Revista Brasileira de Farmacognosia 25 (2015) 455–461

Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.sbfgnosia.org.br/revista

Original Article

Evaluation of limonoid production in suspension cell culture of *Citrus sinensis*

Elisângela Fumagali Gerolino[a], Talita Perez Cantuaria Chierrito[a], Arquimedes Santana Filho[b], Eliezer Rodrigues Souto[c], Regina Aparecida Correia Gonçalves[a], Arildo José Braz de Oliveira[a,*]

[a] *Programa de Pós-graduação em Ciências Farmaceuticas, Departamento de Farmacia, Universidade Estadual de Maringá, Maringá, PR, Brazil*
[b] *Laboratorio de Química de Carboidratos, Departamento de Bioquímica, Universidade Federal do Paraná, Curitiba, PR, Brazil*
[c] *Departamento de Agronomia, Universidade Estadual de Maringá, Maringá, PR, Brazil*

ARTICLE INFO

Article history:
Received 5 November 2014
Accepted 22 May 2015
Available online 23 June 2015

Keywords:
Citrus sinensis
Biotechnology
Limonoid aglycones
Limonin
Plant cell culture

ABSTRACT

The use of cell and plant tissue culture techniques to produce economically important active metabolites has been growing. Among these substances are total limonoid aglycones, which are produced by "pera" orange (*Citrus sinensis* (L.) Osbeck, Rutaceae) and have received considerable attention because of their anticancer actions. The main objective of the present study was to analyze and compare the levels of limonoid aglycones in seeds, callus cultures (originating from seeds), callus cultures (originating from hypocotyls), cell suspensions from hypocotyls cells, and cell suspensions from cotyledons. The cell cultures or *C. sinensis* were obtained by inoculating two strains of callus in MS medium supplemented with 2.0 μM 2,4-dichlorophenoxyacetic acid, 7.0 μM benzyl aminopurine, and 3% (w/v) sucrose in the dark. The highest concentrations of limonoid aglycone that were obtained were observed in cotyledon cell lines (240 mg/100 g dry weight) that were produced on day 21 of culture and hypocotyl cell lines on day 7 (210 mg/100 g dry weight). Explants of different origins under the same culture conditions had different limonoid aglycone content. The present results may suggest strategies for enhancing the productivity of biologically important limonoid aglycones and investigating the complex pathways of these secondary metabolites in plant tissue cultures.

Introduction

Biotechnology uses techniques and processes that involve living organisms to obtain specific products and/or modifications that increase the production of chemical substances of interest in less time and less capital investment (Davies and Deroles, 2014). Secondary metabolites that are found in plants are generally produced in low concentrations compared with primary metabolites. Therefore, different strategies, including *in vitro* culture systems, have been extensively studied to increase the production of secondary metabolites in plants (Smetanska, 2008; Muranaka and Saito, 2010; Gill et al., 2013). *In vitro* cell cultures represent an interesting alternative because secondary metabolites of interest are obtained in a controlled environment that is not influenced by changes in climate or soil conditions (Gonçalves and Romano, 2013; Collin, 2001; Fumagali et al., 2008). Plants that are grown in their natural habitat generally have varying concentrations of compounds of interest, depending on the particular crop season (Salmore and Hunter, 2001; Puricelli et al., 2002; Ralphs and Gardner, 2001). Moreover, their exploitation in their natural environment can cause gradual genetic erosion (Sidhu and Bel, 1996).

Citrus plants are grown worldwide. There are basically four commercial species of *Citrus*, among which *Citrus sinensis* (L.) Osbeck, Rutaceae (sweet orange) is notable. Various metabolites have been isolated from species of the *Citrus* genus. Among the major secondary metabolites are flavonoids and limonoids, and pectins are the primary metabolites (Khalil et al., 2002; Berhow et al., 2000; Okwu, 2008).

Limonoids are highly oxygenated modified triterpenes that are derived from a precursor with a 4,4,8-trimethyl-17-furanylsteroid skeleton that is biosynthesized by the acetate–mevalonate pathway in *Citrus* (Hasegawa and Hoagland, 1977). All naturally occurring *Citrus* limonoids contain a furan ring that is attached to a D-ring at C-17 and oxygen-containing functional groups at C-3, C-4, C-7, C-16, and C-17 (Fig. 1). Oxidative degradation on the C-17 side chain of either of these skeletons results in the loss of four carbon atoms and the formation of β-substituted furan. Further oxidation and skeletal rearrangements in one or more of the four

* Corresponding author.
E-mail: ajboliveira@uem.br (A.J.B. de Oliveira).

