Revista Brasileira de Farmacognosia 27 (2017) 34–39

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

Morpho-anatomical study of *Stevia rebaudiana* roots grown *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 16 May 2016
Accepted 14 August 2016
Available online 7 October 2016

Keywords:
Stevia rebaudiana
Adventitious root cultures
Morpho-anatomical
Chemical profile

ABSTRACT

Stevia rebaudiana (Bertoni) Bertoni, Asteraceae, is used as a food additive because its leaves are a source of steviol glycosides. There are examples of tissue culture based on micropropagation and phytochemical production of *S. rebaudiana* leaves but there are few studies on adventitious root culture of *S. rebaudiana*. More than 90% of the plants used in industry are harvested indiscriminately. In order to overcome this situation, the development of methodologies that employ biotechnology, such as root culture, provides suitable alternatives for the sustainable use of plants. The aim of this study was to compare morpho-anatomical transverse sections of *S. rebaudiana* roots grown *in vitro* and *in vivo*. The *in vitro* system used to maintain root cultures consisted of a gyratory shaker under dark and light conditions and a roller bottle system. Transverse sections of *S. rebaudiana* roots grown *in vitro* were structurally and morphologically different when compared to the control plant; roots artificially maintained in culture media can have their development affected by the degree of media aeration, sugar concentration, and light. GC–MS and TLC confirmed that *S. rebaudiana* roots grown *in vitro* have the ability to produce metabolites, which can be similar to those produced by wild plants.

Introduction

Stevia rebaudiana (Bertoni) Bertoni, Asteraceae (Asterales), has long been known to the Indians, who call it ka'a he'ê ("sweet herb"). *Stevia* is an erect, taproot and perennial herbaceous species that reaches up to 90 cm in height, with small leaves that measure from 3 to 5 cm in length and 1 to 1.5 cm in width, being simple and sessile on opposite and alternate vertices. The flowers are white and the petals are grouped in terminal or axillary racemes (Magalhães et al., 2000).

The herb is native of the Amambay region, in northeastern Paraguay, and is also found in Argentina and Brazil (Tavarini and Angelini, 2013). *Stevia* is known as the sweet herb of Paraguay, sweet leaf, candy leaf, and honey leaf (Soejarto, 2002; Brandle and Telmer, 2007; Madan et al., 2010).

The plant has been used commercially since the 1970s, when the Japanese developed processes to extract and refine stevioside from the leaves (Dacome et al., 2005). The major producers of *Stevia* are China and South Asia. Currently, there is no large-scale *Stevia* farming, however the *Stevia* market has been growing since the steviol glycosides extracted from the leaves were approved as a food additive (sweetener), in 2011 (Tavarini and Angelini, 2013).

There are more than 150 *Stevia* species, however only *S. rebaudiana* has significant sweetening properties, although other species contain chemicals of interest (Soejarto et al., 1982). There are few chemical studies available with regards to the roots. In the main phytochemical studies, the following substances were isolated: longipinene diesters, which were isolated from *S. lucida* (Guerra-Ramírez et al., 1998), *S. serrata* (Sánchez-Arreola et al., 1995), *S. porphyrea* (Sánchez-Arreola et al., 1999), and *S. vicida* (Román et al., 1995), and glycoside stevisalioside, which was isolated from *S. salicifolia* (Mata et al., 1992).

The development of methodologies that employ biotechnology, such as plant tissue culture, micropropagation, root culture, and transformed root culture represent an alternative approach to the search for secondary metabolites with specific properties, and they allow genetic stability to be maintained (Thiyagarajan and Venkatachalam, 2012). Thus, with the development of rapid

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http://dx.doi.org/10.1016/j.bjp.2016.08.007

root growth *in vitro* systems, it would be possible to obtain enough material for the production of extracts without the need for large agricultural areas and the destruction of nature, for the commercial production of compounds of interest.

