Vol. 19, No. 1 (2020) 239-249
Revista Mexicana de Ingeniería Química

PHYTOCHEMICAL PROFILE OF *Coryphantha macromeris* (Engelm.) Britton & Rose (CACTACEAE) OBTAINED FROM *in vitro* CULTURE

PERFIL FITOQUÍMICO DE *Coryphantha macromeris* (Engelm.) Britton & Rose (CACTACEAE) OBTENIDA DE CULTIVOS *in vitro*

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Received: March 6, 2019; Accepted: May 19, 2019

Abstract

In the last years, the demand for natural products has increased. Different cacti species has demonstrated to be a source for the biosynthesis of secondary metabolites, as is the case of *Coryphantha macromeris* (Engelm.) Britton & Rose. The aim of this work was to evaluate the capacity of *C. macromeris* growing under controlled *in vitro* conditions for the obtention of secondary metabolites. *In vitro* cultures of *C. macromeris* plants were established from seeds using Murashige and Skoog culture medium and then the phytochemical profile of aerial and radicular sections was analyzed by means of ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-PDA-HESI-Orbitrap-MS/MS). Under the proposed conditions, 43 metabolites were identified; seven of them are reported here for the first time for this cacti species. Organic acids (citric, gluconic and tianshic acids), and phenolic acids such as piscidic, ferulic and syringic acid and/or their derivatives were found as the metabolites with the highest relative abundance. Our results suggest that the *in vitro* culture of *C. macromeris* represents a potential source for the obtention of selected compounds and serve as the basis for future investigations regarding the culture of *C. macromeris* under different biotechnological systems.

Keywords: Phytochemical analysis, secondary metabolites, cactus phytochemistry, phenolic compounds.

Resumen

En los últimos años, la demanda de productos naturales ha incrementado. Diferentes especies de cactáceas han demostrado ser una fuente para la biosíntesis de metabolitos secundarios, tal es el caso de *Coryphantha macromeris* (Engelm.) Britton & Rose. El objetivo de este trabajo fue evaluar la capacidad de *C. macromeris* cultivada en condiciones *in vitro* para la obtención de metabolitos secundarios. Se establecieron cultivos de plantas *in vitro* a partir de semillas, utilizando medio Murashige y Skoog y posteriormente se analizó el perfil fitoquímico de la sección aérea y radicular, utilizando cromatografía líquida de ultra alta resolución acoplada a espectrometría de masas (UHPLC-PDA-HESI-Orbitrap-MS/MS). Bajo las condiciones propuestas, se identificaron 43 metabolitos; siete de estos compuestos son reportados por primera vez para especies de cactáceas. Algunos ácidos orgánicos (ácido cítrico, glucónico y tianshico) y ácidos fenólicos (ácido piscídico, ferúlico y siríngico y/o sus derivados) se encontraron como los metabolitos de mayor abundancia relativa. Nuestros resultados sugieren que el cultivo *in vitro* de *C. macromeris* representa una fuente potencial para la obtención de compuestos seleccionados y sirve como base para realizar futuras investigaciones relacionadas al cultivo de esta especie en diferentes sistemas biotecnológicos.

Palabras clave: Análisis fitoquímico, metabolitos secundarios, química de cactáceas, compuestos fenólicos.

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Tel. 449-910-7400 ext. 365
https://doi.org/10.24275/rmiq/Bio540
issn-e: 2395-8472

1 Introduction

Biotechnology techniques applied to the *in vitro* growth of cacti species has allowed the production of these plants for commercial and conservation purposes (Lema-Rumińska & Kulus, 2014). Some cacti species are slow growing plants in their natural habitat or under traditional vegetative propagation; thus *in vitro* culture has shown to be a suitable alternative for their propagation (Smith *et al*., 1991; Malda *et al*., 1999; Pérez-Molphe Balch *et al*., 2015). It has been proposed that *in vitro* culture of cacti species enhances growth rate and biomass production by altering the normal Crassulacean Acid Metabolism (CAM) pathway as is the case of Coryphantha minima (Malda *et al*., 1999) and also affects the profile and accumulation pattern of secondary metabolites in plants and their functional properties (Rami & Patel, 2015; Rojsanga *et al*., 2017).

Secondary metabolites are essential for plant surviving, helping to overcome the environmental stressing conditions (Sepúlveda-Jiménez *et al*., 2003). Plant cell tissue and organ culture has become a powerful strategy for plant micropropagation and for the production and study of natural products (Estrada-Zúñiga *et al*., 2016; Ochoa-Villarreal *et al*., 2016; Nieto-Trujillo *et al*., 2017) leading to the discovery of novel compounds with biological activities and commercial applications (Hussain *et al*., 2012). The potential use of *in vitro* culture is immense since the production of specific metabolites may be enhanced through elicitors or precursor treatments (Dias *et al*., 2016; Cortes-Morales *et al*., 2018) and the biosynthesis of compounds under a defined production system avoids variations in crop quality related with geographical and seasonal constrains, allows a continuous supply of phytocompounds with uniform quality and yield, and comprises a step of a multi-stage strategy for the biosynthesis of secondary metabolites on a larger scale such as callus or cell suspension cultures (Smetanska, 2008). Thus, the subsequent dedifferentiation of *in vitro* plant cultures, is a powerful tool to obtain plant active metabolites in greater yield and quality.