http://dx.doi.org/10.1016/j.bjp.2015.05.008
0102-695X/

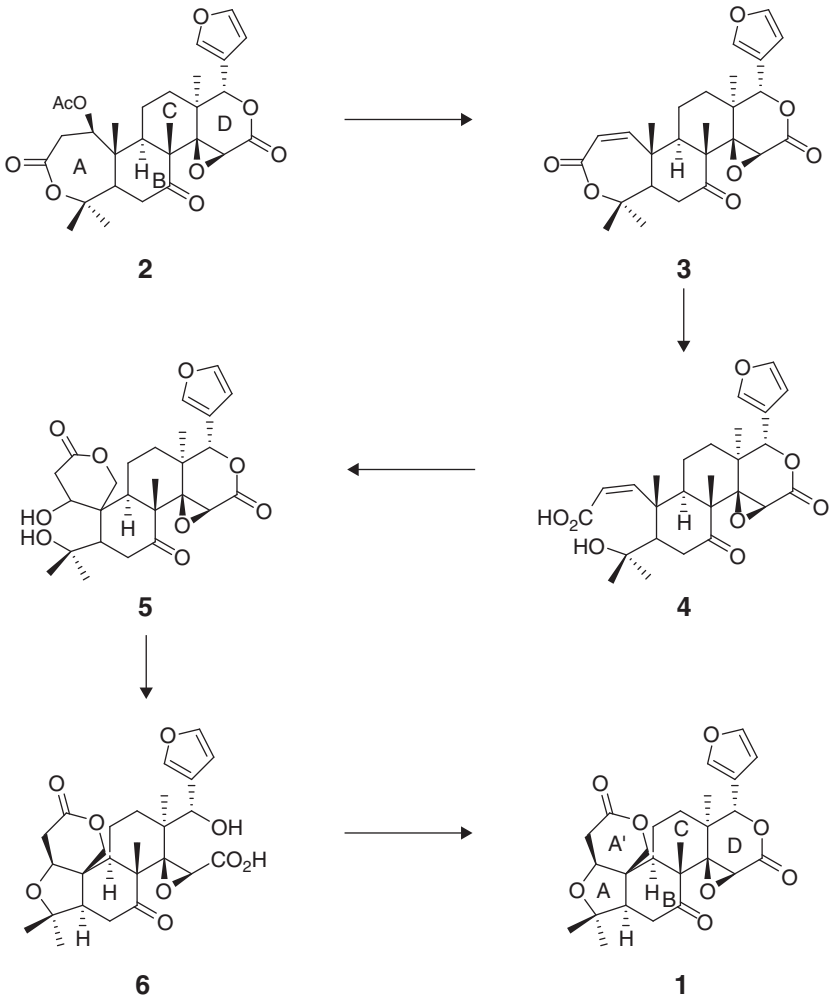


Fig. 1. Limonin biosynthesis pathway proposed by Breska et al. (2007).

rings (designated A, B, C, and D; Fig. 1) give rise to different skeletons of *Citrus* limonoids. Fig. 1 shows the biosynthetic pathway of limonin (**1**), the most abundant aglycone in *Citrus* species.

Limonoids have properties that inhibit the formation of chemically induced tumors in the mouth, stomach, small intestines, colon, lungs, and skin in experimental animals (Berhow et al., 2000). Another interesting property that has been described for limonoid aglycone is its antimalarial activity (Khalil et al., 2002).

Callus cultures and cells in suspension have been used to study the biosynthesis of economically important secondary metabolites (Gonçalves et al., 2010), enabling the propagation of cell lineages that contain alterations in biosynthetic capabilities.

The production of different compounds in plants is generally mediated by environmental factors that vary according to physiological conditions and seasonal variations (Gill et al., 2013). Thus, cell cultures ensure controlled conditions that circumvent environmental changes and allow us to guide, in a controlled manner, the synthesis of these compounds.

Only a few studies have utilized callus and/or cell cultures of *C. sinensis* to obtain secondary metabolites. For example, Niedz et al. (1987) sought to produce volatile compounds from callus cultures, and Endo et al. (2002) used a cell culture that was obtained from embryogenic callus to produce *Citrus* limonoids.

The purpose of the present study was to evaluate the *in vitro* production of limonoid aglycones using non-embryogenic callus cultures and cells in suspension that originated from cotyledons and hypocotyls of Brazilian "laranja-pêra" oranges (*Citrus sinensis*).

Material and methods

Instrumentation and general procedures

Absorption spectra in the infrared region were obtained on a KBr tablet in a Fourier transform infrared spectrometer (FTIR-Bomen, model MB-100C26). The analysis was performed on a disk composed of 1 mg of steeped samples with 250 mg KBr. The one-dimensional (1D)/two-dimensional (2D) ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained using a Varian Mercury Plus spectrometer at 300 MHz with $CDCl_3$ as the solvent and TMS as the internal standard. The spectra of high-resolution masses were obtained using Bruker Daltonics equipment (model micro TOF-QII-ESI-TOF). The plates that were used for analytical thin-layer chromatography (TLC) were 60 F_{254} aluminum silica gel sheets (Merck). The chromatoplates were developed using ultraviolet light (254 and 365 nm) and a sulfuric vanillin solution. The solvents (hexane, acetone, and acetonitrile) that were used for the preparation of the extracts and chromatographic fractionation were of analytical grade.

