

Agrobacterium rhizogenes-Mediated Transformation Enhances Steviol Glycosides Production and Growth in *Stevia rebaudiana* Plantlets

Ángel de Jesús Sánchez-Cordova¹ · Jacqueline Capataz-Tafur² ·
Blanca Estela Barrera-Figueroa² · Adolfo López-Torres³ ·
Paul Mauricio Sanchez-Ocampo^{2,4} · Edgar García-López^{2,4} ·
Ariana Arlene Huerta-Heredia^{2,4}

Received: 19 August 2018 / Accepted: 9 November 2018 / Published online: 23 January 2019
© Society for Sugar Research and Promotion 2019

Abstract *Stevia rebaudiana* accumulates steviol glycosides (SGs) that are used as noncaloric, natural sweeteners in food industry. The most important SGs are rebaudioside A (Reb A) and stevioside (St), and they are markers of the quality of *S. rebaudiana* lines. In this work, the production and physiology of SGs in *Agrobacterium rhizogenes*-transformed plantlets were analyzed. By means of HPLC–DAD, the production of St and Reb A was quantified, resulting in a 1.4- and 1.5-fold production increase of St and Reb A, respectively, in transformed compared to wild-type plantlets. Superior phenotype of transformed plantlets was observed in comparison with wild type, represented as an increase of 25% in biomass of aerial parts, 43% in biomass of roots, 20–30% of leaf area and 24% of chlorophylls content. Moreover, the SG production profiles evaluated in a 20-d period showed higher yield in the transformed line. The results of this work demonstrated the usefulness of *S. rebaudiana* transformation via *A.*

rhizogenes as a strategy to explore new approaches for the improvement of SGs production.

Keywords *Stevia rebaudiana* · Stevioside · Genetic transformation · Rebaudioside A · *Agrobacterium*

Introduction

Stevia rebaudiana, which is commonly known as stevia or honey leaf, is a perennial herbaceous plant belonging to the *Asteraceae* family and is used as a medicinal plant and sweetener. Steviol glycosides (SG) are diterpenoid compounds that accumulate in the leaves of *S. rebaudiana*. The main application of SG is as sweetener, because they are 300 times more intense than sucrose (Yadav and Guleria 2012). In addition, SG are compounds with multiple medicinal properties, such as antibacterial (Gamboa and Chaves 2012), antioxidant (Bender et al. 2015), immunomodulatory and antihyperglycemic effects (Ruiz-Ruiz et al. 2017), antitumor (Gupta et al. 2017), and prevent chronic liver inflammation (Ramos-Tovar et al. 2018). The use of *S. rebaudiana* extracts helps diminishing blood glucose levels in type II diabetes and control high blood pressure in patients that suffer from mild hypertension (Gupta et al. 2013).

Different biotechnological strategies have been employed to increase the production of SG in *S. rebaudiana*, for instance micropropagation using different explants and growth regulators (Razak et al. 2014; Namdari et al. 2015); stress conditions, some of which are salinity (Fallah et al. 2017); incorporation of copper and zinc nanoparticles (Javed et al. 2017a, b); additions of nutrients, including sucrose (Ghorbani et al. 2017) and sources of nitrogen and phosphate (Magangana et al. 2018); elicitation (Bayraktar

✉ Edgar García-López
egarcialo@conacyt.mx

✉ Ariana Arlene Huerta-Heredia
aahuertahe@conacyt.mx

¹ División de Estudios de Posgrado, Universidad del Papaloapan, Av. Circuito Central No. 200 Col. Parque Industrial, CP. 68301 Tuxtepec, Oaxaca, Mexico

² Instituto de Biotecnología, Universidad del Papaloapan, Av. Circuito Central No. 200 Col. Parque Industrial, CP. 68301 Tuxtepec, Oaxaca, Mexico

³ Instituto de Química Aplicada, Universidad del Papaloapan, Av. Circuito Central No. 200 Col. Parque Industrial, CP. 68301 Tuxtepec, Oaxaca, Mexico

⁴ Cátedras CONACyT-UNPA, Universidad del Papaloapan, Av. Circuito Central No. 200 Col. Parque Industrial, CP. 68301 Tuxtepec, Oaxaca, Mexico

et al. 2016); chemical and physical mutagenesis with EMS and gamma radiation (Khan et al. 2016); and *Agrobacterium*-mediated transformation using *A. tumefaciens* (Khan et al. 2014) and *A. rhizogenes* (Pandey et al. 2016).

Agrobacterium rhizogenes induces the formation of roots at infection points by transferring its T-DNA (transfer DNA) from its root-inducing (Ri) plasmid to the genome of various plant species (Tzfira and Citovsky 2006). These induced transformed roots, also known as hairy roots, have been reported to exhibit spontaneous as well as induced, direct and indirect, organogenesis that leads to regenerate whole transgenic plants, often referred as morphogenesis (Christey 2001; Roychowdhury et al. 2013). The morphogenic response in hairy roots is facilitated by the expression of the *rol* oncogenes, mainly *rolA*, *rolB*, *rolC* and *rolD*, present in the Ri plasmid from *A. rhizogenes*, as recently reviewed (Sarkar et al. 2018).

The process of regenerating plants from transformed roots has been observed previously in different species, for instance, *Panax ginseng* (Yang and Choi 2000), *Spinacia oleracea* (Ishizaki et al. 2002), *Catharanthus roseus* (Choi et al. 2004), *Solidago nemoralis* (Gunjan et al. 2013), *Nicotiana tabacum* L. (Gurusamy et al. 2017), where in general there have been found plants with normal or improved phenotypes compared to wild type. The majority of the hairy roots and transformed plants regenerated from them are known to be morphologically stable in long-term culture (Sarkar et al. 2018), given that it has been reported that these transformed plants able to inherit their traits in a Mendelian pattern due to the insertion of the T-DNA in the host genome (Tepfer 1984) can be considered genetically stable.