Considering the promising results from previous studies on *S. rebaudiana* roots, reported by our research group (Reis et al., 2011; Oliveira et al., 2011; Lopes et al., 2015), the existence of few morpho-anatomical studies regarding *S. rebaudiana* roots, and the search for new ways of obtaining primary and secondary metabolites, *S. rebaudiana* roots *in vitro* represent a biotechnological alternative for obtaining these metabolites. Thus, the main aim of this study was to compare *S. rebaudiana* root (*in vitro* and *in vivo*) morpho-anatomical transverse sections and preliminary chemical analysis by GC–MS and TLC. The results of our study provide crucial information for both the optimization and technological development of adventitious roots of *S. rebaudiana* for the production of secondary and mainly primary metabolites in bioreactors.

Materials and methods

Plant material

Stevia rebaudiana Bertoni (Bertoni), Asteraceae, roots and shoots were collected at the Medicinal Plants Teaching Garden, at the State University of Maringa (HDPM-UEM). A voucher specimen (14301-HUEM) was deposited at the Herbarium of the State University of Maringa, Maringa, Brazil, and was identified by Jimi Nakajima (Universidade Federal de Uberlandia).

Seedlings grown in vitro: *S. rebaudiana* shoots were taken from plants from HDPM-UEM. Shoots were subjected to disinfection (Patrão et al., 2007) and transferred to MS media (Murashige and Skoog, 1962) and supplemented with 0.8% agar (w/v) and 30 g l^{-1} sucrose. Shoots were cultivated under light conditions, with a photoperiod of 14 h (45 μmol m^{-2} s^{-1}) at 25 ± 1 °C, obtaining seedlings.

In vitro adventitious roots: Seedlings were grown and their roots (with about 90 and 300 mg fresh weight) were removed and transferred to liquid MS media supplemented with 2.0 mg l^{-1} α-naphthalene acetic acid (NAA) and 30 g l^{-1} sucrose. Seedlings were also cultivated under dark and light conditions, at 25 ± 1 °C, in a gyratory shaker (90 rpm) and in a roller bottle system, under dark conditions (Reis et al., 2011).

Preparation of the extracts

Crude extracts from roots (control): Roots collected at the HDPM-UEM were dried in a circulating air drying oven at 45 °C for 15 days and powdered. The powder (200 g) was extracted using a Soxhlet extractor with 800 ml of hexane for 4 h. The procedure was repeated three times and the hexane extracts were concentrated under reduced pressure at 45–50 °C on a rotary evaporator, yielding 4.13 g of control crude hexane extract (CHEC).

Crude extract from roots grown in vitro in a gyratory shaker, under light and dark conditions: Roots of plants grown in the gyratory shaker were lyophilized after 42 days and subjected (5 g) to Soxhlet extraction using hexane, as described above. 255 mg of gyratory shaker crude hexane extract (CHES) were obtained from roots grown in a gyratory shaker, under light conditions. The same procedure was performed with roots grown in a gyratory shaker in the dark, yielding 230 mg of gyratory shaker crude hexane extract in the dark (CHESD).

Crude extract of in vitro roots grown in the roller bottle system: After 42 days of growth, roots were lyophilized and 5 g were subjected to the same Soxhlet extraction process, yielding 235 mg of roller bottle system crude hexane extract (CHEB).

TLC analysis

Thin-layer chromatography (TLC) was used to compare the chromatographic profiles of root extracts grown in different culture types and in the control (*in vivo*). Silica gel 60 GF254 (Merck®) was used for a stationary phase. Hexane extracts were analyzed using hexane:ethyl acetate 95:5 (v/v) as eluent. Visualization of substance spots on TLC plates was performed using UV light (λ: 254 and 366 nm) and also with 4% vanillin sulfuric acid, followed by heating at 150 °C for 2–4 min (Gibbons and Gray, 1998). Extracts were applied onto TLC plates in known concentrations: 10 mg CHEC, CHES, CHESD and CHEB dissolved in hexane (1 ml).

Morpho-anatomical analysis

Roots were fixed in Bouin solution (saturated picric acid:formaldehyde:glacial acetic acid 7:2:1, v/v/v) (Kraus and Arduin, 1997) and their fragments were kept in the fixative for 5 days. After this period, they were washed with 70% ethanol and put in 60% ethanol for 1 h for dehydration. Afterwards, they were kept in 70% ethanol for preservation.

The roots were embedded in acrylic hystoresin, in accordance with Gerrits (1991) and the manufacturer's guidelines. The embedded material was sectioned into transverse sections with a rotary microtome, and the sections were stained with 0.1% of blue toluidine (O'Brien et al., 1964). Slides were mounted in Permount®.

