Identification and quantitative determination of feruloyl-glucoside from hairy root cultures of *Turbinicarpus lophophoroides* (Werderm.) Buxb. & Backeb. (Cactaceae)

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Abstract

Transformed hairy root cultures have become an alternative for the biosynthesis of plant secondary metabolites with biological activities. In this present work, the effects of liquid Murashige and Skoog (MS) and Gamborg B5 (B5) medium on kinetic behavior, biomass and phenolic metabolite production were analyzed in *Turbinicarpus lophophoroides* (Werderm.) Buxb. & Backeb. hairy root cultures. Liquid MS medium showed the highest biomass production (13.67 g L^{-1} dry weight) after 77 d of culture. For B5 medium, highest biomass was achieved sooner, at day 56, but with lower total biomass (8.10 g L^{-1} dry weight). After isolation, structural elucidation of the major compound present in *T. lophophoroides* hairy roots was determined by nuclear magnetic resonance and mass spectral analysis. As a result, a ferulic acid derivative (feruloyl-glucoside) was isolated from *T. lophophoroides* hairy roots and reported for the first time. Quantitative analysis indicated that feruloyl-glucoside was the major phenolic metabolite at 56 d of growth in MS medium (2.7267 ± 0.041 mg g^{-1} dry weight L^{-1}) and at 7 and 35 d in B5 medium (2.6328 ± 0.108 and 2.4372 ± 0.026 mg g^{-1} dry weight L^{-1}, respectively). The feruloyl-glucoside was not detected in untransformed roots (control). The present results suggested the potential of *T. lophophoroides* hairy roots culture for the production of this phenolic glycoside.

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Introduction

Plant secondary metabolites play an important role in plant defense mechanisms (Madala *et al.* 2014) and represent a source of bioactive molecules (Ludwig-Muller *et al.* 2014). Phenolic glycosides, such as feruloyl-glucoside or its derivates, are constituents of commonly consumed fruits (Lucini *et al.* 2017) and their occurrence has been reported in different sources, such as *Malus* spp. (apple; Pérez-Ilzarbe *et al.* 1991), *Cucumis sativus* L. (cucumber; Abu-Reidah *et al.* 2012), *Opuntia ficus-indica* (L.) Mill. fruits (prickly pear; Kim *et al.* 2016), *Brassica* spp. (Lin and Harnly 2010) and *Vitis vinifera* L. (grape vine; Kovács and Dinya 2000), with proposed functional properties as anti-diabetic, hepatoprotective (Tang *et al.* 2017) and cosmetic whitening agents (Tanimoto *et al.* 2006).

Different cactus species have proven to be valuable sources of plant secondary metabolites (Astello-García *et al.* 2015). *Turbinicarpus lophophoroides* (Werderm.) Buxb. & Backeb.

is a small globular cactus endemic to Mexico that contains alkaloids (Štarha *et al*. 1999) and has been overexploited mainly for ornamental purposes (Smith *et al*. 2013; Villaseñor 2016). *Turbinicarpus lophophoroides* is a slow-growing plant in its natural habitat and is subjected to special protection by the Mexican normativity (NOM-059-SEMARNAT-2010; Semarnat 2010). Thus, the development of *in vitro* cultures by asexual propagation of *T. lophophoroides* has enormous potential, since high-yielding biomass production of cacti may be achieved in shorter periods of time and with minimal space requirements (Pérez-Molphe-Balch *et al*. 2015). *In vitro* culture *via* the generation of transformed hairy roots is a suitable alternative for increased biomass accumulation and improved plant secondary metabolite production through biotechnological approaches and has advantages such as (a) hormone-free medium for root formation, since hairy roots synthesize their own growth factors; (b) faster growth rate compared with that of plants grown *in situ*; (c) biochemical and genetic stability; (d) facilitation of basic research on hairy root-mediated secondary metabolite production coupled with productivity enhancement strategies; and (e) transformed hairy roots-based functional research (Georgiev *et al*. 2007; Georgiev *et al*. 2012; Mehrotra *et al*. 2015).

As part of ongoing efforts towards the conservation and preservation of cactus genetic resources and sustainable management, investigations by the current authors have focused on several aspects of *T. lophophoroides* biotechnology, including *in vitro* conservation of germplasm, induction of transformed hairy roots (Palomeque-Carlín *et al*. 2015; Pérez-Molphe-Balch *et al*. 2015) and phytochemical characterization. The research presented here identified and quantitatively determined the main compound present in hairy root cultures compared with non-transformed roots derived from *in vitro* plants of *T. lophophoroides* and investigated the effect of medium composition on changes in biomass accumulation and feruloyl-glucoside levels during the growth cycle, in order to increase and deepen the knowledge of transformed hairy roots of cacti and to explore their potential applications.

Materials and Methods

Plant material Transformed hairy root cultures of *T. lophophoroides* were previously established by Palomeque-Carlín *et al*. (2015). The root tip segments (1 cm in length) were cultured on Murashige and Skoog (MS; Murashige and Skoog 1962) basal medium (Sigma-Aldrich®, St. Louis, MO) supplemented with 30 g L^{-1} sucrose and 10 g L^{-1} agar (plant cell culture tested; Sigma-Aldrich®), with a final pH of 5.7 adjusted with 1 N NaOH (Thermo Fisher Scientific®, Waltham, MA) and then sterilized by autoclaving at 121°C for 15 min. The hairy root cultures were grown under fluorescent light (General Electric,

Boston, MA) of 40 μmol m^{-2} s^{-1} light intensity with a 16-h photoperiod and a temperature of $25 \pm 2°C$. Non-transformed roots and shoots obtained from *in vitro* plants were grown under the same conditions until enough biomass was obtained (at 273 d) and used as control.