A. rhizogenes-mediated transformation is an approach that has proven useful in a diversity of plants for the study of gene function and biotechnology (Christey 2001).

To our knowledge, there are few reports regarding the genetic transformation mediated by *Agrobacterium* of *Stevia rebaudiana* and the main objectives have been the establishment of transformed root cultures and the evaluation of their capacity to produce SG (Yamazaki et al. 1991; Fu et al. 2015; Pandey et al. 2016). Khan et al. (2014) used *A. tumefaciens* to obtain transformed in vitro plantlets through direct and indirect organogenesis, and they observed somaclonal differences in SG accumulation. Transformed roots established by Yamazaki et al. (1991) and Fu et al. (2015) showed no accumulation of SG. Pandey et al. (2016) correlated the photosynthetic machinery functionality and the expression of the glucosyltransferase UGT85C2 gene with the accumulation of SG using transformed root cultures.

The objective of this work was to compare transformed plants, which were obtained via transformation mediated by *Agrobacterium rhizogenes*, to the plants with a wild

phenotype cultivated in vitro in terms of SG production, total phenols and growth parameters to explore the utility of the transformation as an alternative strategy to increase SG production in *S. rebaudiana*.

Materials and Methods

Plant Material and Growth Conditions

Stevia rebaudiana in vitro plantlet culture was established from foliar explants obtained of 2-month-old plants that were grown in greenhouse. The leaves were washed with water and soap for 5 min to remove dust particles, and then they were subjected to a disinfection cycle with sodium hypochlorite at 5% (v/v) for 2 min and 70% ethanol (v/v) for 1 min, followed by two rinses with sterile distilled water. Induction of *S. rebaudiana* shoots was carried out using leaf explants from plants in Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) in half supplemented with 2% sucrose (w/v), 1 mg/L benzyl amino purine (BAP), 0.5 mg/L indoleacetic acid (IAA) and 8 g/L agar according to Anbazhagan et al. (2010), and pH was adjusted with 1 M sodium hydroxide (NaOH) to 5.8–6.0 before sterilization. After 15 days, the shoots of wild *S. rebaudiana* were transferred to medium for rooting, which contained the MS culture medium supplemented with 2% sucrose (w/v) and 0.5 mg/L indolebutyric acid (IBA). For in vitro propagation of *S. rebaudiana* wild type, the same culture mediums described above were used.

Induction of *S. rebaudiana* transformed shoots was carried out using leaf explants from transformed plantlet with MS culture medium in half supplemented with 2% sucrose (w/v), 8 g/L agar and without growth regulators. The shoots obtained were rooted in MS medium supplemented with 2% sucrose (w/v) and 2.2 g/L of phytagel and without growth regulators, and pH was adjusted with 1 M sodium hydroxide (NaOH) to 5.8–6.0 before sterilization.

All cultures were maintained at 25 ± 2 °C in a photoperiod of 16–8 h at $26 \mu\text{mol s}^{-1} \text{m}^{-2}$ of light intensity and were subcultured every 25 days. Wild type and transformed *Stevia rebaudiana* plantlets have been subcultured for 4 and 2 years, respectively.

Transformation of *S. rebaudiana*

The transformed *S. rebaudiana* plants were obtained by transformation mediated with *Agrobacterium rhizogenes* (strain K599) containing the binary plasmid pCAMBIA 1105.1, which includes the 35S promoter of the cauliflower mosaic virus. For the infection, hypocotyls of in vitro plantlets were punctured with an insulin needle previously submerged in a cellular suspension (0.5 OD at 600 nm) of

A. rhizogenes strain AR4. The infected material was maintained at the same conditions described in the previous section until the emergence of hairy roots from punctured sites, typically after 7 days. These roots were periodically subcultured in semisolid full MS medium added with cefotaxime (400 µg/ml) to eliminate the remaining bacteria (Calderón-Gabriel et al. 2016), and they were subsequently used to regenerate adventitious shoots putatively transformed in semisolid MS medium without growth regulators and with cefotaxime (100 g/l).

Confirmation of Genetic Transformation

For the confirmation of the genetic transformation of the plantlets, the extraction of genomic DNA (gDNA) was performed according to what was reported by Edwards et al. (1991). The gDNA was quantified by spectrophotometry at 260 nm, while its purity regarding proteins was calculated by the 260 nm/280 nm absorbance ratio. Subsequently, the samples were amplified using polymerase chain reaction (PCR). A fragment of the 35S promoter (250 bp) present in the transfer DNA (T-DNA) of the binary vector was amplified using DNA primers designed by the working group with the following sequence: direct (5'-gaactcgccgtaagactgg-3') and reverse initiator (5'-agc-caccttctctctttccact-3'). The PCR conditions used were the following: 94 °C for 10 min, followed by 35 cycles of 15 s at 94 °C, 15 s at 62 °C and 15 s at 72 °C; the amplification was finished with 10 min at 72 °C. The fragments were separated at 80 V for 1 hour in a horizontal electrophoretic chamber in 2% agarose gels and were later visualized by staining with ethidium bromide under ultraviolet light in a photodocumentation device. Binary vector plasmid DNA and gDNA from non-transformed plantlets were used as positive and negative control amplification, respectively.

Kinetics of Metabolite Accumulation and Growth Parameters in Wild and Transformed in Vitro Plantlets of *S. rebaudiana*

For the kinetic comparison of wild and transformed plantlets, a two-factor experimental design was used. Two groups of flasks containing 2-week-old plantlets were incubated under the conditions previously mentioned for 35 days. Samples were taken at 15, 20, 25, 30 and 35 days; three flasks were used for each of the following determinations: SG quantification, total phenols content, chlorophyll and carotenoids contents and growth parameters.

SG Extraction and Quantification

The in vitro plantlets were lyophilized and then mixed with a 30% (v/v) acetonitrile solution followed by sonication

treatment for 30 min at room temperature. During extraction, a biomass–solvent ratio of 20% (w/v) was maintained. The obtained extract was centrifuged at 14,000 rpm for 10 min, and the supernatant was placed in a clean tube.