Illustrations were obtained *via* photomicrograph image capture using a Canon Power Shot A95 (Zoom Browser EX 4.6) digital camera, and illustration scales were obtained using a micrometer scale. The same optical conditions were used for each case.

Gas chromatography–mass spectrometry (GC–MS)

GC–MS analyses were performed using a gas chromatograph (Thermo Electron Focus) equipped with a TR5MSSQC fused

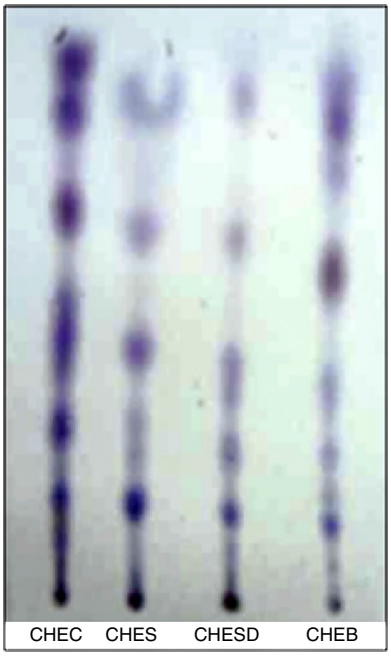

Fig. 1. Thin-layer chromatography of crude hexane extracts of *S. rebaudiana* roots: chromatographic system hexane:ethyl acetate 95:5 (v/v): CHEC, roots of plants grown at HDPM-UEM (control); CHES, roots grown in a gyratory shaker in the presence of light; CHESD, roots grown in a gyratory shaker in the dark; CHEB, roots cultivated in the roller bottle system.