Biomass and phenolic compound accumulation in different culture media For transformed hairy root culture optimization experiments, 0.05 or 0.30 g of root tip segments was transferred into 200-mL Erlenmeyer flasks (narrow neck; DWK Life Sciences GmbH, Mainz, Germany) containing 70 mL liquid MS or Gamborg B5 (B5; Gamborg *et al*. 1968) basal medium (Sigma-Aldrich®), supplemented with 30 g L^{-1} or 20 g L^{-1} sucrose, respectively, and covered with aluminum foil. Each flask was maintained on a rotary shaker at 80 rpm (Sev-Prendo, Puebla City, México) at $25 \pm 2°C$ in dark conditions for 13 wk. For kinetic evaluation, three random samples of independent experiments were collected each week to measure biomass fresh weight (FW) and dry weight (DW). Collected biomass was freeze-dried (FreeZone 4.5; Labconco Corporation, Kansas City, MO) and stored at − 20°C for further analysis.

Nutrient uptake evaluation Nutrient consumption in the medium was evaluated by means of total sucrose concentration determination by the phenol-sulfuric spectrophotometric method proposed by Dubois *et al*. (1956). A standard curve was prepared with sucrose (0, 10, 20, 30, 40, 50, 60 and 70 μg mL^{-1}). Aliquots of 2 mL were taken, and then, 0.5 mL of 5% (v/v) phenol solution (Sigma-Aldrich®) and 2.5 mL of concentrated sulfuric acid (Thermo Fisher Scientific®) were added. After 30 min of reaction, the absorbance of each sample ($n = 3$) was monitored at 490 nm with a Jenway Genova spectrophotometer (Cole-Parmer Instrument Co., Vernon Hills, IL). Additionally, the electric conductivity and pH (Waterproof tester; Hanna® Instruments, Smithfield, RI) of the medium were measured.

Determination of kinetic parameters of *T. lophophoroides* hairy root cultures and phenolic compound accumulation pattern The growth curve of *T. lophophoroides* hairy root cultures was established according to the change in FW and DW each week during the 13-wk growth cycle as described above. Kinetic parameters such as growth rate (μ), duplication time (t_d) and growth index (GI) were calculated as proposed by Gómez-Aguirre *et al*. (2012). The growth rate equation was as follows: $\mu = \ln (X_E \div X_0) \div \Delta t$, where X_0 and X_E are the amounts of root biomass at the beginning and the end of the culture period interval (g L^{-1}), Δt is the culture time interval (days) and μ is the specific growth rate (day^{-1}); duplication time was calculated as follows: $t_d = \ln 2 \div \mu$, where t_d is the doubling time (d). The growth index was calculated as follows: $GI = (X_F − X_0) \div X_0$, where X_F and X_0 are the final and initial root biomass, respectively.

For phenolic compound accumulation analysis, intermediate points of each growth phase were selected. Methanolic extracts were prepared with root samples collected at 21, 56 and 91 d for liquid MS medium and at 7, 35 and 84 d for liquid B5 medium and then, the total content of the major phenolic metabolite was purified and analyzed using chromatographic and spectroscopic approaches, and quantified by high-performance liquid chromatography (HPLC) analysis (see below).

Sample preparation, isolation, and identification of feruloyl-glucoside For phytochemical analysis, *T. lophophoroides* hairy roots growing in MS medium were propagated for 56 d, when the exponential growth phase was achieved (45.25 g DW). Otherwise, non-transformed roots and shoots growing for 273 d were harvested (70 mg DW and 186 mg DW, respectively). All these samples were freeze-dried and then extracted three times with methanol (Thermo Fisher Scientific®), 5 mL of MeOH to 1 g of dried tissue at 60°C for 10 min according to Wagner and Bladt (1996). The resultant extract was concentrated to dryness by rotary evaporation (Heidolph Instruments GmbH & Co. KG., Schwabach, Germany) under reduced pressure (0.3 to 0.5 kPa) at 45°C (Trejo-Moreno *et al.* 2018) and then, the obtained methanolic crude extract (11.2 g DW) was fractionated by preparative Silica Gel 60, Kieselgel 0.063 to 0.200 mm, 70 to 230 mesh ASTM column chromatography (Merck KGaA, Darmstadt, Germany) using a gradient elution system composed of dichloromethane:methanol (100:00 ➔ 00:100). Collected fractions were analyzed by thin-layer chromatography (TLC). Aliquots of each fraction were spotted onto silica gel 60 F_{254} and silica gel 60 RP18 F_{254}S aluminum plates (Merck) and eluted with dichloromethane:methanol (9:1) and water:acetonitrile (7:3), respectively. Plates were examined by ultraviolet (UV) fluorescence (365 nm) and then sprayed with cerium (IV) sulfate solution 0.1 N (Merck) and heated at 105°C for 1 to 2 min (Wagner and Bladt, 1996). The main collected fraction (fraction 22; 35.6 mg DW) was further analyzed using nuclear magnetic resonance (NMR) and mass spectrometry for qualitative analysis of the main chemical entity.

