Industrial Crops & Products 105 (2017) 225–230

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Enhancement of saponins and flavonols by micropropagation of *Agave salmiana*

César A. Puente-Garza, Silverio García-Lara, Janet A. Gutiérrez-Uribe*

Tecnologico de Monterrey, Centro de Biotecnología FEMSA, Avenida Eugenio Garza Sada 2501 Sur, CP 64849 Monterrey, Nuevo Leon, Mexico

ARTICLE INFO

Keywords:
Micropropagation
Flavonols
Saponins
Tigogenin

ABSTRACT

Besides the use of micropropagation to generate massive production of plants, it is an excellent option to produce secondary metabolites under controlled environmental conditions in a large scale. Biological effects have been attributed to phytochemicals found in *Agave* spp. In previous works, *Agave salmiana* Otto ex Salm-Dyck plants have been successfully micropropagated but it is not known how this process and the conditions used during plant acclimation affect the phytochemical profile. A total of 7 flavonols and 5 saponins were quantified in *in vitro* micropropagated plants (IN) as well as in acclimated regenerated plants in open environment (EN). Flavonols were quantified by HPLC-DAD as equivalents of kaempferol or quercetin (aglycone standards). In wild-type plants (WT), only one herbacetin glycoside (HBG1) and one quercetin glycoside (QG1) were detected. HBG1 was the most abundant flavonol found in WT (14.7 mg/100 g dw), IN (16.3 mg/100 g dw) and EN (38.4 mg/100 g). Saponins were quantified by HPLC-ELSD as protodioscin equivalents (PE). Saponins identified were glycosides of chlorogenin, tigogenin and hecogenin. Tigogenin glycoside (TG) was only found in the plants that passed through the *in vitro* process and it was the most abundant saponin. Particularly, tigogenin glycoside (TG) was more abundant in IN (6895.2 mg PE/100 g dw) than in EN (4997.8 mg PE/100 g dw) plants. In summary, *in vitro* micropropagated plants had higher concentration of flavonols and saponins compared with the wild type agave plants.

1. Introduction

The *Agave* species with major revenue produced in Mexico belong to “magueys pulqueros” (SIAP, 2014). This group includes the species: *Agave americana*, *Agave atrovirens*, *Agave mapisaga* and *Agave salmiana* (Cedeño, 1995). *Agave salmiana* Otto ex Salm-Dyck is a domesticated plant with a high rate of exploitation in Mexico (Colunga-GarcíaMarín and Zizumbo-Villarreal, 2007). It is used to produce honeywater or “aguamiel” and pulque (Santos-Zea et al., 2012; Silos-Espino et al., 2007). In some regions, it is used as a source of sugars for the production of mezcal and as a complement to *Agave tequilana* Weber for tequila (Salvador et al., 2012; Michel-Cuello et al., 2008).

The overexploitation of *A. salmiana* limits both its asexual and sexual reproduction (Silos-Espino et al., 2007), so it is necessary the use of *in vitro* micropropagation strategies for massive production of plants (García-Herrera et al., 2010; Silos-Espino et al., 2007). Recent and previous micropropagation work had been successfully done on *A. salmiana* to regenerate plantlets from axillary shoots (Puente-Garza et al., 2015). It was observed that the antioxidant activity varied between regenerated and wild-type plants, suggesting that bioactive compounds were produced during the micropropagation process. In other crops, micropropagation promoted the generation of specific metabolites under controlled environmental conditions for large-scale production (Alonso-Herrada et al., 2016; Arencibia et al., 2017; Basu and Jha, 2013; Chavan et al., 2015; Costa et al., 2012; Santoro et al., 2013; Verma et al., 2016).

Saponins are the most studied molecules in *Agave* spp. with demonstrated health benefits such as anticancer, anti-inflammatory and antifungal activity (Francis et al., 2002; Santos-Zea et al., 2012). Glycosides of hecogenine, diosgenine, chlorogenine, kammogenin, gentrogenin, among others have been found in leaves of mature agave plants (Pérez et al., 2014; Yokosuka and Mimaki, 2007). Similar saponins had been also reported in “aguamiel” from *A. salmiana* (Leal-Díaz et al., 2015).