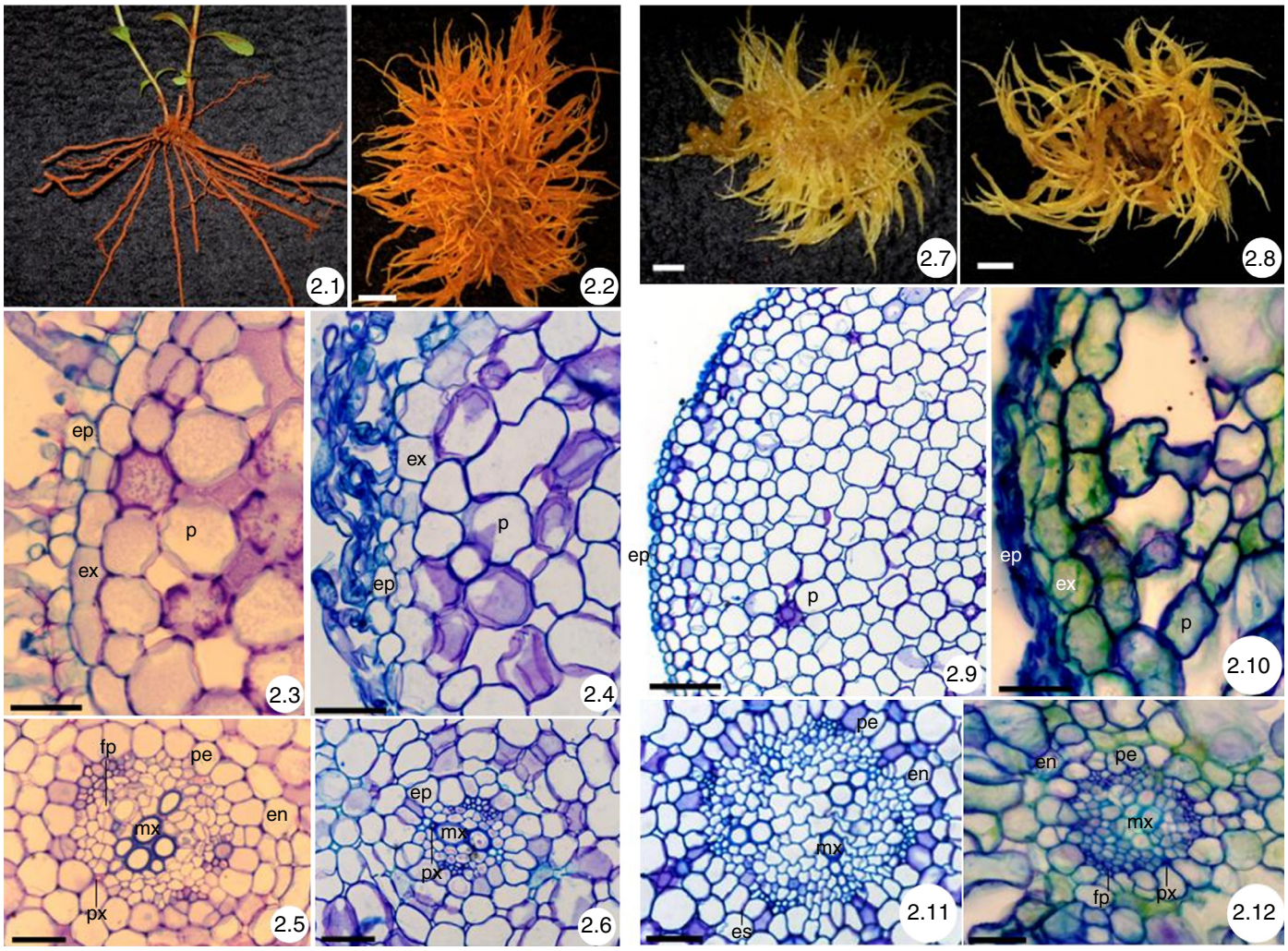

Fig. 2. *Stevia rebaudiana*. (A) (2.1) General aspect of the root specimen grown at HDPM-UEM (control). (2.2) General aspect of the root cultivated in the roller bottle system (bar – 1 cm). (2.3 and 2.5) Detail of the cortex and the central cylinder of the cross section of the root grown at HDPM-UEM. (2.4 and 2.6) Detail of the cross section of the root cultivated in the roller bottle system (bar – 50 μm) (en, endodermis; ep, epidermis; ex, exodermis; fp, primary phloem; mx, metaxylem; p, parenchyma; pe, pericycle; px, protoxylem). (B) (2.7) General aspect of the root grown in a rotary gyratory shaker in the dark. (2.8) General aspect of the root grown in a gyratory shaker in the presence of light (bar – 1 cm). (2.9 and 2.11) Detail of cortex and central cylinder of the cross section of the root grown in a gyratory shaker in the dark. (2.10 and 2.12) Detail of the cortex and central cylinder of the cross section of the root grown in a gyratory shaker in the presence of light (bar – 50 μm) (en, endodermis; ep, epidermis; es, Casparian strips; ex, exodermis; fp, primary phloem; mx, metaxylem; p, parenchyma; pe, pericycle; px, protoxylem).

silica capillary column (20 m × 0.25 mm, 0.25 μm film thickness) and interfaced with a Thermo DSQ-II mass spectrometer. The oven temperature was programmed with an initial temperature of 60 °C, held for 4 min and 60–220 °C at 4 °C min^{-1}, held for 28 min; an injector temperature of 250 °C; a transfer line temperature of 250 °C and an ion source temperature of 250 °C; helium as a carrier gas; a linear flux adjusted to 1 ml min^{-1}; a 1:30 split ratio; 70 eV ionization energy and full scan mode.

Results and discussion

Preliminary TLC analysis

Preliminary characterization of the major compounds present in the extracts was performed using the TLC of hexane extracts obtained from the *S. rebaudiana* roots grown at HDPM-UEM (CHEC) (control) and the roots grown *in vitro* (CHES, CHESD, CHEB). TLC qualitative analysis showed that roots grown *in vitro* have the ability to produce secondary metabolites and can be similar to those produced in parent plants (Fig. 1).

Morpho-anatomical study

The root system of *S. rebaudiana* (*in vivo*) grown at HDPM-UEM (control) is branched (Fig. 2.1), and the roots in the primary structure are cylindrical, as observed in the transverse section. The epidermis is uniseriate with unicellular hairs, a parenchymatous cortex with an exodermis and endodermis (Fig. 2.3), a central cylinder with a one layer pericycle, a xylem with two protoxylem poles (diarch root), and two phloematic strands (Fig. 2.5).