Qualitative analysis of feruloyl-glucoside The main fraction (fraction 22) obtained after preparative chromatography was identified by means of mass spectrometry and NMR analyses. Mass spectroscopic analysis was carried out using an ultra-performance liquid chromatography-electrospray ionization-mass spectrometry (UPLC-ESI-MS) system (UPLC™ ACQUITY-Z-spray™ ESI-APCI-ESCi®; Waters Corp., Milford, MA) equipped with an Acquity UPLC™ BEH C18 column (2.1 mm × 50 mm i.d., 1.7-μm particle size; Waters Corp.) and MassLynx™ (Waters Corp.) software. The mobile phase consisted of 0.05% (v/v) trifluoroacetic acid (Sigma-Aldrich®) aqueous solution (solvent A) and acetonitrile (Merck) (solvent B). The gradient system was as follows: 0 to 4 min, 0% B; 4 to 5 min, 30% B; 5 to 6 min, 50% B; 6 to 8 min, 100% B. The flow rate was maintained at 0.3 mL min^{-1} and the injection volume was 5 μL. The UPLC-ESI-MS analysis was performed in negative ion mode. Finally, the major compound was identified by NMR analysis.

The conformational structure of the main detected metabolite was further elucidated by ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), and two-dimensional hetero-nuclear multiple bond correlation (HMBC) spectroscopy (400 MHz) using a Varian Mercury Plus 400 MHz—ID3 spectrometer (Varian Inc., Palo Alto, CA) and acetone d$_6$:dimethyl sulfoxide-d$_6$ (Sigma-Aldrich®) (95:05) as solvent. Chemical shifts were described in parts per million (ppm) relative to tetramethylsilane (TMS; Sigma-Aldrich®) as internal standard. The resulting spectra were compared with reported data (Kim *et al.* 2016).

Quantitative HPLC analysis of feruloyl-glucoside at different growth phases of *T. lophophoroides* hairy roots For quantitative analysis of feruloyl-glucoside at intermediate points of each growth phase of *T. lophophoroides* hairy roots cultures and *in vitro* non-transformed shoot and root tissues, a high-performance liquid chromatography (HPLC) method using a Waters 2695 separation module equipped with a Waters 996 photodiode array detector and Empower™ Pro software (Waters Corp.) was developed. A Supelcosil LC-F column (4.6 mm × 250 mm i.d., 5-μm particle size) (Sigma-Aldrich®) was used and the mobile phase consisted of 0.5% (v/v) trifluoroacetic acid aqueous solution (solvent A) and acetonitrile (solvent B). The gradient system was as follows: 0 to 1 min, 0% B; 2 to 3 min, 5% B; 4 to 20 min, 30% B; 21 to 23 min, 50% B; 24 to 25 min, 80% B; 26 to 27 100% B; 28 to 30 min, 0% B. The flow rate was maintained at 0.9 mL min^{-1} and the injection volume was 10 μL. The absorbance was measured at 330 nm. The retention time for the most abundant peak (peak 1) was 8.968 min (λ = 242, and 323 nm) and the concentration of this polyphenolic compound was estimated by interpolation of the peak areas and comparison with a calibration curve prepared with feruloyl-glucoside isolated of *T. lophophoroides* as described above. The calibration curve was linear in the range of 31.25 to 250 μg mL^{-1} feruloyl-glucoside in methanol ($y = 35944\times - 278037$; $R^2 = 0.9971$). Feruloyl-glucoside was identified by comparison of the retention times and UV spectra in each sample. All analyses were performed in triplicate and the data were expressed as mean values in milligram per gram of DW of sample.

Statistical analyses Data are expressed as the mean ± standard deviation of three independent experiments. One-way analysis of variance (ANOVA) and Brown-Forsythe tests were used for multiple comparisons among means. The significance

level for all statistical tests was 5%. Statistical analyses were performed with Prism 7 for Mac OS X (GraphPad Software Inc., San Diego, CA).
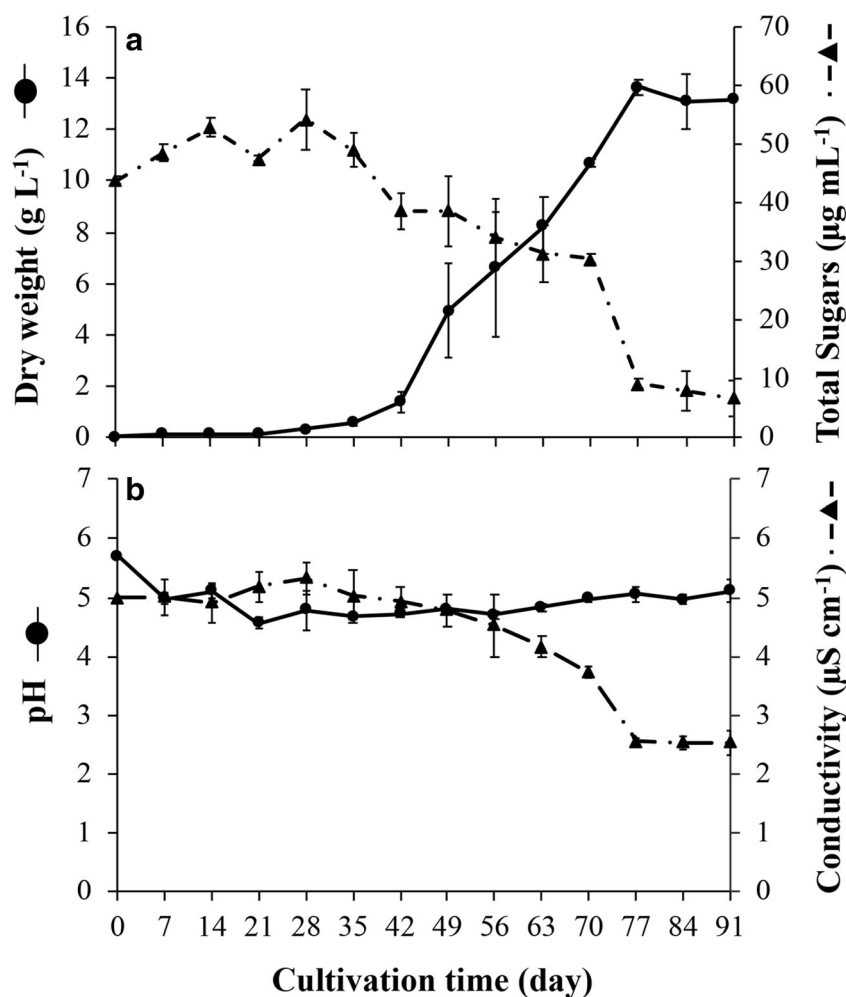
Results