The stevioside (St) and rebaudioside A (Reb A) contents were determined by HPLC–DAD using an Acquity Arc chromatographic system (Waters, MA, USA) following the report by Tada et al. (2013). The separation was carried out in isocratic mode with 32% acetonitrile and 10 mM phosphate buffer (pH 2.6), a flow of 1 mL/min, at a temperature of 30 °C, and in a 5 µm Luna C18 column (250 × 4.6 mm, Phenomenex). Chromatograms were monitored at wavelength of 210 nm, while identifying SG was conducted by comparing the UV absorption spectrum from 200 to 400 nm and the retention time of the standards: 7.6 min for Reb A (1432 Sigma Aldrich) and 8.08 min for St (S3572 Sigma Aldrich). The concentration of Reb A and St in the samples was determined using the calibration curves in the range of 25–500 g/mL.

Extraction and Quantification of Total Phenols

The samples of dry in vitro plantlet were macerated with 80% ethanol (v/v). During the extraction, a biomass–solvent ratio of 10% (w/v) was maintained. Subsequently, the samples were centrifuged at 14,000 rpm for 10 min, and the recovered extract was dried at 60 °C. To determine the total phenol content (TPC), the methodology described by López-Laredo et al. (2009) was followed. The extracts were resuspended in an ethanol: H₂O (80:20 v/v) mixture at a final concentration of 50 mg/mL. The reaction mixture consisted of 100 µL of extract, 100 µL of 1 N Folin–Ciocalteu reagent (F9252 Sigma Aldrich) and 500 µL of 20% sodium carbonate (Na₂CO₃/230952 Sigma Aldrich). This mixture was incubated for 30 min at room temperature, and the absorbance was read at 760 nm in a spectrophotometer (UV/VIS Optizen pop). The calibration curve was generated with gallic acid (C1251 Sigma Aldrich), and the TPC was expressed in microgram equivalents of gallic acid per milligrams of extract (µgGAE/mg ext).

Growth Parameters

To compare the growth parameters of both lines, samples were taken 15, 20, 25, 30 and 35 days after the plantlets were rooted.

The parameters of growth were as follows: the biomass of aerial parts (ps) and roots (pf), the number of leaves, leaf area, the plantlet length and the diameter of the stem. For the foliar area, one leaf was taken from the lower part, one leaf from the intermediate part, and one leaf from the upper part of ten in vitro plantlets that served as sample. Leaf area was analyzed and determined using ImageJ software.

Quantification of Chlorophyll and Carotenoids

The extraction and quantification of chlorophylls A and B, the total chlorophyll and the carotenoids were carried out according to the method described by Lichtenthaler and Wellburn (1983), which consisted of crushing 200 mg of fresh tissue in liquid nitrogen and homogenization with 3 mL of 96% ethanol (v/v). The sample was sonicated for 10 min at room temperature and protected from light. The mixture was centrifuged for 10 min at 4000 rpm. The supernatant was gauged to 5 mL with 96% ethanol (v/v), and the absorbances at 665 (chlorophyll A), 649 (chlorophyll B), 652 (total chlorophylls) and 470 (carotenoids) nm were measured in a spectrophotometer (UV/VIS Optizen pop).

The concentration (mg L^{-1}) of the chlorophylls A and B, A + B and the carotenoids was calculated using the following equations:

$$\text{Chlorophyll A} = 13.95 \times A_{665} - 6.88 \times A_{649}$$

$$\text{Chlorophyll B} = 24.96 \times A_{649} - 7.32 \times A_{665}$$

$$\text{Total chlorophyll} = 27.8 \times A_{652}$$

$$\text{Carotenoids} = 1000 \times A_{470} - 2.05 \times Ca - 114.8 \times Cb / 245$$

where **A** is the absorbance at the indicated wavelength, **Ca** is chlorophylls A and **Cb** is chlorophylls B. The concentration was also calculated in mg/g of fresh tissue using the following equation:

$$\text{mg/g} = C \times V / W$$

where **C** is the concentration (mg L^{-1}), **V** final volume (mL) and **W** weight of the sample (g).

Statistical Analysis

The analysis of variance for all the evaluated factors was carried out by applying a general linear model, while the means were compared using Tukey's multiple range test with $P < 0.05$ to define significance for both analyses using Minitab 16.

Results

Agrobacterium rhizogenes–Transformed *S. rebaudiana* Plantlets

Roots induced by *A. rhizogenes* (strain K599) were recovered one week after infection. After 15 days of growth in MS medium with cefotaxime (100 g/l) and free of growth regulators, some of the cultivated roots began to regenerate the aerial part of the plant producing

adventitious shoots. These shoots were allowed to develop for one more week until they were transferred for growth in semisolid MS medium where they finished the rooting process.

Verification of the Transformation of *S. rebaudiana* Plantlets

The regenerated plantlets were later analyzed by PCR to confirm the genetic transformation by searching for the 35S promoter (transferred by *A. rhizogenes*) in the genomic DNA. The amplification analysis was performed on the original plants and the micropropagated plantlets. Figure 1 shows an example of the recurrent PCR amplification for the plantlets analyzed. The amplification of a 250 bp band was observed, and the results agree with the expected size according to the primers used, which shows the insertion of the T-DNA of the binary vector and, consequently, the genetic transformation. In addition, note the band with a similar size in the lane with the plasmid and template DNA and the absence of amplification of gDNA from the non-transformed plants.

