abstract

The sweetener compounds in Stevia, steviol glycosides (SG), are mainly found in the leaves. The SG content depends on the plant's growth stage and is usually highest just before flowering. In temperate areas, Stevia is a polyannual crop (5 - 7 years) with a vegetative period lasting from April-May until October, during which time the crop can be harvested two or three times. This research focuses on the need for knowledge about Stevia's response to temperate climates in Southern Europe. Two field assays were established from June to October 2013 at two sites in Navarra (Spain). The biomass and content of the two major SG, stevioside (ST) and rebaudioside A (RA), were measured using High Performance Liquid Chromatography (HPLC) in 66 cloned plants, at different developmental stages. Although the results from the two sites showed different SG leaf content dynamics during the plant growth, the optimum harvest date at both sites coincided with the bud-flowering stage at the beginning of September (around 96 days after planting), when a ST yield of 27 $g \cdot m^{-2}$ was reached. These results show Stevia's potential as a commercial crop for stevioside production in northern Spain.

Keywords

Stevia rebaudiana Bert., Crop, Stevioside, Rebaudioside A, Photoperiod

1. Introduction

The number of people suffering cardiovascular diseases, diabetes, hypercholesterolemia and obesity has increased globally [1]. For this reason, the demand for non-caloric sweeteners has increased while sugar consumption has decreased [2]. In this context Stevia plays an important role. Besides being a natural sweetener, stevia leaf extracts have other interesting properties, including decreasing blood pressure, improving digestion and gastrointestinal function and protecting against dental caries [3] [4] [5] [6].

Since ancient times, Stevia has been used in its native area, Paraguay, from where it has been introduced into Asia and North America, and more recently into Europe. Nowadays China is the main producer and Japan the main consumer [7]. Stevia is a relatively new crop, still under domestication, although much work has been done on variety selection, particularly in China, Korea and India.

The European Commission approved the use of SG as a food additive (E-960) in 2011 (Commission Regulation EU No 1131/2011). As this new market opened up, the demand for Stevia increased and its introduction into Europe as a crop was encouraged, especially in the Mediterranean region. In Spain, the area dedicated to Stevia cultivation is estimated to be around 70 - 80 ha.

Among the steviol glycosides (SG), stevioside (ST) and rebaudioside A (RA) are present in the highest concentrations [8]. In the biosynthetic pathways for the main SGs, ST is a direct precursor to RA [9]. ST represents between 60% - 70% of the total SGs and is 300 times sweeter than sucrose, with a slight licorice flavor and a bitter aftertaste [10]. In contrast, RA lacks the bitter aftertaste and is the sweetest SGs, being from 250 up to 400 times sweeter than sucrose [10]. SGs are mainly found in the leaves, with higher levels in younger than older leaves [8] [11] [12] [13], with less in shoots, roots, flowers and seeds [14]. For this reason, the leaves are used for obtaining SG extracts, while shoots are discarded because of the difficulty of extracting SGs from lignified shoots.

SG yield per dried leaf varies from 5% to 22% [15] and SG content depends on plant variety or population [9] [16], propagation methods [17], growing conditions, photoperiod [9] [18], agronomic practices [19] [20] [21] [22], plant age or ontogenetic phase [13] [16] [23].

Stevia is a short-day species with a critical photoperiod of 13 to 14 hours needed to induce flowering [3]. In general, SG content is highest just before flowering, during the flower bud formation phase [24], making this the optimum time for harvesting [25]. After this phase, photoassimilates are allocated to reproductive organs and SG content tends to either stagnate [26] [27] or decrease [28]. Temperate areas, with long summer days (more than 13 - 14 hours of sunlight), provide the ideal conditions for obtaining high yields of SG [8]. A longer vegetative period before flowering and harvest promotes higher leaf biomass production and a higher SG yield.

In various temperate areas, the cultivation of Stevia is long-term (5 - 7 years) and the vegetative period lasts from April-May to October. During winter, above-ground parts of the plant remain inactive, and new stems sprout from the buried rhizome the next spring. In these areas with a temperate climate, Stevia can be harvested two or three times per year [29]. At higher latitudes, with cooler weather, the crop is harvested once a year.