Plant material, callus cultures, and cells

Ripe seeds of *Citrus sinensis* (L.) Osbeck, Rutaceae, were purchased from local shops and sterilized in 0.5% (w/v) sodium hypochlorite solution for 15 min and 0.1% (w/v) Folicur for 15 min and then washed with sterile distilled water. Six units were then

inoculated in bottled MS culture (Murashige and Skoog, 1962) supplemented with 30 g sucrose, 2.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 7.0 μM kinetin, and 0.8% (w/v) agar and incubated at 28 °C in the dark to induce callus formation. After germination and seedling development, the hypocotyls and sterile cotyledons were used as explants. They were transferred to MS medium supplemented with 2.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 7.0 μM kinetin, and 0.8% (w/v) agar and then incubated at 28 °C in the dark to induce callus formation. Afterward, total limonoid aglycones were measured in the third-generation subculture, and the growth profile was studied in a liquid cell culture suspension.

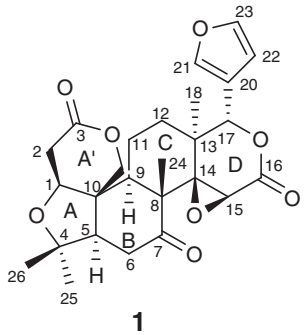
Establishment of cell cultures in suspension

Fresh callus (0.6 g) were transferred to a flask that contained 50 ml of MS liquid medium supplemented with 2.0 μM 2,4-D and 7.0 μM kinetin and then cultivated at 28 °C in the dark on an orbital shaker at 120 rotations per minute for four weeks. The suspended cells were removed every seven days (in triplicate), weighed (fresh weight), and frozen for subsequent lyophilization and verification of the dry weight at intervals of 7, 14, 21, 28, and 35 days of culture.

This procedure was performed for the two types of callus that were induced under laboratory conditions: callus that arose from hypocotyls and callus that arose from seeds.

Obtaining limonoid aglycones

The isolation of limonoid aglycones occurred after extracting seeds from the "laranja-pêra" orange (*C. sinensis*, 287.72 g), which were dried, crushed by turbolysis, and extracted, first under reflux in hexane (to eliminate oils) and then in acetone for 4 h to obtain limonoid aglycones. The acetone extract of the seeds (AES; 1.31 g) was concentrated and resuspended in acetonitrile and filtered. After evaporation of the solvent, we observed crystal formation. The sample was purified using preparative thin-layer chromatography (PTLC) using CH_2Cl_2:acetone (9:1, v/v) as the mobile phase in silica gel 60, which allowed isolation of the limonoid aglycone limonin (**1**). The structure of this limonoid was determined by 1D and 2D ^1H and ^{13}C NMR, mass spectrometry, infrared spectroscopy, and comparisons with the literature (Khalil et al., 2002): limonin (**1**): IV ν_{max} (KBr) 3488, 2986, 1758 (lactone), 1708 (C=O), 1391 cm^{-1}. The ^1H and ^{13}C NMR data were in agreement with the literature (Khalil et al., 2002; EM-IES 493,2071 [M+Na^+], $C_{26}H_{30}O_8$ (calculated mass, 470.19).



1

Extraction of total limonoid aglycones from seeds and callus and suspension cultures of C. sinensis

Callus and suspension cultures of *C. sinensis* were lyophilized and then subjected to extraction by reflux in acetone for 4 h (Ohta et al., 1993). The acetonic extracts were filtered, concentrated, and stored at −20 °C until analysis.

The seeds had previously undergone extraction by reflux in hexane to remove the oil and subsequently subjected to extraction by reflux in acetone to obtain limonoid aglycones extract (Ohta et al., 1993). After evaporation of the solvent, the mass was determined, and the samples were stored at −20 °C until analysis.

Quantification of total limonoid aglycones

Total limonoid aglycones were quantified in a spectrophotometer (Varian Cary 100 UV-VIS). After dilution of the extracts in acetonitrile and reaction with freshly prepared Erlich reagent (0.1 g 4-dimethyl-amino-benzaldehyde, 2.4 ml perchloric acid, and 3.0 ml acetic acid), the samples were homogenized and left undisturbed without stirring for 30 min. Readings were then performed at 503 nm (Breska and Ibarra, 2007).

Limonin (**1**) that was isolated from the seeds was used as the standard to construct the analytical (calibration) curve. From a 500 μg/ml stock solution in acetonitrile, 10–100 μg/ml of the solutions was prepared, and the analytical curve was constructed by linear regression.

The analyses of total limonoid aglycones were performed with the following samples: seeds, callus cultures (originating from seeds), callus cultures (originating from hypocotyls), cell suspension from hypocotyls cells, and cell suspension from cotyledons, which were obtained on days 7, 14, 21, 28, and 35 of culture after lyophilization to obtain the dry weight.

Statistical analysis

The data were analyzed by analysis of variance, and means were compared using Duncan's test and Origin Microcal 9.0 software. Values of $p < 0.05$ were considered statistically significant. For the determination of total limonoids, the results were based on the average of three independent tests with three replications each. The results are expressed as the mean ± standard deviation.

Results

From the acetone extract of the seeds, we used PTLC to isolate and subsequently identify limonoid aglycone (**1**), a procedure that was monitored by an analytical charge-coupled device. The extraction method that was proposed by Ohta et al. (1993) allows one to obtain an extract that is rich in limonoid aglycones and only traces of limonoid glucosides.