Production of Steviol Glycosides (SG) and Phenolic Compounds in Wild and Transformed Plantlets

The quantification of St and Reb A was carried out after 15 days of rooting the plantlets. St and Reb A accumulation was superior by 1.4- and 1.5-fold in comparison with transformed plantlet compared to the wild plantlet over the 20 days of kinetics. The maximum content of St and Reb A of the transformed plantlet was 4.26 ± 0.21 mg St/gDW and 5.36 ± 0.19 mg RebA/gDW, and that of wild plants was 3.22 ± 0.13 mg St/gDW and 3.85 ± 0.16 mg RebA/gDW (Fig. 2a, b). The accumulation of total phenolic contents (TPC) between the two lines was compared, and no significant difference was found (Fig. 3).

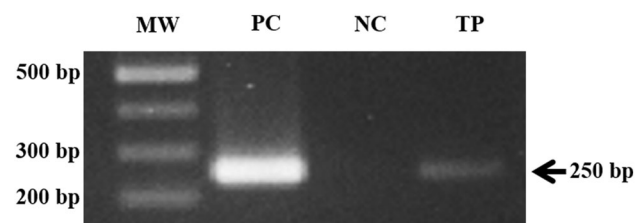


Fig. 1 PCR analysis of 35S promoter in genomic DNA from transformed plantlets. *MPM* molecular weight ladder, *PC* positive control of amplification, *NC* negative control of amplification, *PT* fragment of 35S promoter amplified from genomic DNA from transformed plantlets. The arrow indicates the expected size of amplicons

Fig. 2 Production of steviol glycosides, **a** stevioside and **b** rebaudioside A in wild-type (white square) and transformed (black square) plantlets of *S. rebaudiana*

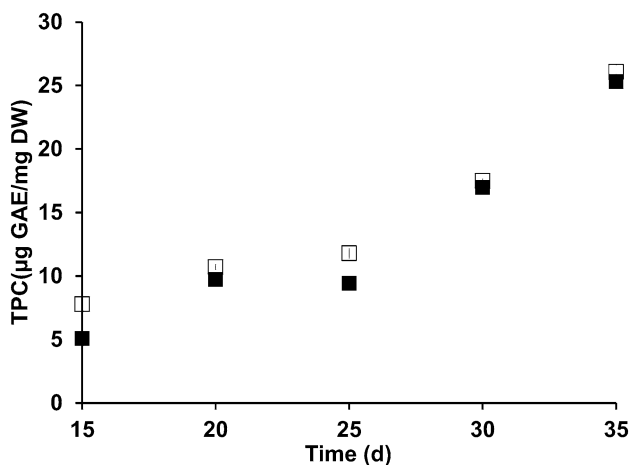
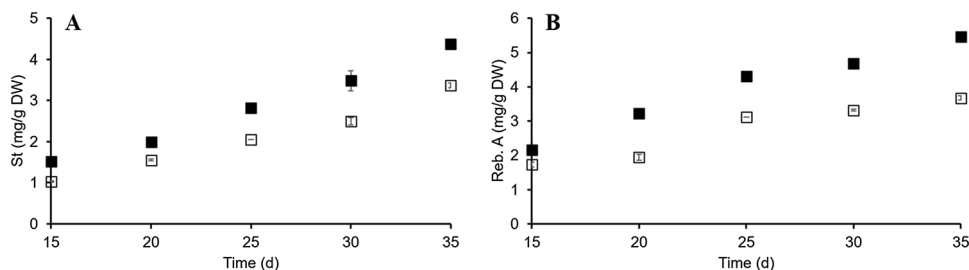


Fig. 3 Production of total phenolic contents (TPC) in wild-type (white square) and transformed (black square) plantlets of *S. rebaudiana*

Plantlet Growth of *S. rebaudiana*

The growth of the transformed plantlets was visibly superior compared to the wild plantlets (Fig. 4a, b). Growth parameters, such as the biomass of aerial parts and roots, the length, the stem diameter and the number of leaves, were significantly greater than the wild plants (Table 1),

where the aerial parts and root biomass at 35 days in the transformed plants was 52.9 ± 1.1 mg dry weight and 49.5 ± 1.3 mg fresh weight, which is an increase of 25 and 43%, respectively, compared with the wild plant. The final length of the plantlets was 16.45 ± 0.3 cm with 114.50 ± 08.01 leaves and a diameter of 1.55 mm, reflected as an increase of 14.2, 18.6 and 30%, respectively, in comparison with the wild plant. Table 2 summarizes the analysis of variance of the effect of the type of plantlet and the time of cultivation. Both factors significantly affected the different parameters of growth, morphology and production of SG. In addition, both factors significantly influenced the leaf area of the plants (Table 3).

The leaves of the transformed plantlets were larger and greener (Fig. 4c–g). The comparison of the lower, middle and upper parts of the leaf area of the plantlet showed that these leaves were significantly larger, between 20 and 30%, compared to wild plantlets, with a content of chlorophyll and carotenoids of 1.03 ± 0.019 and 0.16 ± 0.010 mg/gFW, respectively, which is 24 and 33% greater than the wild plant (Table 1).

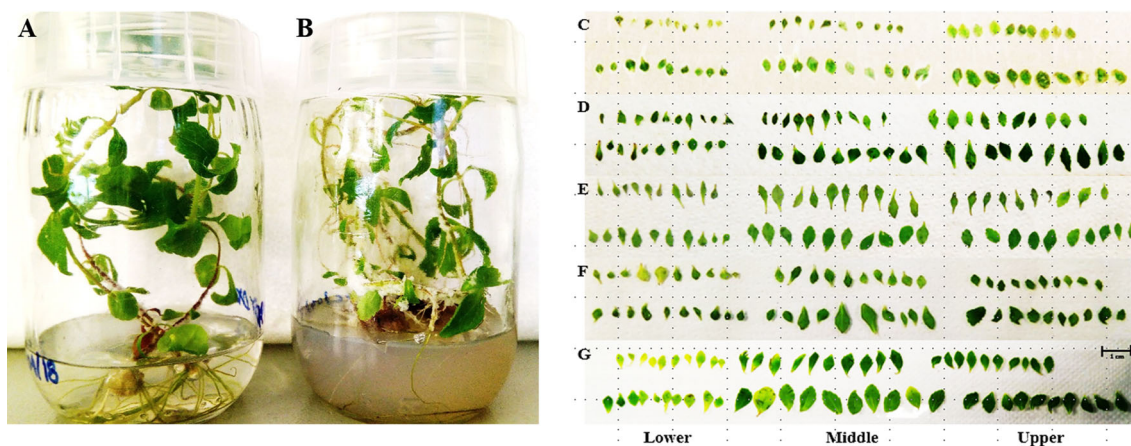


Fig. 4 **a** *S. rebaudiana* transformed plantlet growing in MS without exogenous growth regulators and **b** wild type growing in MS with 1 mg/ml BAP and 0.5 mg/ml IAA. Foliar area from lower, middle

and upper parts of wild-type and transformed plantlet of *S. rebaudiana* at **c** 15, **d** 20, **e** 25, **f** 30 and **g** 35 days after rooting