This research focuses on the need for knowledge about Stevia's response to temperate climates in Southern Europe. Specifically, we elucidate fluctuations in biomass production and SG content per plant during the various developmental stages of a crop cycle at two different sites in northern Spain.

2. Material and Methods

2.1. Study Sites

Field assays were carried out from June to October 2016 at two sites in Navarra, Spain: San Adrián (42˚33'N, 1˚93'E, 318 m asl, mean annual precipitation 503 mm, mean annual temperature 16.5˚C) and Puente la Reina (42˚67'N, 1˚81'E, 344 m asl, mean annual precipitation 605 mm and mean annual temperature 13˚C). The steppe climate of San Adrián is characteristic of the central Ebro Valley, and is classified as dry cold steppe or dry Mediterranean (Bsk in the Köppen climate classification). Puente la Reina has a Mediterranean climate with warm, summers (Csb in the Köppen climate classification). **Table 1** presents climate data (mean temperatures and precipitation) for the two sites during the field assay period. Soils from both sites show similar characteristics: both are classified as sandy-loam with a basic pH and similar N content, although the soil in San Adrián has more P and K content than that of Puente la Reina (**Table 2**).

Table 1. Total cumulative precipitation (TCP), mean maximum temperature (Mmax.) and mean minimum temperature (Mmin.) from June to September 2013 at the two assay sites (source: http://meteo.navarra.es/climatologia/zona_media.cfm).

	San Adrián				Puente la Reina			
2013	June	July	August	Sept.	June	July	August	Sept.
TCP ($L \cdot m^{-2}$)	4.04	2.33	0.50	0.50	34.70	14.50	32.1	32.1
Mmax. (˚C)	31.95	29.53	26.25	26.25	30.35	28.05	23.93	23.93
Mmin. (˚C)	15.91	14.47	11.95	11.95	18.40	17.34	14.26	14.26

Table 2. Physical and chemical parameters of the soil at the study.

Soil parameters	San Adrián	Puente la Reina
pH (1:2.5 V/V)	8	8.2
C:N	10.6	12.5
Organic matter (%)	2.59	3.03
Kjeldahl nitrogen (%)	0.14	0.14
Olsen phosphorus (mg P kg^{-1})	41	26
Extractable potassium (mg K kg^{-1})	589	482
Extractable magnesium (mg Mg kg^{-1})	158	130
Coarse sand (0.2 - 2.0 mm) (%)	8	15.7
Fine sand (0.02 - 0.2 mm) (%)	61.4	40.8
Silt (0.002 - 0.02 mm) (%)	18.3	26.8
Clay (<0.002) (%)	12.4	16.7
Soil texture	SANDY LOAM	SANDY LOAM

2.2. Plant Material and Experimental Design

For the assays were used in vitro plant clones, obtained from a single previously-selected plant. On June 5th, at each site, 10 cm-tall specimens of Stevia were manually planted in one row using a planting pattern of 40 × 35 cm. Black padding was installed to minimize weed growth. Nitrogen, phosphorus and potassium were supplied three times through fertigation (COMPO, 7% N, 5% P_2O_5, 6% K_2O, and microelements).

Every two weeks, four plants were cut five cm above soil level and the shoots and leaves were separated. A subsample of fresh leaves from each plant was used to measure SG content and the rest of the sample was air dried, being turned over every two days. Both the fresh and dried biomass of the shoots and leaves were recorded.

In total, 66 plants (32 in San Adrián and 34 in Puente la Reina) were analyzed over 9 sampling dates corresponding to different developmental stages, from early vegetative growth (V3) to the crop flowering stage (R2) (**Table 3**). The description of plant developmental stages followed Carneiro [30]. The vegetative stages sampled corresponded to: V3, plants with just one main stem and axillary branches with internodes of less than 2 cm in length; V4, plants with more than one stem and axillary branches with internodes of more than 2 cm in length; and late-V4, plants with more than one stem and axillary branches more than 15 cm in length (this stage occurs just before the differentiation of flower buds). The reproductive stages corresponded to: R1, the branch apex has already differentiated into flower buds; and R2, the flowers are in anthesis, the time of flowering to pollination. Plant developmental stages, sampling dates and day length for both sites are shown in **Table 3**. The transition from one developmental stage to the next lasts a few days.