The mass spectra of compound **1** showed a molecular ion $[M+Na]^+$ at $m/z = 493.2171$, equivalent to the molecular formula $C_{26}H_{30}O_8$, and 12 degrees of unsaturation. The spectral data for compound **1** indicated the presence of two lactones, one ketone, four methyls, and one furan ring. The physical and spectral data (infrared spectroscopy, ^1H and ^{13}C NMR, DEPT, and 2D correlation techniques) shown in attachment that were consistent with those reported for limonin compound **1** in Khalil et al. (2002).

The determination of total limonoid aglycones was performed using the method that was developed by Breska and Ibarra (2007), which estimates total limonoid aglycone concentrations in samples of *Citrus* in limonin (**1**) equivalents.

Under the present conditions, the formation of a colorimetric complex was linear with increasing concentrations of limonin, even with concentrations that exceeded 100 μg/ml. The correlation coefficient (R^2) for the analytical curve was 0.9984 (Fig. 2).

Although both types of callus (cotyledons and hypocotyls) from different initial explants have been shown to be effective in initiating callus induction in *C. sinensis*, the callus that was obtained from cotyledons maintained good quality during successive subcultures. Cotyledons are the most suitable source of explants for

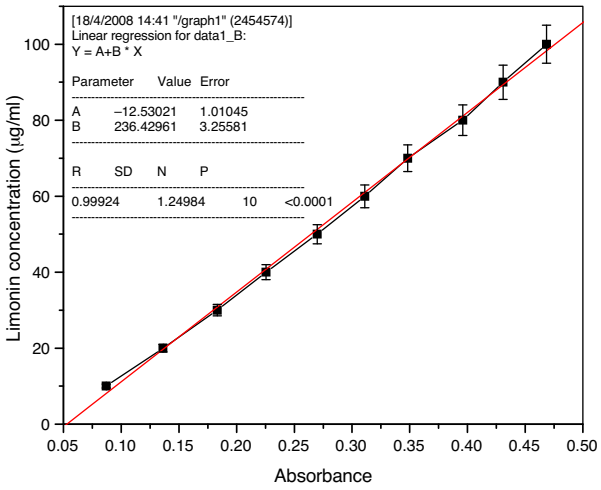


Fig. 2. Analytical curve for the quantification of total limonoid aglycones using limonin (**1**) as the standard. The standard of limonin (10–100 μg/ml) was dissolved in acetonitrile and reacted with a colorimetric reagent.

callus cultures when considering their ready availability and rate of multiplication (Fig. 3).

The determined quantity of limonoid aglycones in the extract of *C. sinensis* (Pera-Rio) seeds (Table 1) was higher than the one found in the seeds of other varieties of *Citrus sinensis*, including Valencia orange (213.6 mg/100 g), Ruby blood orange (219.3 mg/100 g), Queen orange (173.1 mg/100 g), and Jaffa orange (224.2 mg/100 g; Rouseff and Nagy, 1982).

Considering the growth rate and production of limonoid aglycones from the two cell lines in suspension, the resulting cells from cotyledons had a slower growth rate than the cells from hypocotyls but with higher concentrations of limonoid aglycones (Fig. 4).

A greater amount of limonoid aglycone (240 mg/100 g of cell dry weight) was accumulated in the callus cultures from cotyledons, but the difference was small compared with callus cultures from

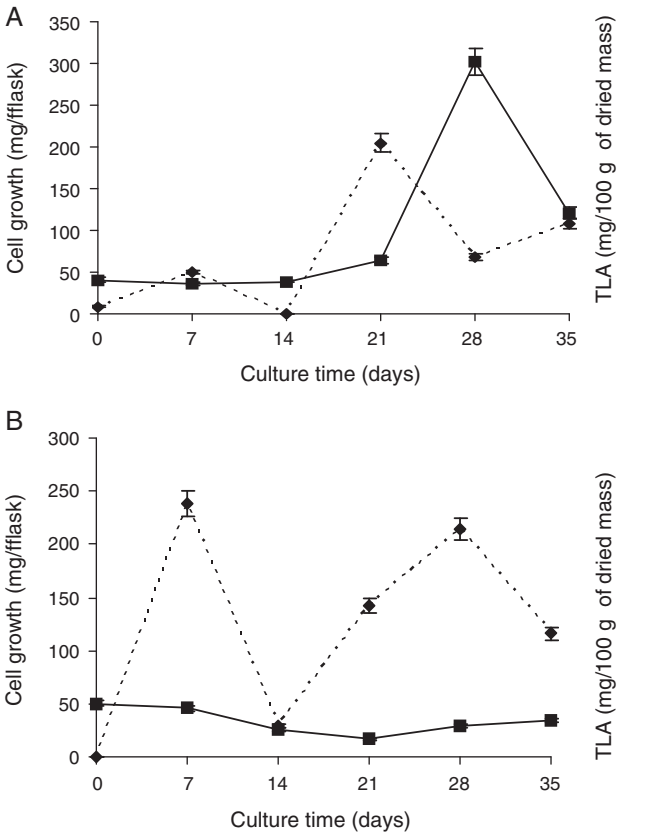


Fig. 4. Cell growth (mg, ——) and production of total limonoid aglycones (mg/100 g dry weight, ------) in callus culture in suspension of *Citrus sinensis* from: (A) hypocotyls and (B) cotyledons. Callus were grown in MS medium (Murashige and Skoog, 1962) supplemented with 2.0 μM 2,4-dichlorophenoxyacetic acid, 7.0 μM kinetin, and 3.0% (w/v) sucrose.