Growth kinetics of _T. lophophoroides_ hairy roots The kinetic behavior of _T. lophophoroides_ hairy root cultures growing in MS and B5 medium (Figs. 1 and 2, respectively) was determined. For MS medium, the lag, exponential, and stationary growth phases (0 to 35, 36 to 77, and 78 to 91 d, respectively) were observed (Fig. 1a). The specific growth rate corresponded to 0.06 d^{-1}, which indicates the increase in the biomass of the root per unit of biomass concentration over time; the growth index was 307.78, and the duplication time was 12.14 d. Changes in total sucrose concentration (Fig. 1a), in pH, and electrical conductivity (Fig. 1b) in the medium were analyzed as a function of culture time and biomass growth in MS medium. The increase in biomass accumulation and the decrease of electrical conductivity in MS medium was in agreement with the consumption of the carbon source. The

pH of the medium was constant along growth kinetics (Fig. 1B).

On the other hand, the hairy roots of _T. lophophoroides_ growing in B5 medium (Fig. 2a) showed shorter growth phases, but lower biomass yield, when compared with those parameters for MS medium: lag phase (0 to 14 d), exponential phase (15 to 49 d), stationary phase (50 to 55 d) and death phase (day 56 to 91). Kinetic parameters as given by growth index, duplication time and growth rate (33.00 d, 15.00 d and 0.05 d^{-1}, respectively) were calculated. The total sugar concentration was measured to assess the agreement between biomass production and the decrease of the carbon source concentration in B5 liquid medium (Fig. 2a). Conductivity and pH (Fig. 2b) showed similar behaviors as observed in MS cultured hairy roots.

The morphological characteristics of _T. lophophoroides_ hairy roots growing in MS medium showed lateral roots developed preferentially (Fig. 3c, d) from inoculum (Fig. 3a) with less pronounced tissue browning (Fig. 3d), whereas in B5 medium after inoculation, the hairy roots proliferated mainly by elongation (Fig. 3f, g) and the biomass turned brown at 84 d (Fig. 3H).

Figure 1. Growth curve of hairy roots of _Turbinicarpus lophophoroides_ (Werderm.) Buxb. & Backeb. in liquid Murashige and Skoog medium (Murashige and Skoog 1962). (_a_) Biomass of the root tissue (in _dry weight g L^{-1}_) and _total sugars_ (_µg mL^{-1}_) in remaining liquid medium over _cultivation time_ (_d_); (_b_) _pH_ and electric _conductivity_ (_µS cm^{-1}_) values of the medium over _cultivation time_ (_d_). Each _value_ represents the mean of three replicates ± standard error.

Figure 2. Growth curve of hairy roots of *Turbinicarpus lophophoroides* (Werderm.) Buxb. & Backeb. in liquid Gamborg B5 medium (Gamborg *et al.* 1968). (*a*) Biomass of the root tissue (in *dry weight g L⁻¹*) and *total sugars* ($\mu g\ mL^{-1}$) in remaining liquid medium over *cultivation time* (*d*); (*b*) *pH* and electric *conductivity* ($\mu S\ cm^{-1}$) values of the medium over *cultivation time* (*d*). Each *value* represents the mean of three replicates ± standard error.
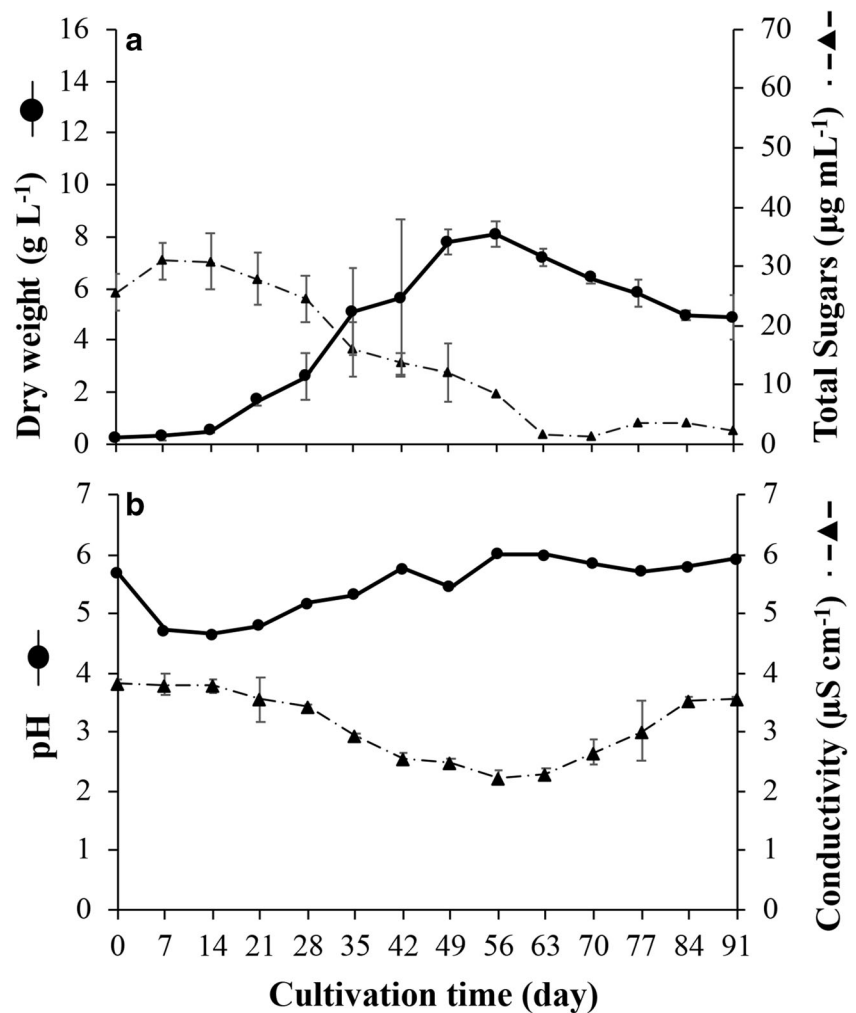