An important richness of biologically active phenolics exists in several species of genus *Agave* (Almaraz-Abarca et al., 2013a). Flavonols, such as kaempferol and quercetin, in different glycosylated forms have been detected in the mature flowers of *A. duranguensis* (Barriada-

Abbreviations: WT, wild-type plants from field; IN, *in vitro* micropropagated plants; EN, *Ex vitro* acclimated plants; masl, meters above sea level; PE, protodioscin equivalents

* Corresponding author.
E-mail address: jagu@itesm.mx (J.A. Gutiérrez-Uribe).

http://dx.doi.org/10.1016/j.indcrop.2017.05.014
Received 15 December 2016; Received in revised form 6 May 2017; Accepted 9 May 2017

Bernal et al., 2014). Kaempferol has also been recovered from the residue of *A. sisalana* leaves after fiber removal (Santos et al., 2015)

Although there are works on micropropagation of *A. salmiana* (Puente-Garza et al., 2015; Ramírez-Malagón et al., 2008; Silos-Espino et al., 2007), there is not enough information about the effects on phytochemical profile. The aim of this study was to evaluate and analyze the presence and concentration of saponins and flavonols in wild-type and new plants generated by micropropagation of maguey, *A. salmiana*, as a strategy to generate metabolites of interest.

2. Materials and methods

2.1. Plant material

Regenerated plants were produced *in vitro* using a protocol for axillary shoots from seeds of *A. salmiana* (Puente-Garza et al., 2015). Two 1-year old plants were collected from a commercial plantation in Ejido Puebla, Saltillo, Coahuila, Mexico (25°24′54″N; 101°18′11″O; 1442 masl) provided by Agmel SA de CV Company (Mexico); the average relative humidity (RH) was 57%, with a precipitation of 16.26 mm during October 2012. According to a previous work from our research group, a voucher specimen was deposited in the herbarium of the Universidad Autónoma de Nuevo León (UANL) in Mexico under the number 025619 (Leal-Díaz et al., 2015). The average temperature registered was 20.5 °C, with maximum of 28.8 °C and minimum of 10.8 °C. The plant samples were pathogen and disease-free and from each plant, 9 samples were taken.

After micropropagation, 10 plants were acclimated by exposing them to open environment conditions during March 2013 in Tecnologico de Monterrey (25°38′43. 93″N; 100°17′01. 07″O; 532 masl), with a RH of 49.2%, 15.5 mm of precipitation, and an average temperature of 20.4 °C, with maximum of 28.9 °C and minimum of 14.4 °C.

2.2. Samples preparation for extracts analysis

Leaf tissue samples were taken from 13-week-old *in vitro* (IN) plants, 18-week-old *ex vitro* acclimated plants in open enviroment conditions (EN) at Tecnologico de Monterrey and wild-type plants (WT) from natural populations in Coahuila. All samples were stored at −80 °C overnight and then lyophilized. Dried leaf tissues were pooled (leaves of 50 IN plants, leaves of 10 EN plants and 9 leaves of two WT plants) and ground using a molecular miller with mixer ball (MM 400, Retsch, Verder Scientific, Germany) to reach a particle size lower than 100 μm. Then, 100 mg of leaves powder and 1 mL of methanol-water 80:20 (v/v) solution were agitated (Vortemp 1550, Labnet Int. Inc., Edison, NJ) during 2 h at 150 rpm and 30 °C. Then, the extract was centrifuged at 3000 rpm for 5 min. The supernatant was then dried at vaccum, re-suspended in 1 mL methanol-water 50:50 (v/v) and stored at −20 °C for further analysis (García-Pérez et al., 2011; Guajardo-Flores et al., 2012). At least three extracts were prepared from each pooled sample.

2.3. Determination and identification of phenolic compounds and saponins

Saponins and phenolic compounds were analyzed using HPLC–MS-TOF (Model G1969A, Agilent 1100, Santa Clara, CA) and a Zorbax Eclipse XDB-C18, 4.6 × 150 mm (5 μm) column (Agilent Technologies, Santa Clara, CA). A gradient elution, as proposed by Leal-Díaz et al. (2015) with modifications to improve the resolution of flavonols, was used. Briefly, the program consisted of phase A (water and 0.1% formic acid) and phase B (acetonitrile with 0.1% formic acid) at a flow rate of 0.8 mL/min. The gradient was set and ran as follows: 82% of phase A was maintained during the first 15 min and then decreased to 25% in the next 10 min, and maintained for 5 min before reducing to 0% in 10 min to keep running only with 100% B during the last 10 min. The mass spectra was collected using electrospray source in positive mode (ESI +) under the following conditions: 300–1500 *m/z* range; nitrogen gas at 350 °C and 9.0 L/min flow rate; 45 psig nebulizer pressure; 4000 V capillary voltage, fragment voltage 135 V and skimmer at 40 V. Saponin and phenolic compounds were identified based on the fragmentation pattern and accurate mass previously reported.