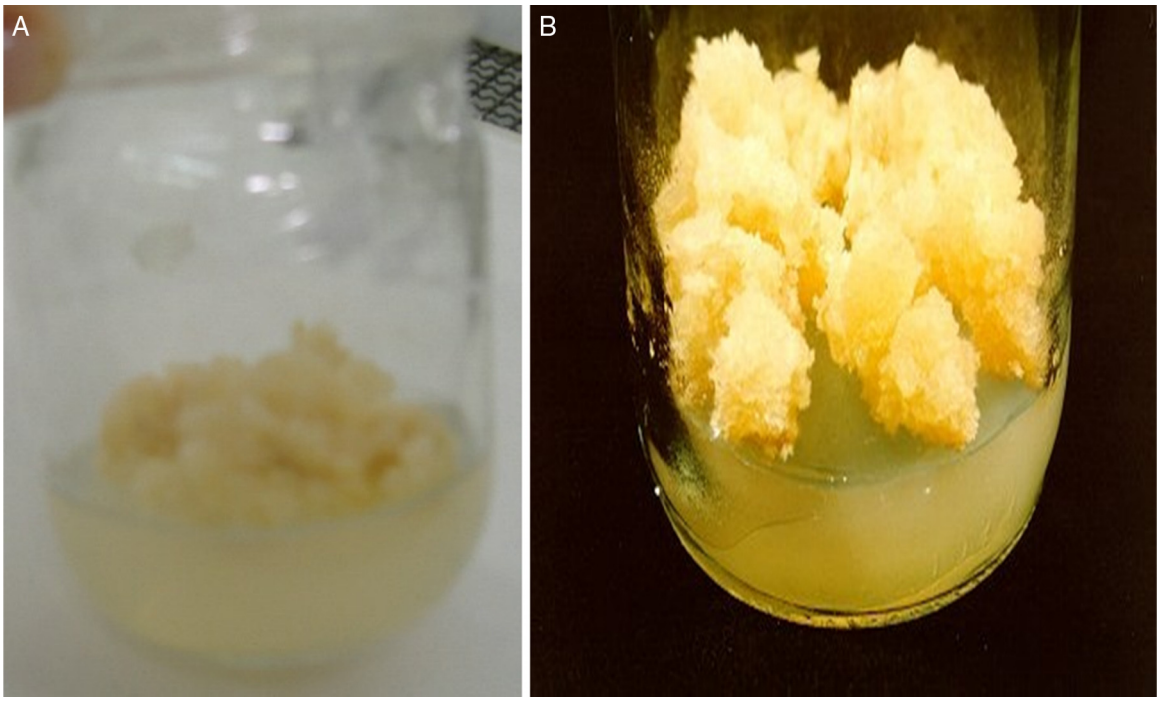


Fig. 3. Callus from *C. sinensis* cultivated in MS medium (Murashige and Skoog, 1962) supplemented with 2.0 μM 2,4-dichlorophenoxyacetic acid, 7.0 μM kinetin, 3.0% sucrose (w/v), and 0.8% (w/v) agar and incubated at 28 °C in the dark. (A) Callus obtained from hypocotyls. (B) Callus obtained from cotyledons.

Table 1
Content of total limonoid aglycones in seeds, callus and cells suspension culture of *Citrus sinensis*.

Sample[a]	LTA (mg/100 g dry weight)
Seeds	400 ± 7.0
Callus (cotyledons)	10.0 ± 0.5
Callus (hypocotyls)	200 ± 1.0
Cell suspension (hypocotyls)[b]	210 ± 3.0
Cell suspension (cotyledons)[b]	240 ± 5.0

[a] Analyzes were performed in triplicate.
[b] The values refer to the peak production of LTA on the growth curve.

hypocotyls (210 mg/100 g cell dry weight), even though this cell line has a greater growth rate.

Discussion

Limonin (**1**) was used as the standard for the spectrophotometric analysis to determine the levels of limonoid aglycones because it is the major component of *Citrus* species, the most abundant limonoid aglycone, and commercially available (Bilal et al., 2013). Furthermore, for most *Citrus* limonoids, the structure of BCD rings (Fig. 1) remains the same, whereas biosynthetic reactions are concentrated on the conversion of the A ring of nomalin (**2**) to the A–A′ two-ring structure of limonin (**1**) (Breska and Ibarra, 2007). Most of the other aglycones that have been isolated from *Citrus* were intermediates, with variations in the structure of the A ring, including obacunone (**3**) and ichangin (**5**) (Roy and Saraf, 2006).