The growing demand of natural products with functional properties has increased in the last years (Khan, 2018) and new sources ought to be investigated and research conducted by protecting wild populations of endangered and non-endangered species. Different cacti species has demonstrated to be a source of secondary metabolites with health promoting effects (Cha *et al*., 2013; El-Mostafa *et al*., 2014; Jiménez-Aguilar *et al*., 2015). These metabolites may serve as the basis for the discovery, design or synthesis of new compounds with biological activities (Veeresham, 2012). For this reason, the establishment of *in vitro* plant cultures becomes a fundamental task with socio-economic scope, generating systems that allow the conservation of the habitat and species with limited reproductive capacity (Malda *et al*., 1999) and, at the same time, the production of secondary metabolites in a greater range than in the wild. Few studies have focused on the phytochemical composition of traditionally used cacti species as is the case of *C. macromeris*. *C. macromeris* is traditionally known as "Dona Ana" and in the regions where it is distributed (northern Mexico and southern United States), it is traditionally used for healing stomach disorders. In previous reports we have shown the phytochemical profiling of aerial and radicular sections of *C. macromeris* growing under greenhouse conditions (Cabañas-García *et al*., 2019), finding the presence of different classes of secondary metabolites with reported functional and potential applications. The aim of this work was to evaluate the potential of *C. macromeris* growing under controlled *in vitro* conditions for the obtention of secondary metabolites in order to increase and deepen in the knowledge of cacti species and their potential applications.

2 Materials and methods

2.1 Plant material, in vitro culture and plant acclimatization

Mature fruits with seeds of *Coryphantha macromeris* (Engelm.) Britton & Rose were collected at the municipality of Concepción del Oro, Zacatecas, México. The plant material was botanically identified by Professor M. Alvarado Rodríguez. A voucher specimen was deposited at the herbarium of the Autonomous University of Aguascalientes (HUAA; Voucher No. 6386). In the laboratory, the seeds were extracted and disinfected with treatments of 70% (v/v) ethanol (1 min), and 1.8% (v/v) sodium hypochlorite (25 min) and rinsed four times with sterile distilled water under aseptic conditions (Dávila-Figueroa *et al*., 2005). Seeds were germinated in culture vessels containing MS medium (Murashige & Skoog, 1962) and 30 g L^{-1} sucrose, solidified with 10 g L^{-1} agar (Sigma-Aldrich, St. Louis, MO,

USA). All culture media were adjusted to pH 5.7 with 1N NaOH and then sterilized by autoclaving at 121 °C for 15 min. Cultures were incubated at 25 ºC under fluorescent light (40 μmol m^2 s^{-1}) and 16/8 (light/dark) photoperiod. Seedlings of 4-6 mm in length obtained from the germinating seeds were used as explant source for *in vitro* multiplication. For this, roots were separated, and the aerial sections were inoculated vertically onto: 1) MS basal medium; 2) MS basal medium supplemented with 6-benzylaminopurine (BA, 4.44 μM) and 1-naphthalenacetic acid (NAA, 0.054 μM); and 3) MS basal medium with activated charcoal (AC, 1.0 g L^{-1}) with the purpose of producing new shoots from the areoles. These culture media were selected based in our experience in *in vitro* culture of Coryphantha and other closely related species (Pérez-Molphe Balch *et al*., 1998). After 45 days of culture, the average number of shoots and roots formed per explant were evaluated, and a culture media was selected based on the physical characteristics and on the number of shoots generated *in vitro*. Experiments was performed in triplicate. The generated shoots were collected and transferred to the medium which produced higher number of shoots and then subcultured each three month for 12 months and used for phytochemical analysis.

For the acclimatization of micropropagated plants to *ex vitro* conditions, each plant was transferred into pots containing a mix of sand and soil (1:1) and covered with plastic bags for 3 weeks, as we reported previously (Pérez-Molphe Balch *et al*., 2002) in order to prevent desiccation.