For metabolites quantification, HPLC (Agilent Technologies, 1200 series, Santa Clara, CA) coupled to Photodiode array detector (PDA) and evaporative light scattering detector (ELSD) were used, following the same chromatographic separation established for the HPLC–MS-TOF. The injection volume for the IN and EN samples was 1 μL and along with WT, the samples were also injected with a volume of 5 μL due to the quantification limits of the less abundant saponins. Data were collected at 340 nm to quantify flavonoids using standards of kaempferol and quercetin (Sigma-Aldrich, St. Louis, MO) with the following equations: $y = -55.302 + 17.794x$ ($R^2 = 0.994$) and $y = -41.660 + 10.177x$ ($R^2 = 0.992$), respectively. Saponins were quantified as protodioscin equivalents (PE) using the equation $y = -143.152 + 9.895\,x$ ($R^2 = 0.999$) (Sigma-Aldrich, St. Louis, MO) detected by ELSD using nitrogen as drying gas, pressure at 3.4 bar and tube temperature of 45 °C as previously reported (Leal-Díaz et al., 2015).

2.4. Statistical analysis

The contents of saponins and flavonols were subjected to analysis of variance (ANOVA) and differences among means were compared by Tukey tests at $P < 0.05$. All the analyses were done using software Minitab 16 of Minitab Inc.

3. Results and discussion

3.1. Identification of phenolic compounds and saponins

In the present study, herbacetin glycosides (HBG1, *m/z* = 757.26 $[M+H]^+$; HBG2, *m/z* = 611.20 $[M+H]^+$), one kaempferol triglycoside (KG1, *m/z* = 727.19 $[M+H]^+$) and one diglycoside (KG2, *m/z* = 581.18 $[M+H]^+$). Quercetin glycosides (QG1-3) were identified based on their UV–vis spectrum (Table 1, Fig. 1). The presence of kaempferol and quercetin was consistent with previous reports for *A. duranguensis* flowers (Barriada-Bernal et al., 2014) and for *Agave victoriae-reginae* foliar tissue (Almaraz-Abarca et al., 2013b); however, the number of sugars attached to aglycone were not previously reported. Hesperidin glycosides were differentiated from those derived from quercetin based on comparison of the UV–vis spectra previously reported (Almaraz-Abarca et al., 2013b).

Five saponins derived from chlorogenin, tigogenin and hecogenin were detected in the *A. salmiana* leaves (Fig. 2, Table 2). Previously, glycosylated forms of these sapogenins had been identified in plants of *Agave* genus (Eskander et al., 2010; Olvera-García et al., 2015; Pant et al., 1986). Glycosilated forms of chlorogenin, different from CG1 (pentasacharide) and CG2 (tetrasaccharide), had been previously reported in other *Agave* species such as *A. fourcroydes* (Ohtsuki et al., 2004). A saponin with mass spectra similar to tigogenin glycoside (TG) had been previously reported by and Ding et al. (1989) in *A. sisalana* and in *A. atrovirens* by Olvera-García et al. (2015). Hecogenin glycoside 1 (HG1) was reported as cantalasaponin 4 from *A. cantala* (Pant et al., 1986). Hecogenin glycoside 2 (HG2) was reported as agavasaponin C' (Bodeiko and Kyntia, 1975) or as cantalasaponin 2 (Pant et al., 1986) and also identified in *A. macrocantha* (Eskander et al., 2010).

3.2. Quantification of flavonols and saponins

The WT plants exhibited less flavonols (19.1 mg/100 g dw), compared with IN and EN (52.0 and 111.2 mg/100 g dw, respectively) (Table 3). In the IN plants, HBG2 was the only flavonol that was not detected. EN plants had the highest content of all flavonols and the