Although this colorimetric method is not currently the most widely used for *Citrus* limonoid analyses (Tian et al., 2003; Vikram et al., 2007; Solemain et al., 2005), we chose this methodology to determine limonoid aglycone content because it does not require expensive equipment, thus increasing the potential for its application in the analysis of limonoid aglycones in callus cultures and cell suspensions of *Citrus* species.

The combinations of phytoregulators that were used in this study were similar to those used by Mansell and McIntosh (1991), who used a combination of 2,4-D and kinetin, which differ from Endo et al. (2002), who used only cytokinin.

The limonoid aglycones concentration was the highest in the *C. sinensis* seed extract compared with extracts of callus that were obtained from hypocotyl and cotyledon explants (Table 1).

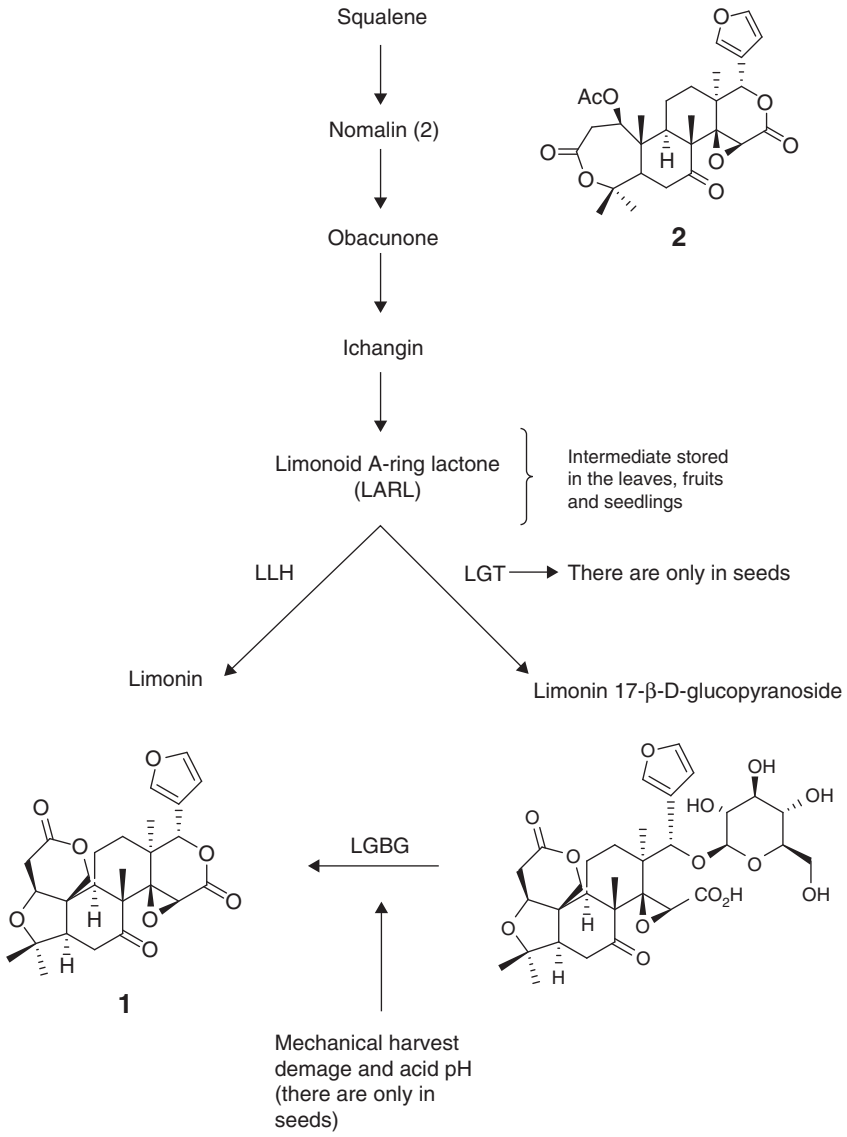


Fig. 5. Major enzymes involved in the keysteps of limonoid biosynthesis in *Citrus*. LGT, limonoid glucosyltransferase; LLH, limonoid D-ring lactone hydrolase; LGBG, limonoid glucoside β-glucosidase.

Importantly, callus from both hypocotyls and cotyledons were able to produce limonoid aglycones. However, callus from hypocotyls had a greater ability to biosynthesize limonoid aglycones. This increased biosynthesis of callus from hypocotyl explants can be explained by the fact that nomalin (**2**) is the most likely initial precursor of all known limonoids in *Citrus*. Nomalin (**2**) has been shown to be biosynthesized by the acetate–mevalonate pathway in the phloem region of the stem, which is present in the hypocotyl explants that are utilized for callus induction (Moriguchi et al., 2003). This precursor then migrates to other tissues, such as leaves, fruit tissues, and seeds, where other limonoids are biosynthesized independently (Fig. 5). This too can explain the smaller quantities of limonoid aglycones in the callus from seeds that require nomalin (**2**) to biosynthesize limonoid aglycones (Endo et al., 2002).