Table 1 Growth parameters of *S. rebaudiana* wild-type and transformed plantlets at 35 days in in vitro conditions

Growth parameters	<i>S. rebaudiana</i> wt	<i>S. Rebaudiana</i> transformed plantlets
Biomass of aerial part (mg DW)	42.1 ± 0.4	52.9 ± 1.1
Seedling length (cm)	14.4 ± 0.7	16.45 ± 0.3
Diameter of the stem (mm)	1.19 ± 0.02	1.55
Number of leaves per seedling	96.50 ± 0.4	114.50 ± 1.8
Biomass of roots (mgFW)	34.6 ± 2.1	49.5 ± 1.3
Leaf area from the lower part (cm ² /leaf)	0.19 ± 0.024	0.24 ± 0.013
Leaf area from the intermediate part (cm ² /leaf)	0.3 ± 0.016	0.4 ± 0.02
Leaf area from the upper part (cm ² /leaf)	0.25 ± 0.016	0.3 ± 0.017
Chl a + b content (mg/gFW)	0.83 ± 0.016	1.03 ± 0.019
Carotenoid content (mg/gFW)	0.12 ± 0.003	0.16 ± 0.010

Table 2 Resumed analysis of variance for growth parameters, SG production and TPC for wild-type and transformed plantlets of *S. rebaudiana* during 35 days of culture

Source of variation	Degree of freedom	(MS) mean of square											
		Biomass of aerial part (mg DW)	Number of leaves	Plantlets length (cm)	Diameter of the stem (mm)	Biomass of roots (mgFW)	Chl a content (mg/gFW)	Chl b content (mg/gFW)	Chl a + b content (mg/gFW)	Carotenoid content (mg/gFW)	TFC (µg GAE/mg DW)	St (mg/gDW)	Reb A (mg/gDW)
Plantlets	1	316.41	765.4	12.880	0.20301	223.78	1.03937	0.18739	1.3382	2.3491	4.70	2.7349	7.3055
Time	4	773.96	2463.6	86.291	0.27735	850.01	1.81076	0.39317	3.4580	1.8703	409.67	4.1517	4.6140
Error	20	0.39	11.6	0.24	0.00344	4.03	0.00061	0.00083	0.0020	0.0002	0.14	0.0292	0.0095
CV	–	0.51	0.35	0.51	0.24	0.66	0.38	0.39	0.52	0.37	0.50	0.39	0.36
R ²	–	0.99	0.98	0.99	0.97	0.98	0.99	0.99	0.99	0.99	0.99	0.98	0.99

Table 3 Resumed analysis of variance for leaf area from lower, intermediate and upper parts from wild-type and transformed plantlets of *S. rebaudiana* during 35 days of culture

Source of variation	Degree of freedom	(MS) mean of square		
		Leaf area from the lower part (cm ² /leaf)	Leaf area from the intermediate part (cm ² /leaf)	Leaf area from the upper part (cm ² /leaf)
Plantlets	1	0.026739	0.143187	0.093391
Time	4	0.078989	0.162544	0.089834
Error	90	0.001017	0.001196	0.000932
CV	–	0.48	0.39	0.39
R ²	–	0.79	0.88	0.84

Discussion

In this work, roots induced due to the infection of *A. rhizogenes* in *S. rebaudiana* demonstrated that it was possible to spontaneously regenerate adventitious shoots that were later propagated for the establishment of a line of transformed in vitro plantlets. The transformed in vitro plantlets

showed greater height and wider leaves compared to the non-transformed plantlets (Fig. 4) in addition to maintaining growth and production stability during the continuous subcultures. Also, these in vitro plantlets are considered homogeneous or nonchimeric, since they come from roots that originated from a single transformation event (Choi et al. 2004; Zhou et al. 2011). Plant

regeneration from transformed roots has been reported before using different species (Gunjan et al. 2013; Gurusamy et al. 2017), and it produces plants with normal or improved phenotypes in comparison with the wild type; this was also the case of the plants regenerated in this work (Table 1). Until now, the effect described above had not been observed in *Stevia rebaudiana*, although there are reports of direct and indirect regeneration from explants and calli transformed with *Agrobacterium tumefaciens* (Khan et al. 2014) and in applications where mutagenic agents, for instance ethyl methanesulfonate (EMS) and gamma radiation, directly on leaves were later used for direct regeneration of genetically modified plants (Khan et al. 2016). Unlike what was observed in our transformed in vitro plantlets, when EMS was used to induce mutations in *S. rebaudiana*, the plants exhibited a reduced height and wider leaves compared to the wild plants. The plants exposed to gamma radiation generally showed a greater size than those modified with EMS but were still smaller than the wild plants in addition to featuring narrower leaves. These events impacted both modification events in terms of the decrease in the foliar area compared to the wild plants (Khan et al. 2016). According to our observations, the transformation of *S. rebaudiana* mediated by *A. rhizogenes* did not negatively affect plant growth parameters, suggesting this as an appropriated strategy for applications in which the production of biomass is a desirable characteristic.