Table 3. Mean daylight hours for two-week periods from June till September 2013 at San Adrián (42˚33'N, 1˚93'E) and Puente la Reina (42˚67'N, 1˚81'E). Harvest day and plant growth stage are also shown.

	San Adrián		Puente la Reina	
	Daylight (hrs: min)	Plant growth stage	Daylight (hrs: min)	Plant growth stage
June 1st-15th	15:10		15:13	
June 16th-30th	15:16		15:18	
July 1st-15th	15:06	V3	15:08	V3
July 16th-31st	14:43	V4	14:45	V3, V4
August 1st-15th	14:10	V4	14:12	V4, late-V4
August 16th-31st	13:31	V4, late-v4	13:32	
Sept. 1st-15th	12:49	Late-V4, R1	12:50	Late-V4, R1
Sept. 16th-30th	12:07	R1	12:07	R1

2.3. Extraction and Analyses of SG

SGs were extracted three times from fresh leaves (0.50 g), each time using 7 mL of ultrapure water. Extractions were performed at room temperature, shaking for 35 m at 300 rpm in an orbital shaker. The extracts were centrifuged at 4500 rpm for 12 m and the supernatant of the three extracts was then pooled and mixed, and brought to a final volume of 50 ml. Finally, the extracts were purified by filtering through a 0.45 μm filter. 20 μL aliquots of extract were injected into a High Performance Liquid Chromatography (HPLC) system coupled with an ultraviolet detector. A Waters Atlantis T3 5 μm column (4.6 × 150 mm) was utilized and the HPLC operating conditions used were as follows: isocratic mobile phase acetonitrile: water (70/30) and a flow of 0.5 $mL \cdot min^{-1}$. The ultraviolet detector was set to monitor at 205 nm. Retention time was 15:05 min for RA and 15:96 for ST. Pure RA (97.5%) was used as standard. Calibration curves were obtained from standard solutions (10 - 500 ppm) for RA. Previous tests showed that the relationship between the response factor of RA and ST was statistically equal to one, and therefore ST was also quantified using the calibration curve of RA.

2.4. Data Analysis

Scatterplots of dry leaf biomass, leaf-to-stem ratio, ST and RA versus time after planting were performed and a polynomial surface for each site was fitted using local polynomial regression fitting with the loess function from the statistical software R [31].

3. Results

Leaf biomass increased with time at both sites but peaked at different dates (**Figure 1(a)**). The plants in San Adrián presented greater leaf biomass and earlier than those at Puente la Reina. In San Adrián, leaf biomass reached a peak (63.3 g/plant) 96 days after planting, between the end of the late-V4 and early R1 stages (grey square point in **Figure 1(a)**). In Puente la Reina, the highest yield (53.6 g/plant) was reached 102 days after planting, at the R1 stage (black square point in **Figure 1(a)**). The leaf to stem ratio decreased throughout the plant growth cycle, from 4.5 down to 0.5 at both sites (**Figure 1(b)**).

The dynamic of ST percentage in leaves throughout plant development differed between the two sites (**Figure 1(c)**). In San Adrián, the ST percentage reached a peak (6.22%) in the late-V4 stage, 87 days after planting (grey square point in **Figure 1(c)**). In Puente la Reina, the ST percentage increased with time and was its highest (8.67%) in the R2 stage, 120 days after planting (black square point in **Figure 1(c)**). Taking into account biomass production, total ST leaf content per plant peaked in San Adrián (3.72 g/plant) at the end of the late-V4 stage, 92 days after planting (grey square point in **Figure 1(d)**), whereas the peak in Puente la Reina (4.03 g/plant) was reached in the R1 stage, 108 days after planting (black square point in **Figure 1(d)**).