2.2 *Sample preparation and phytochemical analysis by UHPLC-PDA-HESI-Orbitrap-MS/MS*

C. macromeris methanolic extracts were prepared as reported previously (Cabañas-García *et al*., 2019). For this, three-month-old plants with one year of subcultures on MS basal medium were collected, sliced and dyed in an oven at 40 ºC during 1 week in dark conditions and then extracted three times with methanol in an ultrasonic bath. Samples were filtered and concentrated under reduced pressure at 40 ºC and the remaining solvent was the eliminated by lyophilization. Each sample was resuspended in HPLC-MS-grade methanol, filtered (0.22 μm) and used for phytochemical analysis. Phytochemical analysis was performed using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific) with a C18 column (ID: 150 x 4.6 mm, 5 μm; Restek Corporation) and equipped with a quaternary Series RS pump and a Dionex Ultimate 3000 Series TCC-3000RS column compartments with an Ultimate 3000 Series WPS-3000RS autosampler (Thermo Fisher Scientific) and a rapid separations PDA detector. The detection wavelengths were 254, 280, 320 and 440 nm, and Photodiode Array Detector (PDA) was recorded from 200 to 800 nm for peak characterization. The separation was performed (flow rate: 1.0 mL min-1, injection volume: 10 μL) in a gradient elution mode composed by 1% formic aqueous solution (A) and acetonitrile (B). The gradient program [time (min), %B] was: (0.00, 5), (5.00, 5), (10.00, 30), (15.00, 30), (20.00, 70), (25.00, 70), (35.00, 5), and 12 min for column equilibration before each injection. The system was controlled by Chromeleon 7.2 Software (Thermo Fisher Scientific, Waltham, MA, USA and Dionex Softron GmbH division of Thermo Fisher Scientific) and hyphenated with a Thermo high resolution Q Exactive focus mass spectrometer (Thermo Fisher Scientific). The chromatographic system was coupled to the mass spectrometer with a Heated Electrospray Ionization Source II (HESI II). Nitrogen (purity > 99.999%) was employed as both the collision and damping gas. Nitrogen was obtained from a Genius NM32LA nitrogen generator (Peak Scientific, Billerica, MA, USA). Mass calibration for Orbitrap was performed once a week, in both negative and positive modes. Caffeine and N-butylamine (Sigma Aldrich, Saint Louis, MO, USA) were the calibration standards for positive ions and buspirone hydrochloride, sodium dodecyl sulfate, and taurocholic acid sodium salt were used to calibrate the mass spectrometer. These compounds were dissolved in a mixture of acetic acid, acetonitrile, water and methanol (0.1:1:1:1; Merck Darmstadt, Hesse, Germany) and infused using a Chemyx Fusion 100 syringe pump. XCalibur 2.3 software and Trace Finder 3.2 (Thermo Fisher Scientific, San Jose, CA, USA) were used for UHPLC control and data processing, respectively. Q Exactive 2.0 SP 2 (Thermo Fisher Scientific) was used to control the mass spectrometer.

2.2.1 *MS parameters*

The HESI parameters were optimized as follows: sheath gas flow rate 75 units; auxiliary gas unit flow rate 20; capillary temperature 400 ºC; auxiliary gas heater temperature 500 ºC; spray voltage 2500 V (for ESI-); and S lens RF level 30. Full scan data in negative mode was acquired at a resolving power

of 70,000 full width half maximum (FWHM) at m/z 200. For the compounds of interest, a scan range of m/z 100-1000 was chosen; the automatic gain control (AGC) was set at 3×10^6 and the injection time was set to 200 ms. Scan-rate was set at 2 scans s^{-1}. External calibration was performed using a calibration solution in positive and negative modes before each sample series. In addition to the full scan acquisition method, for confirmation purposes, a targeted MS/MS analysis was performed using the mass inclusion list and expected retention times of the target analytes, with a 30s-time window, with the Orbitrap spectrometer operating both in positive and negative mode at 17,500 FWHM (m/z 200). The AGC target was set to 2×10^5, with the maximum injection time of 20 milliseconds. The precursor ions were filtered by the quadrupole operating at an isolation window of m/z 2. The fore vacuum, high vacuum and ultrahigh vacuum were maintained at approximately 2 mbar, from 105 to below 1010 mbar, respectively. Collision energy (HCD cell) was operated at 30 eV. Detection was based on calculated exact mass and on retention time of target compounds presented in Table 1. Metabolite identification was achieved by comparing spectrometric data with evidences existing in literature or analyzing the fragmentation pattern of each molecule. The mass tolerance window was set to 5 ppm.

2.3 Statistical analysis

Statistical analysis was performed using SigmaPlot software (Systat software, version 11.0). Sample differences were determined using one-way ANOVA. Statistical significance of means was considered at $p \leq 5\%$.