Chemical identification of the major compounds: identification of feruloyl-glucoside After HPLC analysis, two peaks were detected (peak 1 at retention time (Rt) 8.979 min and peak 2 at Rt 10.225 min) from extracts of tissues in the exponential growth phase of *T. lophophoroides* hairy roots. The main peak (peak 1) was isolated and tentatively identified by means of mass spectrometry and then, the conformational structure was elucidated by NMR analysis. Adduct ion of peak 1 ([M-H]⁻ at mass/charge number of ions (m/z 355.1047)) (Fig. S1) yielded fragments at m/z 175, 193 and 295, suggesting the presence of a feruloyl-glucoside derivative as proposed by Chougui *et al.* (2015). The mass accuracy between theoretical and measured mass was 3 mg L⁻¹. This structure was corroborated by NMR analysis. The ¹H and ¹³C NMR chemical shifts and the coupling constants of this compound, which displayed very similar data to those previously described for feruloyl-glucoside (Table 1; Kim *et al.* 2016). The ³J correlation between the anomeric proton of glucose (δ 6.33 d, *J* = 8.0) and the acetate carbonyl group at 167.41 in the HMBC spectrum indicated the position of the sugar in the ferulic acid skeleton. This is the first report

of the presence of feruloyl-glucoside in *T. lophophoroides* hairy roots.

Quantitative HPLC analysis The accumulation pattern of feruloyl-glucoside in *T. lophophoroides* hairy root cultures was determined using HPLC analysis. The present results suggested that the induction of *T. lophophoroides* hairy roots increased the accumulation of feruloyl-glucoside (peak 1, Rt 8.979 min, λ = 242 and 323 nm; Fig. 5e) at different growth stages in both MS and B5 culture medium (Fig. 5). The detected signals for nontransformed roots and shoots harvested at 273 d were too low for quantification (Fig. 5a, Table 2).

The highest accumulation of feruloyl-glucoside in *T. lophophoroides* hairy roots growing in MS medium was achieved at the exponential growth phase (2.7267 ± 0.041 mg g⁻¹ DW L⁻¹; Fig. 5c), followed by the stationary (2.0498 ± 0.010 mg g⁻¹ DW L⁻¹; Fig. 5d) and the lag phase (1.2900 ± 0.054 mg g⁻¹ DW L⁻¹; Fig. 5b) (Table 2). For B5 medium, the content of feruloyl-glucoside was slightly higher at the lag phase (2.6328 ± 0.108 mg g⁻¹ DW L⁻¹; Fig. 5f),
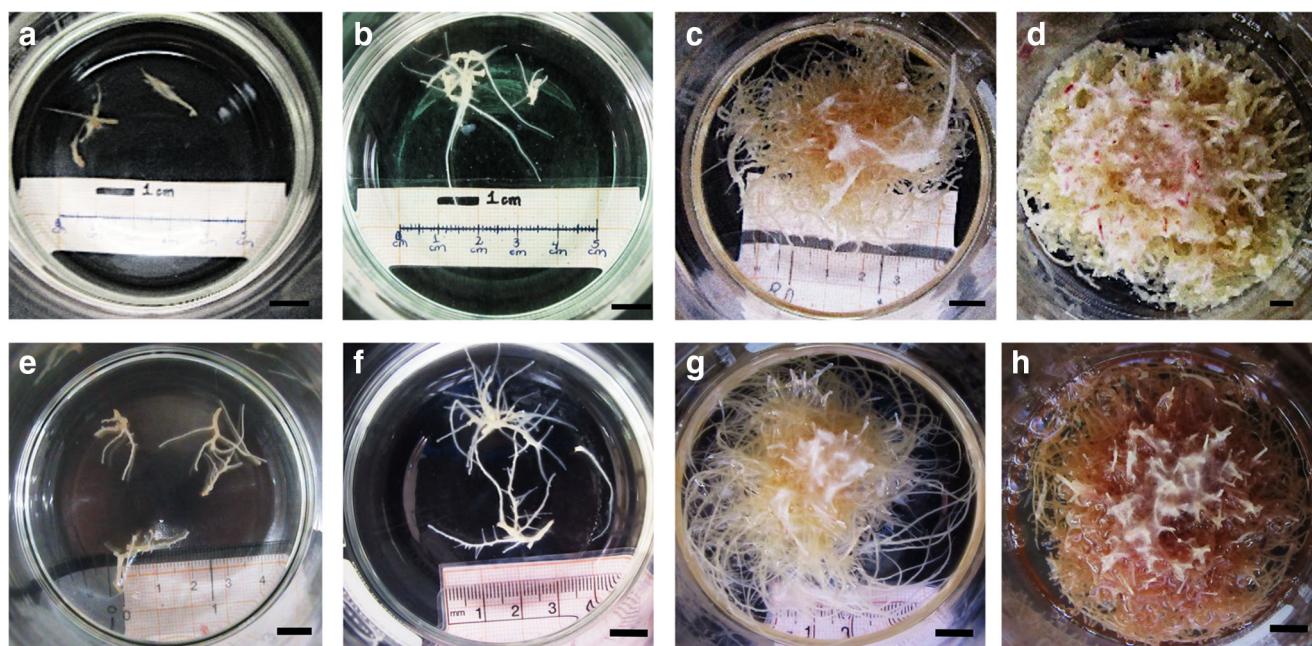
Figure 3. Phenotype of *Turbinicarpus lophophoroides* (Werderm.) Buxb. & Backeb. hairy root cultures. (*a–d*) Hairy roots cultivated in Murashige and Skoog medium (Murashige and Skoog 1962) through time; (*a*) 0 d; (*b*) 21 d; (*c*) 56 d; (*d*) 91 d. (*e–h*) Hairy roots cultivated in Gamborg B5 medium (Gamborg *et al.* 1968) through time; (*e*) 0 d; (*f*) 7 d; (*g*) 35 d; (*h*) 84 d. *Scale bars* 1 cm.