Particularly, in the case of regeneration of whole plants from transformed roots, the integration of T-DNA that is present in wild-type root-inducing plasmids (pRi) and in binary expression vectors favors the correct cell differentiation (David et al. 1984) often without the need for exogenous plant growth regulators, as observed in the current study (Fig. 4a), in addition to a lower prevalence of aberrant phenotypes, which is frequently observed in regeneration events from *A. tumefaciens* and other mutagenesis processes.

Conversely, both genetically modified plants (EMS and gamma rays), as well as *Agrobacterium*-transformed plants, have increased the production of SG compared to wild plants. Low doses of EMS increased St and Reb A by 1.5- and twofold, respectively. However, the use of gamma rays increased the content of Reb A twofold with a decrease in the St content compared to the control (Khan et al. 2016). Our transformed plantlets showed similar behaviors to the aforementioned: 1.4- and 1.5-fold greater St content and Reb A, respectively, as shown in Fig. 2a, b. Khan et al. (2014) observed that in transformed plantlets generated by direct organogenesis, there was an increase in the St and steviol content and that the clones generated by indirect organogenesis (S2) were able to accumulate greater St, while the rest of the clones kept a profile similar

to the mother plant. In contrast, in the case of regenerated plantlets via *A. rhizogenes*-mediated transformation (Fig. 2), the production profile of St and Reb A was similar in all the in vitro plantlets and maintained a similar proportion (Reb A/St) over the time evaluated. The increased accumulation of secondary metabolites can be attributed to the variation in the pattern and copy number of T-DNA integration within the genome of the host plant which can cause the expression of biosynthetic regulators (Amselem and Tepfer 1992; Bulgakov 2008). In addition, the induction effect of the secondary metabolism in Ri-transformed roots, plants and calli has been associated with the “rol effect,” due to the expression of *rol* oncogenes present in the Ri plasmid T-DNA (Matveeva et al. 2015), particularly the *rolB* gene (Bulgakov 2008), which has been detected in the genome of transformed *S. rebaudiana* in vitro plantlets (Fig. 1). The expression of *rolB* gene has been co-related to the enhanced secondary metabolite accumulation in transformed calli cultures and plants with respect to their non-transformed counterparts (Shkryl et al. 2007; Dilshad et al. 2016). Furthermore, it has been also reported that there is a synergistic effect over the secondary metabolism due to the simultaneous expression of the *rol* A, B, C and D genes (Bonhomme et al. 2000; Shkryl et al. 2007), similarly to the case of the currently transformed in vitro plantlets of *S. rebaudiana*.

Ladygin et al. (2008) showed a positive correlation between the development of plastid membranes and the photosynthetic capacity with an accumulation of SG, and this production was compared between plants grown in a greenhouse and in vitro, in vitro etiolated, green calli and etiolated. Those plants or cultures with chloroplasts and photosynthetic pigments showed a greater capacity to produce SG. Note that the in vitro transformed plantlets in the present work accumulated 24 and 33% more chlorophylls and carotenoids, respectively, compared to the wild plantlets suggesting a better development of chloroplasts in the transformed plantlets. Therefore, these plantlets were able to produce 1.36 times more SG than in vitro wild plantlets. The mutated plants developed by Khan et al. (2016) had a lower photosynthetic capacity that resulted in varying contents of the main accumulated SG among the lines, suggesting there was a negative effect on the chloroplast membrane structure, which is unlike the SG accumulation determined in our plantlets using genetic transformation mediated by *A. rhizogenes*. Libik-Konieczny et al. (2018) also observed a strong correlation between the efficiency of photosynthesis and the concentration of SG in lines of stevia plants grown in vivo, suggesting that these metabolites could participate in the protection of the photosynthetic apparatus against adverse environmental conditions.

In the present study, the spontaneous generation of adventitious shoots and transformed plantlets of *S. rebaudiana* from roots induced by *A. rhizogenes* without growth regulators was demonstrated. According to our results, in vitro plantlets, regenerated from *A. rhizogenes*-transformed roots, had a superior phenotype mainly reflected in greater leaf area and higher chlorophyll and carotenoid content compared to in vitro wild-type plantlets, which in turn are closely involved with higher SG accumulation. These plants have still important potential to improve their levels of SG production once they are cultivated *ex vitro* and increase their photochemical efficiency. This, besides the stability of production observed, remarks the potential of transformation of *S. rebaudiana* via *A. rhizogenes* as an interesting approach for the study of alternatives for SG production.

Acknowledgements The authors are grateful to the National Council of Science and Technology (Consejo Nacional de Ciencia y Tecnología—CONACyT) for scholarship No. 615648 to AJSC and to Catedras-CONACyT 3212 (235307) and INFR201501 (255514).