3 Results and discussion

3.1 in vitro culture of C. macromeris

Seeds of *C. macromeris* were germinated *in vitro* and then based on our experience in plant cell tissue and organ culture of cacti species, and using the effective growth regulator combinations proposed by our research group for Coryphantha spp. and other closely related species (Pérez-Molphe Balch *et al*., 1998), three different treatments were evaluated in order to find possible improvements on shoot and root formation. The generated explants into the different treatments showed similar morphological characteristics. Nevertheless, treatment composed by MS + BA (4.4 μM) + NAA (0.054 μM) or AC did not contribute to shoot or root formation since a maximum of 2 new shoots or roots were generated (Fig. 1d). Statistically ($p \leq 5\%$), MS basal medium without plant growth regulators (PGR) or AC is considered as the best of the evaluated treatments, generating an average of 3.5 new and vigorous shoots per explant after 45 days of incubation (Fig. 1d). The number of shoots produced is similar to that reported previously by our research group for *C. duranguensis*, *C. clavata* and *C. radians* cultivated under similar conditions (Pérez-Molphe Balch *et al*., 1998); nevertheless, it is slightly lower when is compared with that reported by Smith *et al*. (1991) for *C. macromeris* plantlets regenerated from 4 years old callus cultures, yielding as many as 20 shoots per culture tube and formed sporadically after 42 to 56 days of culture. Thus, considering that in our investigation each explant produced an average of 3.5 new shoots *in vitro*, an average of 17.5 new shoots per flask containing 5 explants may be achieved after 45 days of culture.

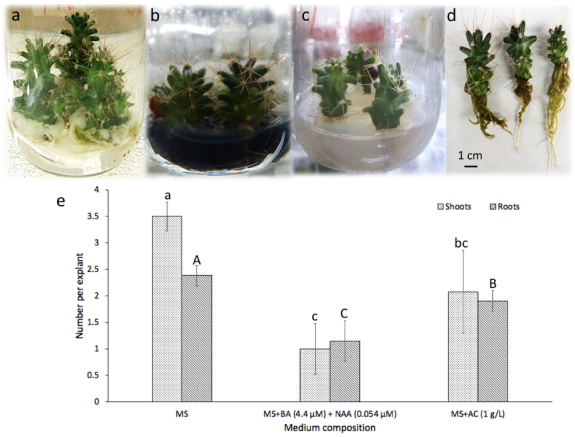

Fig. 1. *In vitro* culture and propagation of *Coryphantha macromeris*. (a) Shoots produced in medium with BA (4.44 μM) + NAA (0.054 μM); (b) shoots in medium with AC; (c) plants transferred to MS basal medium; (d) Complete plants generated *in vitro* and used for phytochemical analysis; e) number of shoots and roots produced per explant of *C. macromeris* after 45 days of culture using different treatments. Each value represents the average (n=12) and error bars the standard deviation. Histobars with same letters are not significantly different ($p \leq 5\%$). Lowercase letters refer to differences among shoots and uppercase among roots.

These shoots generated *in vitro* can be used as a source of explants for new multiplication cycles, due to this, shoots can be generated permanently without the need to collect and establish new plant material *in vitro*. The *in vitro* generated shoots with this medium successfully rooted (Fig. 1d) and showed an acclimatization efficiency to *ex vitro* conditions of 72% (data no shown). The lower number of shoots and roots formed by the treatment composed by the combination of MS basal medium with BA and NAA may be related with the formation of callus tissue in the basal area of the explants (Clayton *et al*., 1990) (Fig.1a) or a possible antagonistic interaction between PGR (Wang & Irving, 2011). These results are similar to that reported by Giusti *et al*. (2002) for shoot proliferation in other closely related species such as *Escobaria minima*, *Mamillaria pectinifera* and *Pelecyphora aselliformis*, finding that a combination between the NAA and BA at different concentrations, inhibited the formation of shoots, suggesting the antagonistic effects between both PGR for shoot formation in some cacti species. Thus, based in our results, for the *in vitro* culture of *C. macromeris*, we recommend using MS medium without the evaluated PGR or AC.

3.2 Phytochemical profiling of aerial and radicular sections of C. macromeris in vitro

In this work, we also analyzed the phytochemical profile of *C. macromeris* growing under controlled *in vitro* conditions (Fig. 1d) in order evaluate its biosynthetic capacity and to increase and deepen in the phytochemical knowledge of cacti species and their potential applications for the obtention of secondary metabolites. Under the proposed chromatographic conditions, 44 metabolites were detected and 43 of them were tentatively identified by tandem mass spectrometry. Within this diversity, 18 metabolites were exclusively detected in shoots, 20 in roots and 6 in both plant sections namely citric, piscidic and tianshic acids, nordihydrocapsiate isomers and p-hydroxynonanophenone (compounds 4, 7, 34, 36, 40 and 43; Table 1). Among detected compounds, 37 metabolites were assigned as we reported previously for *C. macromeris* plants growing under greenhouse conditions (peaks 2 to 25, 27-30, 32-34, 36, 37, 40, 42-44) (Cabañas-García *et al*., 2019), and 7 metabolites were exclusively biosynthesized by the *in vitro* plants, including gluconic acid (compound 1), one ferulic acid derivative (compound 26), two tianshic acid derivatives (compounds 38 and 41) and other polar compounds such as D-myo-Inositol, 2-butanoate 4,5-dihexanoate (compound 31), 6,8-dihydroxy-3-(penta-1,3-dien-1-yl) isochroman-1-one (compound 35) and 4-methyl-5-phenylpentanoate (compound 39). The occurrence of the above-mentioned metabolites is reported here for the first time for cacti species growing under controlled *in vitro* conditions. The elution profile of *C. macromeris* aerial and radicular sections is shown in Figures 2a and 2b, respectively; peak characteristics as is retention time, theoretical and measured mass, UV (λ_{max}) and tentative identification of each compound are summarized in Table 1. All the proposed metabolites showed an accuracy smaller than 5 ppm.