Plant cell suspensions cultures normally are initiated with callus culture lines that produce high amounts of interesting secondary metabolite, such as limonoids (Raval et al., 2003). Callus or cell cultures can be under catabolic repression, which prevents them from producing limonoids or causes them to produce limonoids in very small amounts. Under favorable conditions, however, they can produce limonoids again (Raval et al., 2003). The callus cultures from hypocotyls of *C. sinensis* exhibited this characteristic (Table 1). Thus, we also evaluated limonoid aglycone production in callus cultures from cotyledons and cell suspensions.

As shown in Fig. 4, both cultures had a lag phase of 14–21 days after inoculation, followed by a rapid growth phase that stabilized after 28 days of cultivation. In the callus culture that was obtained from hypocotyls, the levels of total limonoid aglycones increased, together with an increase in biomass. Thus, the concentrations of these compounds remained at constitutively low levels for the biosynthesis of these compounds under culture conditions (Mansell and McIntosh, 1991).

The suspension culture from cotyledons however showed a dramatic increase in limonoid aglycone concentrations on day 7, which may be attributable to the conversion of limonin-17-β-D-glucopyranoside (**7**) to limonin aglycone (**1**) by limonoid glucoside β-glucosidase (Fig. 5) that probably was induced by adaptation to suspension culture stress through a shear effect or aeration, mimicking the same conditions and damage that occur with mechanical harvest in orange juice processing or seed germination. This conversion of limonoid glucosides to limonoid aglycones occurred only in the seeds cells (Berhow et al., 2000).

In this first moment occurred a conversion of limonoid glycosides stocked to limonoid aglycones, showed by increment of TLA concentration from cotyledons cell line (on day 7), after the TLA concentration was drastically reduced but with measurable concentrations by colorimetric assay (day 14) as shown in Fig. 5. However in the hypocotyls cells suspension at day 14, the TLA concentration was reduced to a level below the detection limit of the assay (day 14).

The results suggest that de-differentiated callus tissues from cotyledons have a limited capacity to produce limonoid aglycones. However, callus from hypocotyls explants appeared to be more promising for this purpose. On the other hand when working with suspension cells lines that are obtained from cotyledons callus are the most appropriate because they have the capacity to produce TLA by direct biosynthesis and from hydrolyze of limonoid glucosides to TLA. Clearly, tissue cultures and cell suspensions are valuable tools for studying the biosynthesis of limonoid aglycones from *Citrus*. The analysis and evaluation of techniques that are used to suppress or enhance limonoid aglycones biosynthesis in plants are possible. The results that were obtained using these techniques, combined with molecular techniques, will aid in the development of practical uses of these cultures.

Authors' contributions

EFG (Ph.D. student) and ERS contributed to the running of the plant cell culture laboratory work and analysis. EFG and APSF contributed to MS, NMR and colorimetric date analysis and discussion. TCPC (undergraduate student) contributed to maintenance and cultivation of plant cell cultures and phytochemical work. AJBO wrote manuscript. RACG contributed to critical reading of the manuscript. AJBO and RACG designed the study, supervised the laboratory work and contributed to the critical reading of the manuscript. All the authors have read the final manuscript and approved submission. All authors read and approved the final manuscript submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

We thank CNPq and CAPES for financial support. We also thank Dr. Eliezer Rodrigues Souto, Department of Agronomy, State University of Maringá, Maringá, PR, Brazil, for allowing us to use his laboratory for the induction and maintenance of callus cultures of *C. sinensis*.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2015.05.008.

References

Bilal, H., Akram, W., Hassan, S.A., Sahar, S., Iqbal, M.M., 2013. Determination of limonin and nomilin contents in different citrus cultivars using high performance liquid chromatography. Pak. J. Sci. Ind. R. B 56, 36–40.

Berhow, M.A., Hasegawa, S., Manners, G.D., 2000. *Citrus limonoids*. Functional Chemicals in Agriculture and Food, 2000. American Chemical Society, Washington, DC, pp. 253–260.

Breska, A.P., Ibarra, P., 2007. Colorimetric method for the estimation of total limonoid aglycones and glucoside contents in *Citrus* juices. J. Agric. Food Chem. 13, 5013–5017.

Collin, H.A., 2001. Secondary product formation in plant tissue cultures. Plant Growth Regul. 34, 119–134.

Davies, K.M., Deroles, S.C., 2014. Prospects for the use of plant cell cultures in food biotechnology. Curr. Opin. Biotechnol. 26, 133–140.

Endo, T., Kita, M., Shimada, T., Moryguchi, T., Hidaka, T., Matsumoto, R., Hasegawa, S., Omura, M., 2002. Modification of limonoid metabolism in suspension cell cultures of *Citrus*. Plant Biotechnol. 19, 397–403.

Fumagali, E., Gonçalves, R.A.C., Machado, M.F.P.S., Vidoti, G.J., Oliveira, A.J.B., 2008. Produção de metabólitos secundários em cultura de células e tecidos de plantas: O exemplo dos gêneros *Tabernaemontana* e *Aspidosperma*. Rev. Bras. Farmacogn. 18, 627–641.