Author's Contributions A.J.S-C carried out laboratory work and data analysis. J.C-T contributed to the equipment and B.E.B-F edited the manuscript. P.M.S-O. and A.L-T. contributed to the data interpretation and statistical analysis. A.A.H-H. and E.G-L. designed and analyzed the experiments and wrote the manuscript. All authors reviewed and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Amsellem, J., and M. Tepfer. 1992. Molecular basis of novel root phenotypes induced by *Agrobacterium rhizogenes* A4 on cucumber. *Plant Molecular Biology* 19: 421–432.
- Anbazhagan, M., M. Kalpana, R. Rajendran, V. Natarajan, and D. Dhanavel. 2010. In vitro production of *Stevia rebaudiana* Bertoni. *Emirates Journal of Food Agriculture* 2: 216–222.
- Bayraktar, M., E. Naziri, I. Hakki, A. Fatih, K. Esra, I. Begum, A. Erdal, and B.A. Gurel. 2016. Elicitor induced stevioside production, in vitro shoot growth, and biomass accumulation in micropropagated *Stevia rebaudiana*. *Plant Cell, Tissue and Organ Culture* 127: 289–300.
- Bender, C., S. Graziano, and B.F. Zimmermann. 2015. Study of *Stevia rebaudiana* Bertoni antioxidant activities and cellular properties. *International Journal of Food Sciences and Nutrition* 66: 553–558.
- Bonhomme, V., D. Laurain-Mattar, and M.A. Fliniaux. 2000. Effects of the *rolC* gene on hairy root: Induction development and tropane alkaloid production by *Atropa belladonna*. *Journal of Natural Products* 63: 1249–1252.
- Bulgakov, V.P. 2008. Functions of *rol* genes in plant secondary metabolism. *Biotechnology Advances* 26: 318–324.
- Calderón-Gabriel, L., A. Jiménez-Brigada, A.A. Huerta-Heredia, J. Capataz-Tafur, and E. García-López. 2016. Effect of three strains of *Agrobacterium rhizogenes* and explant type on genetic transformation of *Stevia rebaudiana*. *Mexican Journal of Biotechnology* 1: 34–41.
- Choi, P.S., Y.D. Kim, K.M. Choi, H.J. Chung, D.W. Choi, and J.R. Liu. 2004. Plant regeneration from hairy-root cultures transformed by infection with *Agrobacterium rhizogenes* in *Catharanthus roseus*. *Plant Cell Reports* 22: 828–831.
- Christey, M.C. 2001. Use of Ri-mediated transformation for production of transgenic plants. *Vitro Cellular & Developmental Biology-Plant* 37: 687–700.
- David, C., M.D. Chilton, and J. Tempé. 1984. Conservation of T-DNA in plants regenerated from hairy root cultures. *Nature Biotechnology* 2: 73–76.
- Dilshad, E., H. Ismail, R.M. Cusido, J. Palazon, K. Ramirez-Estrada, and B. Mirza. 2016. *rol* genes enhance the biosynthesis of antioxidants in *Artemisia carvifolia* Buch. *BMC Plant Biology* 16: 125.
- Edwards, K., C. Johnstone, and C. Thompson. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research* 19: 1349.
- Fallah, F., F. Nokhasi, M. Ghaheri, D. Kahrizi, A. Beheshti Ale Agha, T. Ghorbani, E. Kazemi, and Z. Ansarypour. 2017. Effect of salinity on gene expression, morphological and biochemical characteristics of *Stevia rebaudiana* Bertoni under in vitro conditions. *Cellular and Molecular Biology* 63: 102–106.
- Fu, X., Z.P. Yin, J.G. Chen, X.C. Shangguan, X. Wang, Q.F. Zhang, and D.Y. Peng. 2015. Production of chlorogenic acid and its derivatives in hairy root cultures of *Stevia rebaudiana*. *Journal Agricultural and Food Chemistry* 63: 262–268.
- Gamboa, F., and M. Chaves. 2012. Antimicrobial potential of extracts from *Stevia rebaudiana* leaves against bacteria of importance in dental caries. *Acta Odontológica Latinoamericana* 25: 171–175.
- Ghorbani, T., D. Kahrizi, M. Saeidi, and I. Arji. 2017. Effect of sucrose concentrations on *Stevia rebaudiana* Bertoni tissue culture and gene expression. *Cellular and Molecular Biology* 63: 33–37.
- Gunjan, S.K., J. Lutz, A. Bushong, D.T. Rogers, and J. Littleton. 2013. Hairy root cultures and plant regeneration in *Solidago nemoralis* transformed with *Agrobacterium rhizogenes*. *American Journal of Plant Sciences* 4: 1675–1678.
- Gupta, E., S. Kaushik, S. Purwar, R. Sharma, A.K. Balapure, and S. Sundaram. 2017. Anticancer potential of steviol in MCF-7 human breast cancer cells. *Pharmacognosy Magazine* 13: 345–350.
- Gupta, E., S. Purwar, S. Sundaram, and G.K. Rai. 2013. Nutritional and therapeutic values of *Stevia rebaudiana*: A review. *Journal of Medicinal Plants Research* 7: 3343–3353.
- Gurusamy, P.D., H. Schäfer, S. Ramamoorthy, and M. Wink. 2017. Biologically active recombinant human erythropoietin expressed in hairy root cultures and regenerated plantlets of *Nicotiana tabacum* L. *PLoS ONE* 12 (8): e0182367.
- Ishizaki, T., Y. Hoshino, K. Masuda, and K. Oosawa. 2002. Explants of Ri-transformed hairy roots of spinach can develop embryogenic calli in the absence of gibberellic acid, an essential growth regulator for induction of embryogenesis from non-transformed roots. *Plant Science* 163: 223–231.
- Javed, R., A. Mohamed, B. Yücesan, E. Gurel, R. Kausar, and M. Zia. 2017a. CuO nanoparticles significantly influence in vitro culture, steviol glycosides, and antioxidant activities of *Stevia rebaudiana* Bertoni. *Plant Cell, Tissue and Organ Culture* 131: 611–620.
- Javed, R., M. Usman, B. Yücesan, M. Zia, and E. Gürel. 2017b. Effect of zinc oxide (ZnO) nanoparticles on physiology and steviol glycosides production in micropropagated shoots of *Stevia rebaudiana* Bertoni. *Plant Physiology and Biochemistry* 110: 94–99.