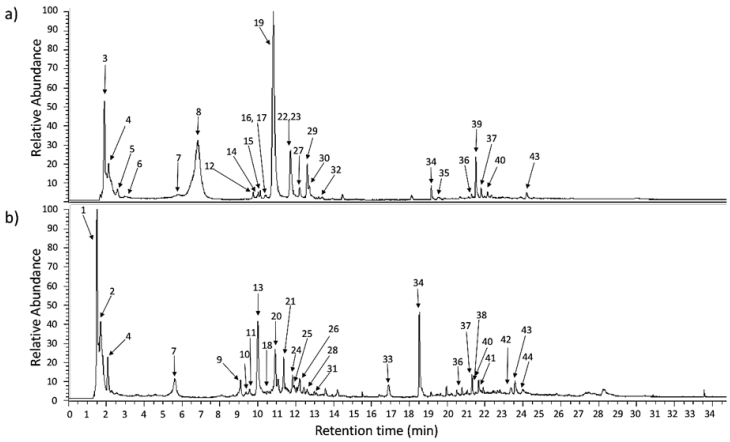

Fig. 2. UHPLC chromatogram of *C. macromeris* methanolic extracts prepared whit aerial (a) and root section (b) of plants growing in vitro. Peak numbers refer to that metabolites indicated in Table 1; repeated numbers in each (a) or (b) refers to that metabolites found in both sections.

Table 1. Metabolites identified in aerial and root sections of *C. macromeris* growing *in vitro* by UHPLC-PDA-HESI-Orbitrap-MS/MS data using HESI in negative ion mode.