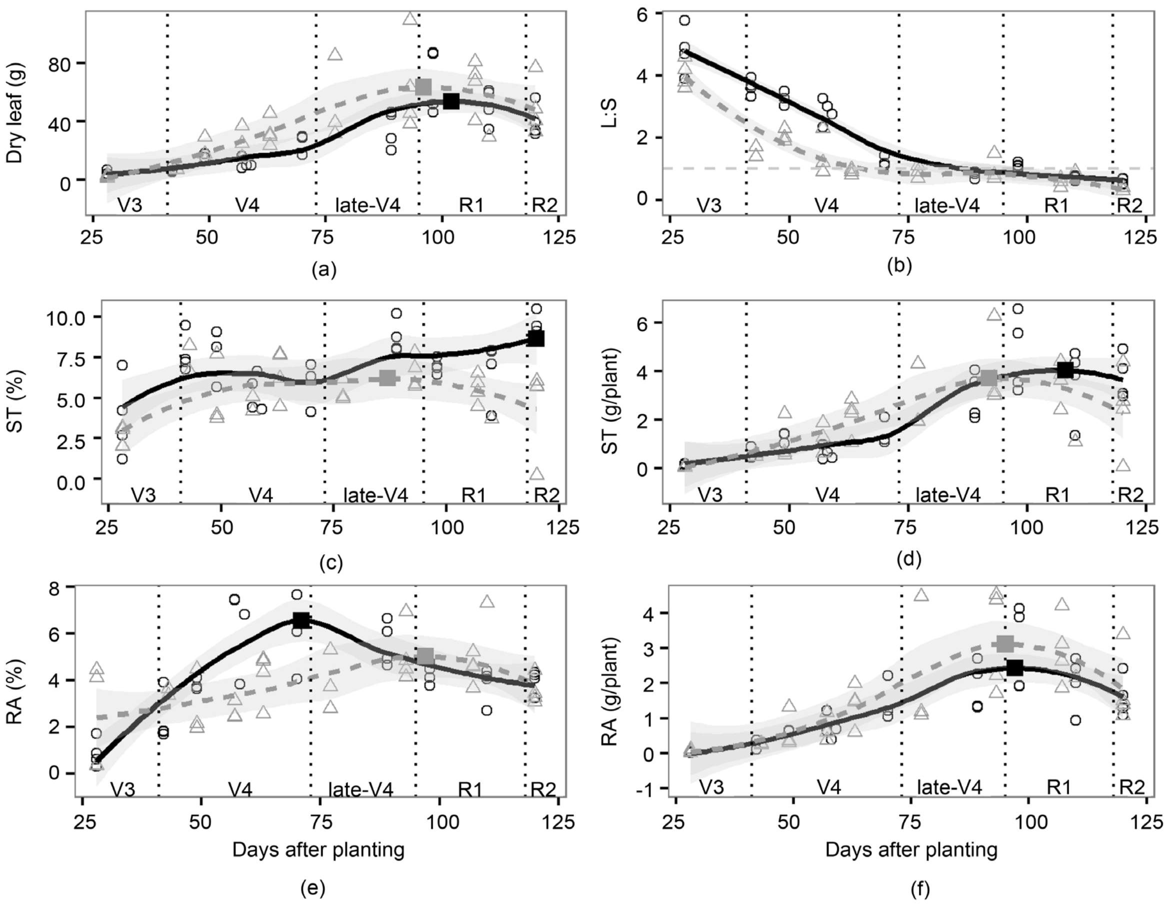

Figure 1. Dynamics of dry leaf biomass (g), leaf-to-stem ratio (L:S), percentage of stevioside in leaves (ST(%)), total ST content per plant (g/planta), percentage of rebaudioside A (RA (%)) and total RA content per planta (g/planta), at both sites, San Adrián (grey triangles) and Puente la Reina (black circles). Local polynomial regression fitting curves are represented with a grey dashed line for San Adrián data and a black solid line for Puente la Reina data. Standard error is represented as a light grey shaded area. The maximum predicted values are marked with rectangles (grey for San Adrián and black for Puente la Reina). Vertical dotted lines separate developmental stages. The transition between stages can last for a few days.