followed by the exponential phase (2.4372 ± 0.026 mg g^{-1} DW L^{-1}; Fig. 5*G*); the stationary growth phase showed the lowest feruloyl-glucoside values with 0.3498 ± 0.0004 mg g^{-1} DW L^{-1} (Fig. 5*H*; Table 2).

Discussion

In recent years, the production of therapeutic bioactive molecules in biotechnological systems has gained attention, due to

Table 1. Carbon and proton chemical shifts (δ, mg L^{-1}) of *feruloyl-glucoside* (acetone d_6)

Carbon position[b]	Feruloyl-glucoside[a]		Kim *et al.* 2016	
	δH	δC	δH	δC
1	-	126.3	-	125.5
2	8.13 (1H, *d*, 1.6, H-2′)	111.8	7.34 (1H, *d*, 1.6, H-2)	111.4
3		146.9	-	147.9
4		146.9	-	149.6
5	7.68 (1H, *d*, 8.0, H-5′)	114.6	6.80 (1H, *d*, 8.2, H-5)	115.5
6	7.98 (1H, *dd*, 8.0, 1.4 H-6′)	123.8	7.15 (1H, *dd*, 8.2, 1.5 H-6)	123.3
7	8.5 (1H, *d*, 15.6)	146.9	7.63 (1H, *d*, 15.9)	146.3
8	6.33 (1H, *d*, 15.6)	114.8	6.48 (1H, *d*, 16.0)	113.9
9		167.41	---	165.4
1′	6.33 (1H, d, 8.0, H-1′)	95.06	5.46 (1H, *d*, 8.1, H-1′)	94.2
2′	3.1 (1H, *m*, C-2′)	74.2	3.20 (1H, *m*, C-2′)	72.5
3′	3.37 (1H, *m*, C-3′)	77.4	3.23 (1H, *m*, C-3′)	76.5
4′	2.88 (1H, *m*, C-4′)	70.4	3.07 (1H, *m*, C-4′)	69.5
5′	3.37 (1H, *m*, C-5′)	74.2	3.22 (1H, *m*, C-5′)	77.8
6′	4.1 (1H, *m*, C-6′a)	61.6	3.66 (1H, *m*, C-6′a)	60.6
	3.9 (1H, *m*, C-6′b)		3.46 (1H, *m*, C-6′b)	
Me	4.03 (3H, *s*, O-CH₃)	56.26	3.82 (3H, *s*, O-CH3)	55.7

[a] Data in ^{1}H (400 MHz) and ^{13}C (100 MHz)