Peak	Retention time (min.)	UV Max (λ_{max})	Tentative identification	Elemental composition $[M-H]^-$	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Accuracy (ppm)	MSn ions	Plant section
1	1.51	270	Gluconic acid	$C_6H_{11}O_7^-$	195.05103	195.05101	0.10	129.018801	Root
2	1.71	222, 274	Citric acid*	$C_6H_7O_7^-$	191. 01973	191.01945	1.46	111.00801	Root
3	1.92	200	Vaccihein A*	$C_{18}H_{17}O_9^-$	377.08778	377.08572	4.72	361.09021, 347.07446, 319.07958, 289.06955, 125.02381	shoot
4	2.08	218, 273	Iso citric acid isomer*	$C_6H_7O_7^-$	191. 01973	191.01945	1.46	111.00790	Root/shoot
5	2.63	220, 275	Iso citric acid isomer*	$C_6H_7O_7^-$	191. 01973	191.01935	1.98	111.00790	shoot
6	3.02	220, 275	Piscidic acid isomer*	$C_{11}H_{11}O_7^-$	255.05103	255.05092	0.43	193.05013, 165.05515, 135.04445, 119.04955, 107.04945	Shoot
7	5.87	222, 275	Piscidic acid isomer*	$C_{11}H_{11}O_7^-$	255.05103	255.05095	0.31	193.05009, 165.05511, 135.04472, 119.04937, 107.04933	Root/shoot
8	6.88	223, 275	Piscidic acid isomer*	$C_{11}H_{11}O_7^-$	255.05103	255.05084	0.74	193.05009, 165.05515, 135.04449, 119.04932, 107.04932	Shoot
9	9.09	225, 276	Hyrtioerectine C*	$C_{11}H_{12}NO_4^-$	222.07718	222.07692	1.17	206.08186, 198.07663, 180.06618, 178.08684,	Root
10	9.38	233, 273	Piscidic acid derivative*	$C_{21}H_{27}O_{13}^-$	487.14571	487.14587	0.32	255.05106, 193.05022, 165.05505, 135.04446, 107.04950	Root
11	9.66	231	Sinapic acid*	$C_{11}H_{11}O_5^-$	223.05997	223.06097	4.48	208.03757, 179.07094, 164.04730	Root
12	9.81	222, 272	Lucuminic acid*	$C_{19}H_{25}O_{12}^-$	445.13515	445.13525	0.22	163.03951, 119.04940, 107.04953	Shoot
13	10.01	225, 276	Syringic acid acetate*	$C_{11}H_{11}O_6^-$	239.05611	239.05594	0.71	197.04456, 195.06580, 179.03436, 149.06012, 135.04456, 107.04944	Root
14	10.08	223, 275	Hyrtioerectine C*	$C_{11}H_{12}NO_4^-$	222.07718	222.07692	1.17	206.08183, 198.07677, 180.06609, 178.08684,	Shoot
15	10.18	228	Piscidic acid derivative*	$C_{21}H_{27}O_{13}^-$	487.14571	487.14572	0.02	255.05087, 193.05019, 165.05513, 135.04459, 107.04922	Shoot
16	10.44	230	Sinapic acid derivative*	$C_{22}H_{29}O_{14}^-$	-	517.15637	-	223.06093, 208.03735, 179.07088, 164.04736,	Shoot
17	10.49	230	Sinapic acid*	$C_{11}H_{11}O_5^-$	223.05997	223.06096	4.43	208.03757, 179.07094, 164.04730	Shoot
18	10.50	236, 283	Propanedioic acid, [5-[[2-[(6-deoxy-α-L-galactopyranosyl) oxy] cyclohexyl] oxy]-Pentyl]*	$C_{20}H_{33}O_{10}^-$	433.20792	433.20819	0.62	417.21247, 387.20291, 287.14960, 245.13959, 131.07077	Root
19	10.88	225, 275	Syringic acid acetate*	$C_{11}H_{11}O_6^-$	239.05611	239.05589	0.92	197.04605, 195.06590, 179.03441, 149.06023, 135.04465, 107.04942	Shoot
20	10.92	236, 326	Ferulic acid*	$C_{10}H_9O_4^-$	193.05063	193.05028	1.80	179.03455, 149.06018, 163.03961, 147.04457, 135.03998	Root
21	11.37	237, 286	Cinnamic acid derivative*	$C_8H_{14}O_6^-$	-	206.08197	-	147.04456, 103.05459, 135.04437	Root
22	11.78	236, 327	Ferulic acid*	$C_{10}H_9O_4^-$	193.05063	193.05022	2.12	163.03963, 149.06049, 135.03993	Shoot
23	11.79	236, 327	Ferulic acid derivative (Fertaric acid) *	$C_{14}H_{13}O_9^-$	325.05651	325.05649	0.06	193.05032, 163.03928, 149.06001, 135.03989	Shoot
24	11.83	237, 321	Ferulic acid isomer*	$C_{10}H_9O_4^-$	193.05063	193.05040	1.19	163.03963, 149.06050, 135.04468	Root
25	12.06	241	Ferulic acid derivative*	$C_{20}H_{29}O_{10}^-$	429.17662	429.17649	0.30	193.05081, 163.03934, 149.06033, 135.04446,	Root
26	12.19	237, 282	Ferulic acid derivarive	$C_{18}H_{33}O_6^-$	-	345.22845	-	193.04984, 149.06029, 135.04456	Root
27	12.27	236, 290	Cinnamic acid derivative*	$C_8H_{14}O_6^-$	-	206.08195	-	147.04454, 103.05433, 135.04437	Shoot
28	12.59	282, 328	Azelaic acid*	$C_9H_{15}O_4^-$	187.09850	187.09732	0.96	125.09640	Root
29	12.69	236, 326	Ferulic acid isomer*	$C_{10}H_9O_4^-$	193.05063	193.05023	2.07	163.03963, 149.05992, 135.03996	Shoot