The dynamic of RA percentage in leaves throughout plant development also differed between the sites (Figure 1(e)). In San Adrián, the RA percentage was highest (5.03%) between the end of the late-V4 stage and the beginning of R1, 97 days after planting (grey square point in Figure 1(e)). In Puente la Reina, the RA percentage peaked (6.55%) in the V4 stage, 70 days after planting (black square point in Figure 1(e)). Taking into account biomass production, total RA leaf content per plant was at its highest level between the end of the late-V4 and beginning of the R1 stages at both sites. In San Adrián, RA content reached 3.12 g/plant 95 days after planting (grey square point in Figure 1(f)) and in Puente la Reina, RA content reached 2.43 g/plant 97 days after planting (black square point in Figure 1(f)).

4. Discussion

The observed decrease in leaf biomass during the reproductive stages (R1 and

R2) was due to senescence and the dropping of basal leaves. Mean maximum temperature (MMax) throughout the assay period was higher in San Adrián than Puente la Reina (**Table 1**). Warmer temperatures could have facilitated earlier and more luxuriant plant growth in the specimens in San Adrián than those in Puente la Reina. Plant growth during summer may be affected by prolonged exposure to temperatures greater than 35˚C [32]; however, temperatures did not get this high during the assay period.

A leaf to stem ratio decrease was observed throughout the plant growth cycle. In the early stages, the stems were green and soft with a high water content and leaf weight was greater than stem weight (L:S > 1). In later stages, the stems enlarged and lignified, and consequently their dry weight increased considerably. This meant that stem weight was greater than leaf weight during the reproductive stages. Decreased leaf biomass in the later stages due to reproductive development has been observed previously [33]. Although a similar L:S pattern was seen at both sites throughout the growth stages, L:S was greater in Puente la Reina plants than in San Adrián plants during the vegetative stages. Similar studies in Israel also showed a decreased L:S ratio with time and a leaf biomass peak in September [22].

In choosing the optimum time for harvest, the most important factors to consider are leaf yield and content of the main SGs. Other factors with commercial implications could also play an important role. Previous studies have shown that the stevioside content of inflorescences is very low [22], therefore, increasing the proportion of leaves in the plant biomass to be processed increases the efficiency of industrial processing and decreases its cost. The peaks of leaf biomass and ST and RA percentages are more or less coincident in San Adrián whereas in Puente la Reina they are out of sync: the ST peak occurs after the leaf biomass peak and the RA peak occurs before. Thus, the optimum harvest time in San Adrián would be around 96 days after planting when the plants are initiating their flowering stage, at the beginning of September (26.43 ST $g{\cdot}m^{-2}$ and 22.29 RA $g{\cdot}m^{-2}$). In Puente la Reina, ST yield reached a peak in the middle of the reproductive stage R1 a few days after the RA peak. However, the curve of ST maximum yield is fairly plateau-shaped, so similar ST yields can be achieved earlier, when the proportion of inflorescences versus leaves is lower. Therefore, the optimum time for harvesting in Puente la Reina would also coincide with the beginning of the flowering stage, at the start of September (27.36 g ST m^{-2} and 17.36 g RA m^{-2}). The ST yields recorded at both sites are similar to those reported in other studies, between 23.5 and 30.9 $g{\cdot}m^{-2}$ in Israel [22], 28 and 34 $g{\cdot}m^{-2}$ in India [20], and 31.5 $g{\cdot}m^{-2}$ in Canada [25].

5. Conclusion

In conclusion, this paper addresses the viability of growing Stevia under the climatic conditions found in northern Spain. The results show that Stevia can be successfully cultivated in this region, good glycoside yields are obtained under the long daylight conditions, and there is a long vegetative growth period (from spring to late summer).

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References

[1] Jeria, D.M., Jeria, D.M. and Pozo, A.A. (2011) Estudio Del Secado Convectivo De Hojas De Stevia Rebaudiana Y Factibilidad Técnico-Económica De Una Planta Elaboradora De Edulcorante a Base De Stevia. Ph.D. Dissertation, Facultad de Ciencia Químicas y Farmacéuticas, Universidad de Chile, Santiago.

[2] Marti, N., Funes, L.L., Saura, D. and Micol, V. (2008) An Update on Alternative Sweeteners. *International Sugar Journal*, **110**, 425-429.

[3] Kinghorn, A.D. and Soejarto, D.D. (1991) Stevioside. In: *Economic and Medical Plant Research*, Academic Press, New York, 157-171. .