Roots grown *in vitro* showed morphological and anatomical alterations that were more significant in the gyratory shaker under dark and light conditions than for those grown in the roller bottle system. The roots cultivated in the roller bottle system have many secondary roots when compared to the control and it was not possible to distinguish the main or primary root (Fig. 2.2). The primary growth roots had a uniseriate epidermis with unicellular hairs, a parenchymatous cortex with an exodermis and endodermis (Fig. 2.4), a central cylinder with a one layer pericycle, a xylem with only one protoxylem pole, and phloematic strands (Fig. 2.6). In fact, a small reduction in the number of cells in the vascular tissue in the root system was confirmed.

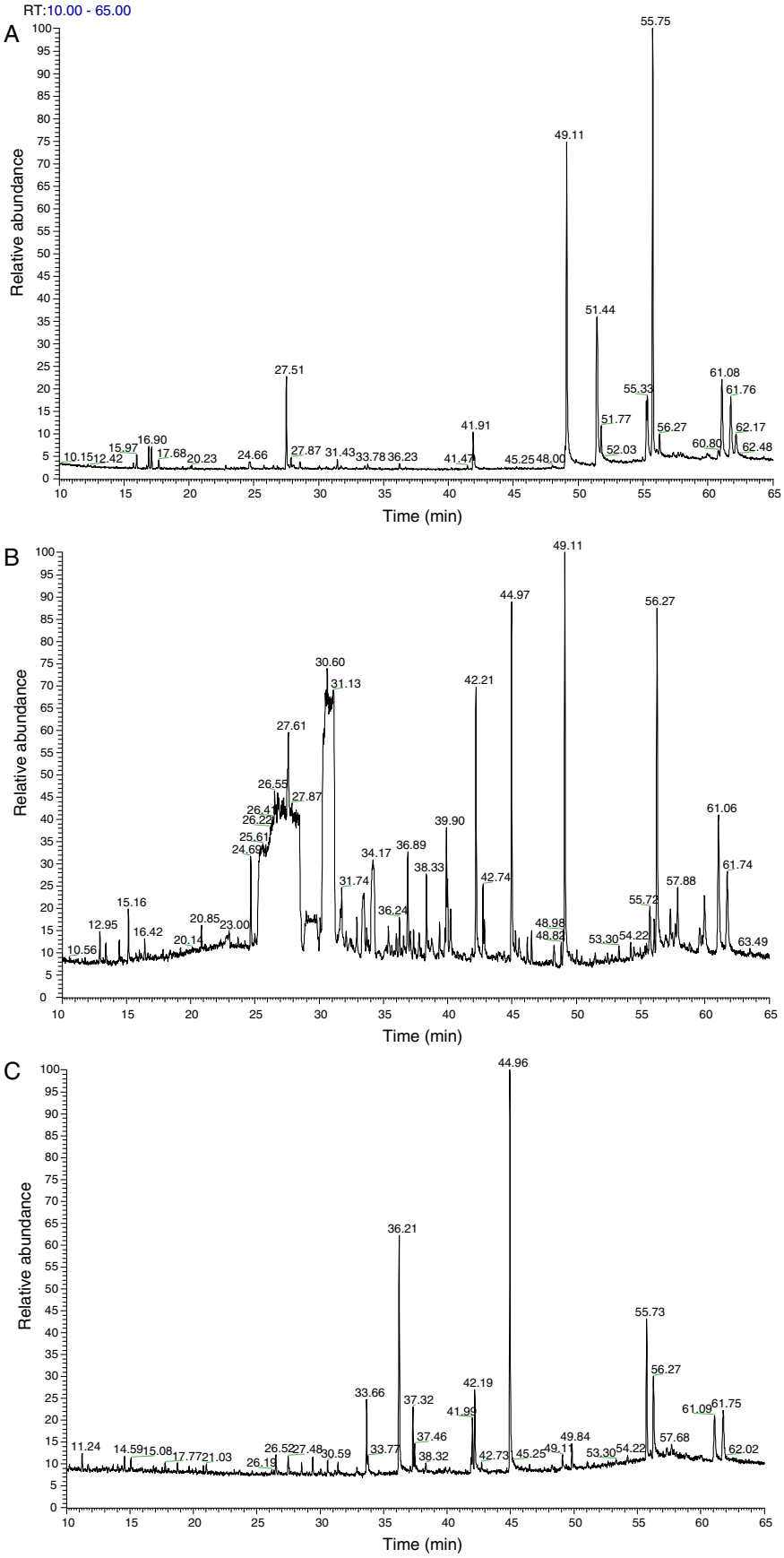

Fig. 3. GC–MS chromatogram of hexane extracts: (A) roots of plants grown at the HDPM-UEM (control); (B) roots grown in a gyratory shaker in the dark; (C) roots cultivated in the roller bottle system.

The roots had also branched in the gyratory shaker under dark conditions (Fig. 2.7), in the primary structure, and the epidermis was glabrous, comprising a layer of regular isodiametric cells (Fig. 2.9). The cortex was parenchymatous, with an exodermis and endodermis with Casparian strips (Fig. 2.11). The central cylinder showed a uniseriate pericycle and a vascular system consisting of four protoxylem poles and a root tetrarch, which is different from the control plant.

The root system was equally branched under light conditions (Fig. 2.8), and the roots showed a lower transverse section diameter, the exodermis and epidermis were slightly different, and the cortical parenchyma had large intercellular spaces (Fig. 2.10). The central cylinder showed a poorly developed vascular system, characterized by triarch root (Fig. 2.12).

The *in vitro* plants are subjected to an environment with high relative humidity and low light intensity. According to Wetzstein and Sommer (1982), these conditions could possibly lead to structural changes, which can be observed when comparing the transverse sections of the roots grown in a gyratory shaker under dark and light conditions. In the presence of light, the root is triarch and the cortical parenchyma showed large intercellular spaces, while in the absence of light, the roots are tetrarch and there are no intercellular spaces in the cortical parenchyma (Fig. 2.9 and 2.10).