- Khan, S.A., L.U. Rahman, K. Shanker, and M. Singh. 2014. *Agrobacterium tumefaciens* mediated transgenic plant and somaclone production through direct and indirect regeneration from leaves in *Stevia rebaudiana* with their glycoside profile. *Protoplasma* 251: 661–670.
- Khan, S.A., L.U. Rahman, R. Verma, and K. Shanker. 2016. Physical and chemical mutagenesis in *Stevia rebaudiana*: Variant generation with higher UGT expression and glycosidic profile but with low photosynthetic capabilities. *Acta Physiologiae Plantarum* 38: 1–12.
- Ladygin, V.G., N.I. Bondarev, G.A. Semenova, A.A. Smolov, O.V. Reshetnyak, and A.M. Nosov. 2008. Chloroplast ultrastructure, photosynthetic apparatus activities and production of steviol glycosides in *Stevia rebaudiana* in vivo and in vitro. *Biologia Plantarum* 52: 9–16.
- Libik-Konieczny, M., E. Capecka, E. Kąkol, M. Dziurka, A. Grabowska-Joachimiak, E. Sliwinska, and L. Pistelli. 2018. Growth, development and steviol glycosides content in the relation to the photosynthetic activity of several *Stevia rebaudiana* Bertoni strains cultivated under temperate climate conditions. *Scientia Horticulturae* 234: 10–18.
- Lichtenthaler, H.K., and A.R. Wellburn. 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochemical Society Transactions* 11: 591–592.
- López-Laredo, A.R., F.D. Ramírez-Flores, G. Sepúlveda-Jiménez, and G. Trejo-Tapia. 2009. Comparison of metabolite levels in callus of *Tecoma stans* (L.) Juss. ex Kunth. cultured in photoperiod and darkness. *Vitro Cellular & Developmental Biology-Plant* 45: 550–558.
- Magangana, T.P., M.A. Stander, and N.P. Makunga. 2018. Effect of nitrogen and phosphate on *in vitro* growth and metabolite profiles of *Stevia rebaudiana* Bertoni (Asteraceae). *Plant Cell, Tissue and Organ Culture* 134: 141–151.
- Matveeva, T.V., S.V. Sokornova, and L.A. Lutova. 2015. Influence of *Agrobacterium* oncogenes on secondary metabolism of plants. *Phytochemistry Reviews* 14: 541–554.
- Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–497.
- Namdari, N., L. Shooshtari, and A. Qaderi. 2015. In vitro micropropagation of *Stevia rebaudiana* Bertoni. *Biological Forum—An International Journal* 7: 1750–1754.
- Pandey, H., P. Pandey, S.S. Pandey, S. Singh, and S. Banerjee. 2016. Meeting the challenge of stevioside production in the hairy roots of *Stevia rebaudiana* by probing the underlying process. *Plant Cell, Tissue and Organ Culture* 126: 511–521.
- Ramos-Tovar, E., E. Hernández-Aquino, S. Casas-Grajales, L.D. Buendia-Montaño, S. Galindo-Gómez, J. Camacho, V. Tsutsumi, and P. Muriel. 2018. Stevia prevents acute and chronic liver injury induced by carbon tetrachloride by blocking oxidative stress through Nrf2 upregulation. *Oxidative Medicine Cellular Longevity* 2018: 3823426. <https://doi.org/10.1155/2018/3823426>.
- Razak, U.N.A.A., C.B. Ong, T.S. Yu, and L.K. Lau. 2014. *In vitro* micropropagation of *Stevia rebaudiana* Bertoni in Malaysia. *Brazilian Archives of Biology Technology* 57: 23–28.
- Roychowdhury, D., A. Majumder, and S. Jha. 2013. *Agrobacterium rhizogenes*-mediated transformation in medicinal plants: Prospects and challenges. In *Biotechnology for medicinal plants: Micropropagation and improvement*, ed. S. Chandra, H. Lata, and A. Varma, 29–68. Berlin/Heidelberg: Springer.
- Ruiz-Ruiz, J.C., Y.B. Moguel-Ordoñez, and M.R. Segura-Campos. 2017. Biological activity of *Stevia rebaudiana* Bertoni and their relationship to health. *Critical Reviews in Food Science and Nutrition* 57: 2680–2690.
- Sarkar, S., I. Ghosh, D. Roychowdhury, and S. Jha. 2018. The Effects of *rol* genes of *Agrobacterium rhizogenes* on morphogenesis and secondary metabolite accumulation in medicinal plants. In *Biotechnological approaches for medicinal and aromatic plants*, ed. N. Kumar, 27–51. Singapore: Springer.
- Shkryl, Y.N., G.N. Veremeichik, V.P. Bulgakov, G.K. Tchernoded, N.P. Mischenko, S.A. Fedoreyev, and Y.N. Zhuravlev. 2007. Individual and combined effects of the *rolA*, *B* and *C* genes on anthraquinone production in *Rubia cordifolia* transformed calli. *Biotechnology and Bioengineering* 100: 118–125.
- Tada, A., K. Ishizuki, J. Iwamura, H. Mikami, Y. Hirao, I. Fujita, T. Yamazaki, H. Akiyama, and Y. Kawamura. 2013. Improvement of the assay method for steviol glycosides in the JECFA specifications. *American Journal of Analytical Chemistry* 4: 190–196.
- Tepfer, D. 1984. Genetic transformation of several species of higher plants by *Agrobacterium rhizogenes*: Phenotypic consequences and sexual transmission of transformed genotype and phenotype. *Cell* 37: 959–967.
- Tzifira, T., and V. Citovsky. 2006. *Agrobacterium*-mediated genetic transformation of plants: Biology and biotechnology. *Current Opinion in Biotechnology* 17: 147–154.
- Yadav, S.K., and P. Guleria. 2012. Steviol glycosides from Stevia: Biosynthesis pathway review and their application in foods and medicine. *Critical Reviews in Food Science and Nutrition* 52: 988–998.
- Yamazaki, T., H.E. Flores, K. Shimomura, and K. Yoshihira. 1991. Examination of steviol glucosides production by hairy root and shoot cultures of *Stevia rebaudiana*. *Journal of Natural Products* 54: 986–992.
- Yang, D.C., and Y.E. Choi. 2000. Production of transgenic plants via *Agrobacterium rhizogenes* mediated transformation of *Panax ginseng*. *Plant Cell Reports* 19: 491–496.
- Zhou, M.L., X.M. Zhu, J.R. Shao, Y.X. Tang, and Y.M. Wu. 2011. Production and metabolic engineering of bioactive substances in plant hairy root culture. *Applied Microbiology and Biotechnology* 90: 1229–1239.