[4] Lee, C.N., Wong, K.L., Liu, J.C., Chen, Y.J., Cheng, J.T. and Chan, P. (2001) Inhibitory Effect of Stevioside on Calcium Influx to Produce Antihypertension. *Planta Medica*, **67**, 796-799. https://doi.org/10.1055/s-2001-18841

[5] Jeppesen, P.B., Gregersen, S., Rolfsen, S.E.D., Jepsen, M., Colombo, M., Agger, A., Xiao, J., *et al.* (2003) Antihyperglycemic and Blood Pressure-Reducing Effects of Stevioside in the Diabetic Goto-Kakizaki Rat. *Metabolism*: *Clinical and Experimental*, **52**, 372-378. https://doi.org/10.1053/meta.2003.50058

[6] Woelwer-Rieck, U., Lankes, C., Wawrzun, A. and Wüst, M. (2010) Improved HPLC Method for the Evaluation of the Major Steviol Glycosides in Leaves of *Stevia rebaudiana*. *European Food Research and Technology*, **231**, 581-588. https://doi.org/10.1007/s00217-010-1309-4

[7] Fischer, J.C. (2012) Tercer Simposio Nacional de la estevia realizado en Santa Cruz en el marco de Agropecruz.

[8] Ramesh, K., Singh, V. and Megeji, N. (2006) Cultivation of Stevia: A Comprehensive Review. *Advances in Agronomy*, **89**, 137-177. https://doi.org/10.1016/S0065-2113(05)89003-0

[9] Ceunen, S. and Geuns, J.M.C. (2013) Influence of Photoperiodism on the Spatio-Temporal Accumulation of Steviol Glycosides in *Stevia rebaudiana* (Bertoni). *Plant Science*, **198**, 72-82. https://doi.org/10.1016/j.plantsci.2012.10.003

[10] Kinghorn, A.D. (2002) Overview. In: Kinghorn, A.D., Ed., *Stevia*, *the Genus of Stevia*, *Medicinal and Aromatic Plants Industrial Profiles*, Taylor and Francis, London, 2.

[11] Goettemoeller, J. and Ching, A. (1999) Seed Germination in *Stevia rebaudiana*. In: Janick, J., Ed., *Perspectives on New Crops and New Uses*, ASH Press, Alexandria, VA, 510-511.

[12] Bondarev, N.I., Sukhanova, M.A., Reshetnyak, O.V. and Nosov, A.M. (2004) Steviol Glycoside Content in Different Organs of *Stevia rebaudiana* and Its Dynamics during Ontogeny. Bio*logia Plantarum*, **47**, 261-264. https://doi.org/10.1023/B:BIOP.0000022261.35259.4f

[13] Yadav, A.K., Singh, S., Dhyani, D. and Ahuja, P.S. (2011) A Review on the Improvement of Stevia [*Stevia rebaudiana* (Bertoni)]. *Canadian Journal of Plant Science*, **91**, 1-27. https://doi.org/10.4141/cjps10086

[14] Rajasekaran, T., Giridhar, P. and Ravishankar, G.A. (2007) Production of Steviosides in *Ex Vitro* and *In Vitro* Grown *Stevia rebaudiana* Bertoni. *Journal of the Science of Food and Agriculture*, **87**, 420-424. https://doi.org/10.1002/jsfa.2713

[15] Kennelly, E.J. (2001) Sweet and Non-Sweet Constituents of *Stevia rebaudiana*. In: Kinghorn, A.D., Ed., *Stevia. The Genus Stevia*, Taylor and Francis, London, 68-85.

[16] Geuns, J.M.C. (2003) Stevioside. *Phytochemistry*, **64**, 913-921. https://doi.org/10.1016/S0031-9422(03)00426-6

[17] Tamura, Y., Nakamura, S., Fukui, H. and Tabata, M. (1984) Clonal Propagation of *Stevia rebaudiana* Bertoni by Stem-Tip Culture. *Plant Cell Reports*, **3**, 183-185. https://doi.org/10.1007/BF00270195

[18] Metivier, J. and Viana, A.M. (1979) The Effect of Long and Short day Length upon the Growth of Whole Plants and the Level of Soluble Proteins, Sugars, and Stevioside in Leaves of *Stevia rebaudiana* Bert. *Journal of Experimental Botany*, **30**, 1211-1222. https://doi.org/10.1093/jxb/30.6.1211

[19] Shock, C. (1982) Experimental Cultivation of Rebaudi's Stevia in California. University of California, Oakland, 1-9.