[b] See Fig. 5 for carbon position numbering

Splitting patterns and the coupling constant (*J*, values in Hz) are shown in *parentheses*
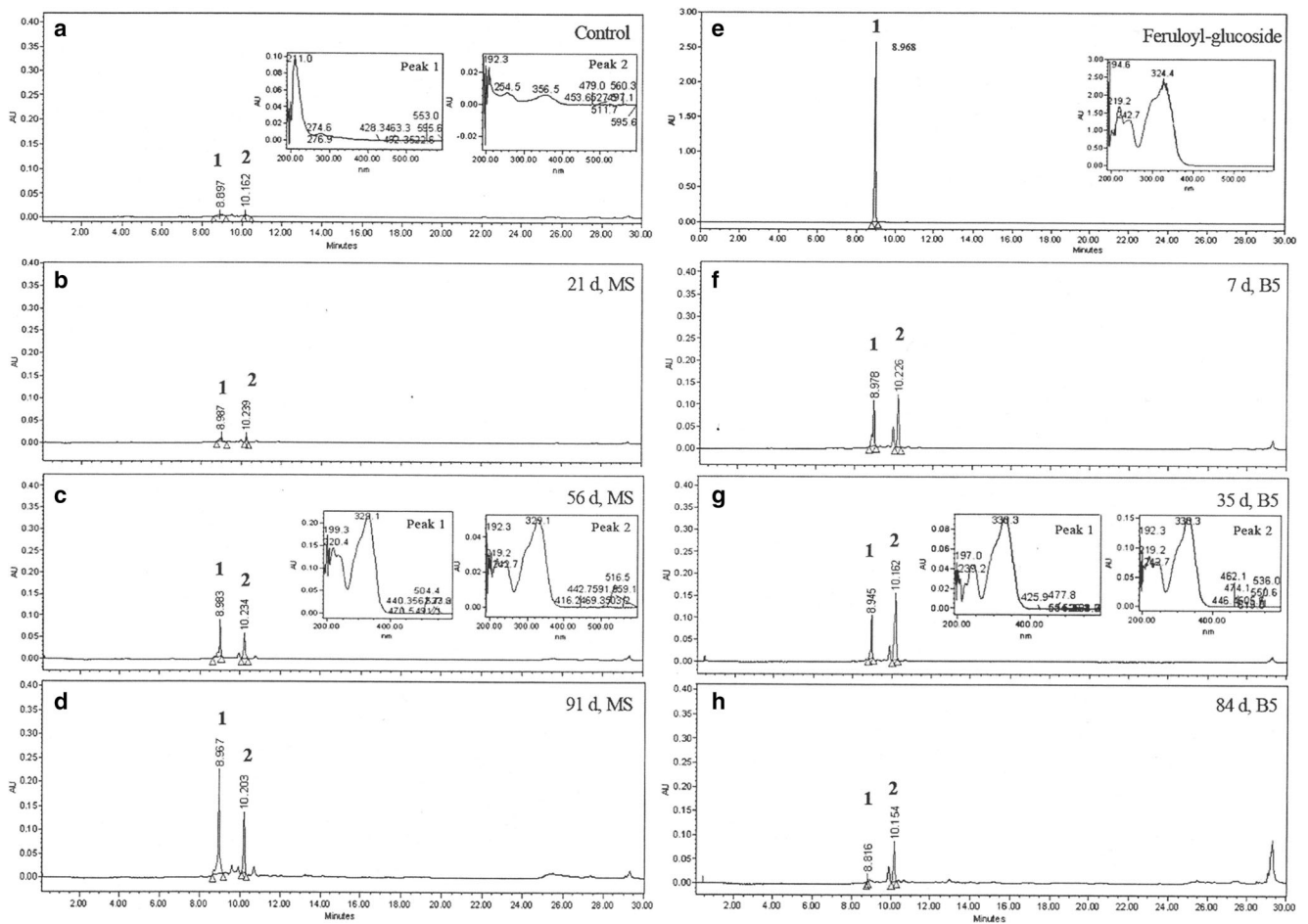
Figure 4. Quantitative high-performance liquid chromatography (HPLC) and ultraviolet absorption spectrum (*inserts*) analyses of *feruloyl-glucoside* detected in *Turbinicarpus lophophoroides* (Werderm.) Buxb. & Backeb. hairy root cultivated in Murashige and Skoog (*MS*; Murashige and Skoog 1962) or Gamborg B5 (*B5*; Gamborg *et al.* 1968) medium. (*a*) Untransformed roots (*control*) at *273 d*; (*b–d*) hairy roots in *MS* medium at *21*, *56*, and *91 d*, respectively; (*e*) *feruloyl-glucoside* isolated; (*f–h*) hairy roots in *B5* medium at *7*, *35*, and *84 d*, respectively. *Peaks* (*1*) feruloyl-glucoside and (*2*) unidentified compound.

Table 2. Concentrations of *feruloyl-glucoside* in *Turbinicarpus lophophoroides* (Werderm.) Buxb. & Backeb. hairy root cultures grown in liquid Murashige and Skoog (*MS*; Murashige and Skoog 1962) or Gamborg B5 (*B5*; Gamborg *et al.* 1968) medium compared with control non-transformed *roots* and *shoots*

| Culture medium	Cultivation time[1] (d)	Biomass* (g L^{-1}) DW	Feruloyl-glucoside* (mg g DW^{-1} L^{-1})
MS	21	0.1429 ± 0.00[a]	1.2900 ± 0.054[a]
	56	6.6190 ± 2.70[b]	2.7267 ± 0.041[b]
	91	13.1905 ± 0.082[c]	2.0498 ± 0.010[c]
B5	7	0.2857 ± 0.1429[a]	2.6328 ± 0.108[b]
	35	5.0952 ± 1.7043[b]	2.4372 ± 0.026[d]
	84	4.9524 ± 0.1650[b]	0.3498 ± 0.0004[e]
Shoot	273	0.186 g**[d]	ND
Root	273	0.070 g**[d]	ND

[1] *Cultivation time* (in *d*) of hairy root culture in lag, exponential, and stationary growth phases, respectively, for *MS* and *B5*; *DW*, dry weight

*Values represent the mean (*n* = 3) ± *standard error*

**Values represent the mean (*n* = 8)

ND, not detected. *Values* followed by the *same letter* within *biomass* and *concentration columns* are not significantly different at the level of *P* < 0.05