Gill, S.S., Anjum, N.A., Hasanuzzaman, M., Gil, R., Trivedi, D.K., Ahmad, I., Pereira, E., Tuteja, N., 2013. Glutathione and glutathione reductase: a boon in disguise for plant abiotic stress defense operations. Plant Physiol. Biochem. 70, 204–212.

Gonçalves, S., Romano, A., 2013. *In vitro* culture of lavenders (*Lavandula* spp.) and the production of secondary metabolites. Biotechnol. Adv. 31, 166–174.

Gonçalves, R.A.C., Cunha, A.C., Oliveira, J.B.O., Machado, M.F.P., Santos, R.A.M., 2010. Aplicações da cultura de células e tecidos de plantas em reações de biotransformação. In: Marsaioli, A.J., Porto, A.L.M. (Eds.), Biocatálise e Biotransformação: Fundamentos e Aplicações. Editora Schoba, São Paulo, pp. 102–157, 2010.

Hasegawa, S., Hoagland, J.E., 1977. Biosynthesis of limonoids in *Citrus*. Phytochemistry 16, 469–471.

Khalil, A.T., Maatooq, G.T., El Sayed, K.A., 2002. Limonoids from *Citrus reticulate*. Z. Naturforsch. 58c, 165–170.

Mansell, R.L., McIntosh, C.A., 1991. *Citrus* spp. Vitro Culture the Production of Naringin Limonin. In: Bajaj, Y.P.S. (Ed.), Medicinal and Aromatic Plants III, Biotechnology in Agriculture and Forestry, vol. 15. Spring-Verlag, Berlin, Heildelberg, pp. 193–210.

Moriguchi, T., Kita, M., Hasegawa, S., Omura, M., 2003. Molecular approach to citrus flavonoid and limonoid biosynthesis. J. Food Agric. Environ. 1, 22–25.

Murashige, T., Skoog, F.A., 1962. Revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Muranaka, T., Saito, K., 2010. Production of pharmaceuticals by plant tissue cultures. In: Mander, L., Liu, H.W. (Eds.), Comprehensive Natural Products II: Chemistry and Biology, vol. 3. Elsevier, Oxford, pp. 615–628.

Niedz, R.P., Moshonas, M.G., Peterson, B., Shapiro, J.P., Shaw, P.E., 1987. Analysis of sweet orange (*Citrus sinensis* (L.) Osbeck) callus cultures for volatile compounds by gas chromatography with mass selective detector. Plant Cell Tissue Org. 51, 181–185.

Ohta, H., Fong, C.H., Berhow, M., Hasegawa, S., 1993. Thin-layer and high performance liquid chromatographic analyses of limonoids and limonoid glucosides in *Citrus* seeds. J. Chromatogr. 639, 295–302.

Okwu, D.E., 2008. Citrus fruits: a rich source of phytochemical and their roles in human health. Int. J. Chem. Sci. 6, 451–471.

Puricelli, L., Innocenti, G., Delle Monache, G., Caniato, R., Filippini, R., Cappelletti, E.M., 2002. In vivo and in vitro production of alkaloids by *Haplophyllum patavinum*. Nat. Prod. Lett. 16, 95–100.

Raval, K.N., Hellwig, S., Prakash, G., Ramos-Plasencia, A., Srivastava, A., Büchs, J., 2003. Stage process for the production of azadirachtin-related limonoids in suspension culture of *Azadirachta indica*. J. Biosci. Bioeng. 96, 16–22.

Ralphs, M.H., Gardner, D.R., 2001. Distribution of norditerpene alkaloids in tall larkspur plant parts through the growing season. Biochem. Syst. Ecol. 29, 117–124.

Rouseff, R.L., Nagy, S., 1982. Distribution of limonoids in *Citrus* seeds. Phytochemistry 21, 85–90.

Roy, A., Saraf, S., 2006. Limonoids: overview of significant bioactive triterpenes distributed in plants kingdom. Biol. Pharm. Bull. 29, 191–201.

Salmore, A.K., Hunter, M.D., 2001. Environmental and genotypic influences on isoquinoline alkaloid content in *Sanguinaria canadensis*. J. Chem. Ecol. 27, 1713–1747.

Sidhu, O.P., Bel, H.M., 1996. Seasonal variation in azaradirachtin in seeds of *Azadirachta indica*. Curr. Sci. 70, 1084–1085.

Solemain, A., Parvin, Z., Esmaeil, M., 2005. Quantification of limonin in Iranian orange juice concentrates using high-performance liquid chromatography and spectrophotometric methods. Eur. Food Res. Technol. 221, 202–207.

Smetanska, I., 2008. Production of secondary metabolites using plant cell cultures. Adv. Biochem. Eng. Biotechnol. 111, 187–228.

Tian, Q., Li, D., Barbacci, D., Schwartz, S.J., Patil, B.S., 2003. Electron ionization mass spectrometry of citrus limonoids. Rapid Commun. Mass Espectrom., 2517–2522.

Vikram, A., Jayaprakasha, G.K., Patil, B.S., 2007. Simultaneous determination of citrus limonoids aglycones and glucosides by high performance liquid chromatography. Anal. Chem. Acta 590, 180–186.