30	12.80	237, 313	Ferulic acid isomer*	$C_{10}H_9O_4^-$	193.05063	193.05028	1.80	163.03963,149.0602, 135.03969	Shoot
31	12.98	282	D-myo-Inositol, 2-butanoate 4,5-dihexanoate	$C_{22}H_{37}O_9^-$	445.24210	445.24460	0.33	375.20310, 343.21277, 245.13945, 269.13974, 229.14453, 131.07074	Root
32	13.45	282	Azelaic acid*	$C_9H_{15}O_4^-$	187.09850	187.09729	1.12	125.09640	Shoot
33	16.92	284	Corchorifatty acid F*	$C_{18}H_{31}O_5^-$	327.21770	327.21786	0.48	309.20665, 291.19690, 173.11787, 157.12346 125.09642	Root
34	18.53	283, 368	Tianshic acid*	$C_{18}H_{33}O_5^-$	329.23335	329.23349	0.42	165.12788, 127.11205	Root/Shoot
35	19.66	283, 403	6,8-dihydroxy-3-(penta-1,3-dien-1-yl) isochroman-1-one	$C_{14}H_{13}O_4^-$	245.08190	245.08174	0.65	151.03943, 137.02382, 121.02871, 111.08681, 109.02865	Shoot
36	20.79	282	Nordihydrocapsiate*	$C_{17}H_{25}O_4^-$	293.17583	293.17606	0.78	263.16559, 247.16969, 157.12259, 141.12785	Root/Shoot
37	21.32	283	Decyl gallate (Gallic acid derivative) *	$C_{17}H_{25}O_5^-$	309.17075	309.17102	0.87	293.17578, 169.01399, 153.01965, 125.02386	Root
38	21.52	282	Tianshic acid derivative	$C_{18}H_{33}O_4^-$	-	313.23859	-	165.12788, 127.11205, 125.09650	Root
39	21.61	283	4-methyl-5-phenylpentanoate	$C_{12}H_{15}O_2^-$	191.10775	191.10742	1.72	175.07587	Shoot
40	21.67	282	Nordihydrocapsiate*	$C_{17}H_{25}O_4^-$	293.17583	293.17609	0.88	277.18082, 263.16519, 247.17030, 153.05557,	Root/shoot
41	21.91	283	Tianshic acid derivative	$C_{18}H_{31}O_4^-$	-	311.22290	-	165.12788, 127.11205, 125.09650	Root
42	23.37	283, 336	13-hydroxyoctadecadienoic acid*	$C_{18}H_{31}O_3^-$	295.22787	295.22815	2.87	281.21198, 279.23285,	Root
43	23.58	283, 333	p-Hydroxynonanophenone*	$C_{15}H_{21}O_2^-$	233.1547	233.15462	0.34	219.17459, 135.04431, 121.02875	Root/shoot
44	24.00	283, 335	Unknown*	$C_{13}H_{27}O_8^-$	-	311.16888	-	-	Root

*Previously detected and identified in *C. macromeris* greenhouse plants (Cabañas-García *et al*., 2019)

Compound 1 was assigned as gluconic acid as proposed by Taamalli *et al*. (2015). Gluconic acid showed the highest relative abundance in the chromatogram (Fig. 2b) and was only detected in radicular section. For other systems it has been proposed that this metabolite may be involved in the mechanisms of phosphorus solubilization in the medium (Oteino *et al*., 2015), which may explain its exclusive presence in roots of *in vitro* plants, rather than in radicular sections of plants growing under greenhouse conditions (Cabañas-García *et al*., 2019). Signals revealed for compounds 2, 4 and 5 indicated the presence of isocitric acid isomers and signals for compound 3, the presence of vaccihein A; compounds 6, 7 and 8 were assigned as piscidic acid isomers and compounds 10 and 15 as two of their derivatives (Table 1). According to the number of peaks detected for piscidic acid and the relative abundance shown in the chromatogram (Fig. 2a and 2b), our results suggest that piscidic acid or its derivatives may correspond to one of the main constituents of *C. macromeris* growing under controlled conditions, similar to that for greenhouse plants (Cabañas-García *et al*., 2019). This is interesting since *in vitro* plants of *C. macromeris* shows the capacity for the biosynthesis of several metabolites found in plants cultivated *ex vitro* and offers a controlled system for the obtention and elicitation of selected compounds. On the other hand, compounds 9 and 14 were assigned as the alkaloid hyrtioerectine C. Our results suggest that this metabolite may exist as constitutive, rather than adaptative since no stressing conditions were given to *in vitro* plants; further studies are required to asses this hypothesis. Compounds 11 and 17 were assigned as sinapic acid isomers (Martínez-Las Heras *et al*., 2016) and compound 16 as their derivative since characteristic fragment ions of sinapic acid were detected (Table 1). On the other hand, compound 12 was assigned as lucuminic acid and compounds 13 and 19 as syringic acid acetate (Table 1). According to the relative abundance shown in the chromatogram (Fig. 2a and 2b), syringic acid acetate may also correspond to one of the main constituents of *C. macromeris* aerial and root section, similar to that found for piscidic acid isomers and their derivatives (Table 1 and Fig. 2a and 2b).

Compound 18 was assigned as a propanedioic acid derivative and compounds 20, 22, 24, 29 and 30 as ferulic acid isomers (Sulaiman *et al*., 2016). Compounds 23, 25 and 26 were proposed as ferulic acid derivatives, since characteristic fragment ions of ferulic acid were detected (Table 1). Among these metabolites, the presence of compound 26 is reported for the first time in *in vitro* cultures of *C. macromeris*; further studies are required to characterize the structure of compound 26. Compounds 21 and 27 were assigned as cinnamic acid derivatives and compounds 28 and 32 as azelaic acid isomers.