Figure 5. Molecular structure of major feruloyl-glucoside isolated from hairy roots of *Turbinicarpus lophophoroides* (Werderm.) Buxb. & Backeb., with carbon positions labeled.
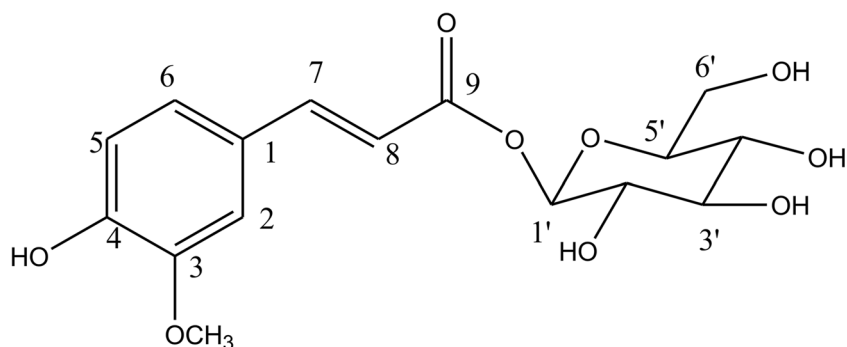
the capacity for enhanced biomass and metabolite yields (Matkowski 2008). One such biotechnological approach, transformed hairy roots, provides a plant-based method for the biosynthesis of bioactive molecules (Ludwig-Muller *et al.* 2014) such as phenolics.

Different species of cacti have been recognized as sources of bioactive metabolites (Jiménez-Aspee *et al.* 2014; Kim *et al.* 2016); nevertheless, reports dealing with the organ culture of cacti are scarce (Pérez-Molphe-Balch *et al.* 2015). In the current study, the main compound present in transformed hairy root cultures of *T. lophophoroides* was identified and quantified for the first time; in contrast to the non-transformed roots and shoots derived from *in vitro* plants where feruloyl-glucoside was not detected. The effect of MS and B5 culture medium on biomass accumulation and feruloyl-glucoside levels during the growth cycle was investigated.

The biomass production in *T. lophophoroides* hairy roots was influenced by the use of different culture medium. The maximum biomass production was achieved at 77 d (13.7 g L^{-1}; Fig. 1*a*) using MS liquid medium and at 56 d (8.1 g L^{-1}; Fig. 2*a*) for liquid B5 medium. For other plant systems, it has been proposed that biomass growth can be influenced by different factors, such as inoculum size, carbon source availability, ammonium/nitrate ratio or pH (Wu *et al.* 2006). According to the kinetic parameters calculated for *T. lophophoroides* hairy roots growing in MS or B5 medium, the MS medium (DT = 12.14 and μ = 0.06 d^{-1}) generated a 307.78-fold increase in 77 d, compared with inoculum. On the other hand, inoculum of hairy roots growing in B5 medium (DT = 15.00 and μ = 0.05 d^{-1}) yielded a 33.00-fold increase in 56 d; thus, MS medium was proposed as the best basal salt mixture for *T. lophophoroides* hairy roots biomass production.

After the isolation of the major metabolite of *T. lophophoroides* hairy root cultures, the identification was carried out. Mass spectral (see electronic supplementary material Fig. S1) and NMR analyses indicated the presence of feruloyl-glucoside (Table 1). Feruloyl-glucoside is a glycosylated hydroxycinnamic acid found in natural sources and such functional properties as whitening (Tanimoto *et al.* 2006), antioxidant, hypolipidemic, and anti-inflammatory activities (Tang *et al.* 2017) have been proposed. Quantitative

analysis indicated that B5 medium yielded the highest accumulation of feruloyl-glucoside after 7 d of culture (2.6328 ± 0.108 mg g^{-1} DW L^{-1}), when compared with that of MS medium (2.7267 ± 0.041 mg g^{-1} DW L^{-1}) after 56 d of culture (Table 2); thus, B5 medium was proposed as optimal for the production of this phenolic glycoside in the lag phase of the culture (day 7). The observed differences in biomass and metabolite accumulation found in the current study for MS and B5 medium (see Table 2) might be attributed to the differences in medium composition, since it has been proposed that the ammonium/nitrate ratio in the culture medium affects phenolics and biomass production (Russowski *et al.* 2006; Wu *et al.* 2006). The present results suggested that the accumulation pattern of feruloyl-glucoside in MS and B5 medium was independent of biomass accumulation (see Fig. 5 and Table 2), similar to that found for rosmarinic acid production in *Dracocephalum moldavica* L. hairy roots (Weremczuk-Jeżyna *et al.* 2013). In contrast, the detected signals for non-transformed roots and shoots harvested after 273 d were too low for quantification (see Fig. 5*a*, Table 2). It has been proposed that ferulic acid may participate in the lignin biosynthesis (Hamada *et al.* 2003); thus, based in these findings, the feruloyl-glucoside may participate in the lignin formation in non-transformed roots and shoots of *T. lophophoroides* obtained from *in vitro* cultures. Further studies are required to asses this hypothesis. Information regarding the accumulation pattern of feruloyl-glucoside in biotechnological systems is scarce and limited to cell suspension cultures of *Chenopodium rubrum* L. (Bokern *et al.* 1991), where nevertheless information related to feruloyl-glucoside concentration is not given; thus, the current research substantiated the potential applications of *T. lophophoroides* hairy roots for the production of biomass and for the biosynthesis and study of this phenolic glycoside (or related compounds) after 7 d of culture in B5 medium (Fig. 5).

Conclusions

The present study demonstrated the capacity of *T. lophophoroides* hairy roots for the biosynthesis of

feruloyl-glucoside, a ferulic acid derivative with functional properties. Among evaluated conditions, B5 medium proved to be suitable for feruloyl-glucoside biosynthesis after 7 d of culture; meanwhile, MS medium was better for biomass production under the given conditions. The results reported herein should contribute to the overall knowledge of cactus species and hairy root culture conditions. The identified metabolite feruloyl-glucoside was reported here for the first time in *T. lophophoroides*.

Compliance with ethical standards

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

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