According to Mayer et al. (2008), similar results were found for *Cymbidium* plants Hort. (Orchidaceae) *in vitro*, which also showed a less developed cortex with intercellular spaces, unlike plants grown in their natural environment. The vascular bundles of *S. rebaudiana* grown *in vitro* are less developed than those of the control plant, as was also noted for the vascular systems of *Rollinia mucosa* leaves (Albarello et al., 2001). Street and Mcgregor (1952) reported that roots artificially maintained in culture media could have their development affected by the degree of media aeration, sugar concentration, light, and other factors.

The modification of diarch to triarch root conditions noticed in the gyratory shaker was reported by Torrey (1955) for pea roots grown in culture media. Roots of *S. rebaudiana* grown in a gyratory shaker went from diarch (control) to triarch root in the presence of light, and from diarch (control) to tetrarch root in the absence of light.

Gas chromatography–mass spectrometry (GC–MS)

The GC–MS analyzes of hexane extracts (Fig. 3) show the chromatographic profile of HDPM (Fig. 3a) and the *in vitro* root cultures of *S. rebaudiana* (Fig. 3b and c). It is possible to observe that the *in vitro* root culture has a greater variety of compounds than the HDPM and that the hexane extracts from the gyratory shaker in the dark (Fig. 3b) have a higher diversity of compounds when compared to the chromatogram of roots grown in the roller bottle system. GC-MS analysis of the hexane extracts of HDPM (Fig. 3a) and the *in vitro* root cultures of *S. rebaudiana* (Fig. 3b and c) shows a complex mixture of compounds with typical fragmentation of longipinene derivatives (Sánchez-Arreola et al., 1999; Cerda-García-Rojas et al., 2006), the data for which is not shown. Similar results were obtained by Reis (2009), who isolated and established a longipinene derivative from *S. rebaudiana* roots grown at HDPM-UEM (CHEC) using spectroscopic and chemical methods.

Higher metabolite production variability may be the consequence of the culture conditions employed (Murthy et al., 2016) and/or morphological and anatomical alterations, which were more significant in the gyratory shaker (in the dark) than in the roller bottle system (Reis et al., 2011). Further phytochemical studies are necessary for the complete identification of these unknown compounds.