[20] Megeji, N.W., Kumar, J.K., Singh, V., Kaul, V.K. and Ahuja, P.S. (2005) Introducing *Stevia rebaudiana*, a Natural Zero-Calorie Sweetener. *Current Science*, **88**, 801-804.

[21] Macchia, M., Morelli, I., Angelini, L.G. and Flamini, G. (1999) Agronomic Characteristics and Quantitative Analysis of Stevioside in *Stevia rebaudiana* Bert. a New Source of Sweet Compounds. *Proceeding of* 4*th European Symposium on Industrial Crops and Products*, Bonn, 23-25 March 1999, 331-332.

[22] Serfaty, M., Ibdah, M., Fischer, R., Chaimovitsh, D., Saranga, Y. and Dudai, N. (2013) Dynamics of Yield Components and Stevioside Production in *Stevia rebaudiana* Grown under Different Planting Times, Plant Stands and Harvest Regime. *Industrial Crops and Products*, **50**, 731-736. https://doi.org/10.1016/j.indcrop.2013.08.063

[23] Nakamura, S. and Tamura, Y. (1985) Variation in the Main Glycosides of Stevia (*Stevia rebaudiana* Bertoni). *Japanese Journal of Tropical Agricultural*, **29**, 109-115.

[24] Kang, K.H. and Lee, F.W. (1981) Physio-Ecological Studies on Stevia (*Stevia rebaudiana* Bertoni). *Korean Journal of Crop Science*, **26**, 69-89.

[25] Brandle, J. and Rosa, N. (1992) Heritabilrty for Yield, Leaf : Stem Ratio and Stevioside Content Estimateil from a Landrace Cultivar of *Stevia rebaudiana*. *Canadian Journal of Plant Science*, **72**, 1263-1266. https://doi.org/10.4141/cjps92-159

[26] Chen, K., Chang, T.R. and Chen, S.T. (1978) Studies on the Cultivation of Stevia and Seasonal Variation of Stevioside. *China Gartenbau*, **24**, 34-42.

[27] Zaidan, L., Dietrich, S. and Felippe, G. (1980) Effect of Photoperiod on Flowering and Stevioside Content in Plants of *Stevia rebaudiana* Bertoni. *Japanese Journal of Crop Science*, **49**, 569-574. https://doi.org/10.1626/jcs.49.569

[28] Tavarini, S. and Angelini, L.G. (2013) *Stevia rebaudiana* Bertoni as a Source of Bioactive Compounds: The Effect of Harvest Time, Experimental Site and Crop Age on Steviol Glycoside Content and Antioxidant Properties. *Journal of the Science of Food and Agriculture*, **93**, 2121-2129. https://doi.org/10.1002/jsfa.6016

[29] Angelini, L.G. and Tavarini, S. (2014) Crop Productivity, Steviol Glycoside Yield, Nutrient Concentration and Uptake of *Stevia rebaudiana* Bert. under Mediterranean Field Conditions. *Communications in Soil Science and Plant Analysis*, **45**, 2577-2592. https://doi.org/10.1080/00103624.2014.919313

[30] Carneiro, J.W.P. (2007) *Stevia rebaudiana* (Bert.) Bertoni: Stages of Plant Development. *Canadian Journal of Plant Science*, **87**, 861-865.

https://doi.org/10.4141/P06-040

[31] R Development Core Team (2015) R: A Language and Environment for Statistical Computing.

[32] Midmore, D.J. and Rank, A.H. (2002) A New Rural Industry—Stevia—To Replace Imported Chemical Sweeteners.

[33] Kienle, U. (2010) Welches Stevia hätten Sie denn gerne? Anbau und Herstellung Perspektiven weltweit. *Journal fur Verbraucherschutz und Lebensmittelsicherheit*, **5**, 241-250. https://doi.org/10.1007/s00003-010-0558-2