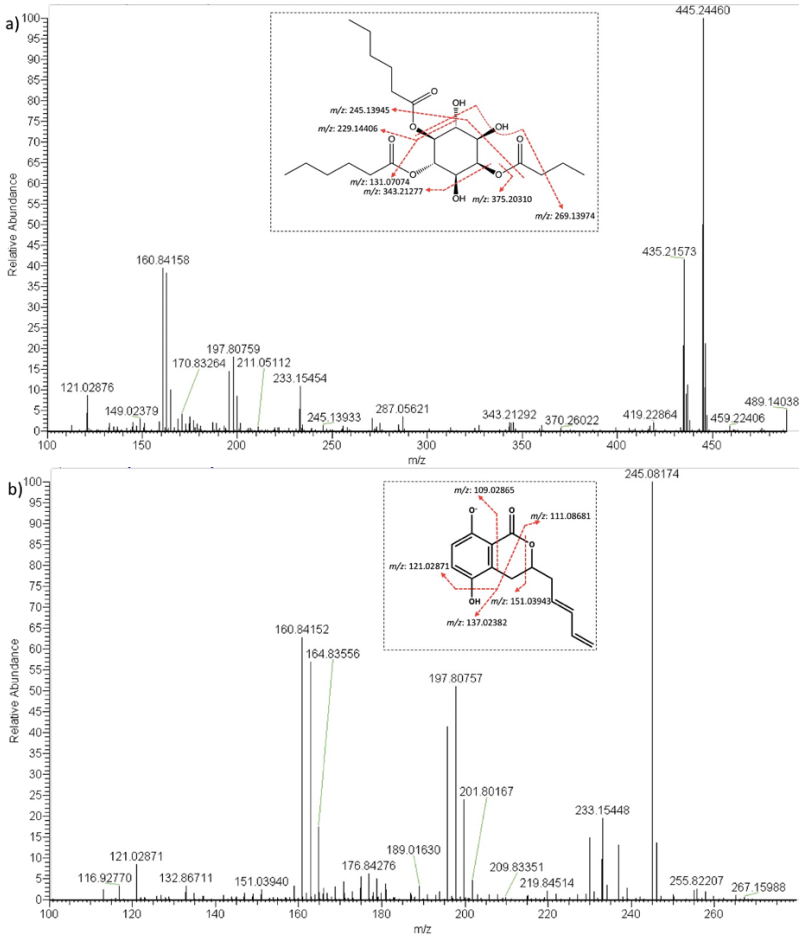

Fig 3. Full scan spectra of peaks 31 (a) and 35 (b), two metabolites identified for the first time for cacti species. Dotted inset represents the molecule and red dotted lines in each inset, the proposed fragmentation pattern.

Azelaic acid was detected in both sections of *in vitro* plants, similar to that previously reported for plants growing under greenhouse conditions (Cabañas-García *et al*., 2019). It has been proposed that the presence of organic acids in radicular sections may be related to mechanisms of phosphorus uptake and heavy metals tolerance (Hocking, 2001) thus we hypothesized that this metabolite may occur as constitutive and a translocation or radicular secretion mechanisms may exist in *C. macromeris*.

Our results suggests that the phytochemical profile of *C. macromeris* is slightly affected by the *in vitro* culture since the diversity of metabolites was lower compared with that reported for greenhouse plants (Cabañas-García *et al*., 2019), in which, 69 metabolites were detected. Nevertheless, several compounds with functional properties are present in *C. macromeris* plants growing under controlled conditions *in vitro*. It has been shown that the *in vitro* culture of plants enhances the biomass production in shorter periods of time (Malda *et al*., 1999), especially for slow growing cacti species, suggesting the feasibility of the system for the obtention of *C. macromeris* metabolites. Compounds detected under *in vitro* conditions may occur as constitutive rather than adaptative, since no stressing conditions were given to plants in the culture media and compounds which were exclusively detected under controlled *in vitro* conditions (compounds 1, 26, 31, 35, 38, 39 and 41) may be present due to the highest nutrimental availability in the medium when compared with plants growing under greenhouse conditions (Cabañas-García *et al*., 2019).

In conclusion, our results contribute to the phytochemical knowledge of cacti species and suggest the potential of *C. macromeris* growing *in vitro* as a valuable source for the biosynthesis and study of selected metabolites. The quantification of target

compounds is required to assess the suitability of the system for the *in vitro* obtention of different metabolites and its commercial feasibility. Our investigation also offers a useful system for future investigations related with plant, cell tissue and organ culture of cacti species.

Conclusions

We thank the Universidad Autónoma de Aguascalientes (México) for their financial support. The first author thanks to the National Council of Science and Technology (CONACYT) for the grant awarded (CVU: 366290). We also thanks to Dr. Jorge Bórquez and Ruben Muñoz, from the University of Antofagasta, Chile, for technical assistance and at the National Fund of Scientific and Technological Development of Chile (Fondecyt No. 1150745).

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