Conclusion

After comparing *S. rebaudiana* root cross sections, it was possible to report that roots grown *in vitro* are structurally and morphologically different when compared to control plants. Factors such as light, degree of aeration, nutrient concentration and others, which are in accordance with several reports in the literature, make *S. rebaudiana in vitro* roots different. Chromatographic profiling by TLC and GC–MS analysis showed that roots of plants grown *in vitro* have the ability to produce secondary metabolites, which can be similar to those produced by plants in nature (*in vivo*), but in a sustainable way.

Authors' contributions

TFOS and RVR (MSc students) carried out the laboratory work (preparing herbaria samples, chemical extraction, obtaining chromatograms, and plant anatomy studies). TPCC contributed to carrying out the root culture laboratory work and analysis. ALMA and LAS contributed in plant anatomy studies. JEG contributed to the chromatographic analysis. AJBO and RACG designed the study, supervised the laboratory work, and wrote the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors are grateful to the CNPq and CAPES, Brazilian funding agencies.

References

Albarello, N., Figueiredo, S.F.L., Viana, V.R.C., Neves, L.J., 2001. Anatomia foliar de *Rollinia mucosa* Jacq. Baill. (Annonaceae) sob condições de cultivo in vivo e in vitro. Rev. Bras. Plant Med. 4, 35–46.

Brandle, J.E., Telmer, P.G., 2007. Steviol glycoside biosynthesis. Phytochemistry 68, 1855–1863.

Cerda-García-Rojas, C.M., Guerra-Ramírez, D., Román-Marín, L.U., Hernández-Hernández, J.D., Joseph-Nathan, P., 2006. DFT molecular modeling and NMR conformational analysis of a new longipinenetriolone diester. J. Mol. Struct. 789, 37–42.

Dacome, A.S., Silva, C.C., Costa, S.C., Fontana, J.D., Costa, C.E., Adelmann, J., 2005. Sweet diterpenic glycosides balance of a new cultivar of *Stevia rebaudiana* (Bert.) Bertoni. Isolation and quantitative distribution by chromatographic, spectroscopic, and electrophoretic methods. Process Biochem. 40, 3587–3594.

Gerrits, P.O., 1991. The Application of Glycol Methacrylate in Histotechnology – Some Fundamental Principles. University Groningen, Netherlands.

Gibbons, S., Gray, A.I., 1998. Isolation by planar chromatography. In: Cannell, R.J.P. (Ed.), Methods in Biotechnology, Natural Products Isolation, vol. 4. Humana Press, New Jersey, pp. 209–245.

Guerra-Ramírez, D., Cerda-García-Rojas, C.M., Puentes, A.M., Joseph-Nathan, P., 1998. Longipinene diesters from *Stevia lucida*. Phytochem. Rev. 48, 151–154.

Kraus, J.E., Arduin, M., 1997. Manual básico de métodos em morfologia vegetal. Editora Universidade Rural, Seropédica.

Lopes, S.M.S., Krausov, G., Rada, V., Gonçalves, J.E., Gonçalves, R.A.C., Oliveira, A.J.B., 2015. Isolation and characterization of inulin with a high degree of polymerization from roots of *Stevia rebaudiana* (Bert.) Bertoni. Carbohydr. Res. 411, 15–21.

Madan, S., Ahmad, S., Singh, G.N., Kohli, K., Kumar, Y., Singh, R., Garg, M., 2010. *Stevia rebaudiana* (Bert.) Bertoni – a review. Indian J. Nat. Prod. 1, 267–286, IPC code; int.cl A61K 36/00, A23L 1/09.

Magalhães, P.M., de Martínez, A.J.V., Yesid, B.H., Cáceres, A., 2000. Agrotecnología para el cultivo de estévia o hierba dulce. In: CAB, CYTED (Eds.), Fundamentos de agrotecnologia de cultivo de plantas medicinales iberoamericanas. Colômbia, pp. 441–450.

Mata, R., Rodríguez, V., Pereda-Miranda, R., Kaneda, N., Kinghorn, A.D., 1992. Stevisalioside A, a novel bitter-tasting ent-antisene glycoside from the roots of *Stevia salicifolia*. J. Nat. Prod. 55, 660–666.

Mayer, J.L.S., Ribas, L.L.F., Bona, C., Quoirin, M., 2008. Anatomia comparada das folhas e raízes de *Cymbidium* Hort (Orchidaceae) cultivada *ex vitro* e *in vitro*. Acta Bot. Bras. 22, 323–332.

Murashige, T., Skoog, F.A., 1962. Revised medium for rapid growth and bio-assays with tobacco tissue culture. Physiol. Plant. 15, 473–497.
Murthy, H.N., Dandin, V.S., Paek, K.-Y., 2016. Tools for biotechnological production of useful phytochemicals from adventitious root cultures. Phytochem. Rev. 15, 129–145.
O'Brien, T.P., Feder, N., Mccully, M.E., 1964. Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59, 368–373.
Oliveira, A.J.B., Gonçalves, R.A.C., Chierrito, T.P.C., Santos, M.M., Souza, M.S., Gorin, P.A.J., Sassak, G.L.I., Iacomini, M., 2011. Structure and degree of polymerisation of fructooligosaccharides present in roots and leaves of *Stevia rebaudiana* (Bert.) Bertoni. Food Chem. 129, 305–311.
Patrão, A.P., Cunha, A.C., Celloto, V.R., de Souto, E.R., Gonçalves, R.A.C., de Oliveira, A.J.B., 2007. Comparação de diferentes metodologias para obtenção de cultura de calos de *Stevia rebaudiana* (Bertoni) Bertoni. Acta Sci. Health Sci. 29, 121–124.
Reis, R.V., Dissertação de Mestrado 2009. Estudo químico, avaliação da atividade biológica e comparação morfo-anatômica das raízes de *Stevia rebaudiana* (Bertoni) Bertoni. Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Estadual de Maringá, Maringá, pp. 83.
Reis, R.V., Borges, A.P.P.L., Chierrito, T.P.C., de Souto, E.R., de Souza, L.M., Iacomini, M., Gonçalves, R.A.C., 2011. Establishment of adventitious root culture of *Stevia rebaudiana* Bertoni in a roller bottle system. Plant Cell Tissue Organ Cult. 106, 329–335.
Román, L.U., Morán, G., Hernández, J.D., Cerda-García-Rojas, C.M., Joseph-Nathan, P., 1995. Longipinane derivatives from *Stevia viscida*. Phytochem. Rev. 38, 1437–1439.
Sánchez-Arreola, E., Cerda-García-Rojas, C.M., Joseph-Nathan, P., Román, L.U., Hernández, J.D., 1995. Longipinene derivatives from *Stevia serrata*. Phytochem. Rev. 39, 853–857.
Sánchez-Arreola, E., Cerda-Garcıá-Rojas, C.M., Román, L.U., Hernández, J.D., Joseph-Nathan, P., 1999. Longipinene derivatives from *Stevia porphyrea*. Phytochem. Rev. 52, 473–477.
Soejarto, D.D., 2002. Ethnobotany of *Stevia* and *Stevia rebaudiana*. In: inghorn, A.D. (Ed.), Stevia: The Genus *Stevia*. Taylor and Francis, London and New York, pp. 40–67.
Soejarto, D.D., Kinghorn, A.D., Farnsworth, N.R., 1982. Potencial sweetening agents of plant origin. III. Organoleptic evaluation of *Stevia* leaf herbarium samples for sweetness. J. Nat. Prod. 45, 590–599.
Street, H.E., Mcgregor, S.M., 1952. The carbohydrate nutrition of tomato roots. III. The effects of external sucrose concentration on the growth and anatomy of excided roots. Ann. Bot. London 16, 185–205.
Tavarini, S., 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. J. Sci. Food Agric. 93, 2121–2129.
Thiyagarajan, M., Venkatachalam, P., 2012. Large scale *in vitro* propagation of *Stevia rebaudiana* (Bert.) for commercial application: pharmaceutically important and antidiabetic medicinal herb. Ind. Crops Prod. 37, 111–117.
Torrey, J.G., 1955. On the determination of vascular patterns during tissue differentiation in excised pea roots. Am. J. Bot. 42, 183–198.
Wetzstein, H.Y., Sommer, H.E., 1982. Leaf anatomy of tissue-cultured *Liquidambar styraciflua* (Hamamelidaceae) during acclimatization. Am. J. Bot. 69, 1579–1586.