SUSTAINABLE AND INTEGRAL EXPLOITATION OF AGAVE

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EFFECTS ON SAPONIN, FLAVONOL AND ANTIOXIDANT ACTIVITY IN *in vitro* PLANTS OF *Agave salmiana*

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**ABSTRACT**

Maguey, *Agave salmiana*, is an important plant for “pulque” industry and functional foods but has several constraints of elite germplasm availability and homogeneous nutraceutical properties. A micropropagation protocol was established to generate *in vitro* plants from young germinated plantlets by axillary shoots. At the same time we evaluated the impact of this process in the phytochemical profile of the new plants. The optimal induction of axillary shoots was observed in plantlets incubated on a solidified Murashige and Skoog (MS) medium supplemented with L2 vitamins and 0.04 mg l⁻¹ 2,4-Dichlorphenoxyacetic acid (2, 4-D) and 10 mg l⁻¹ 6-benzylaminopurine (BAP). The *in vitro* protocol took 16 weeks, obtaining an efficiency of 87.5% after acclimatization under controlled conditions. The total phenolic (TP) content, antioxidant (AOX) capacity and identification of phenolic compounds and saponins were performed in wild type (WT) plants, *in vitro* (IN), *ex vitro* with irrigation (EXW) and *ex vitro* at normal environmental conditions (EXN).

The highest TP content were in IN and EXN plants, however AOX capacity for IN plants was 3-fold higher than EXN and EXW plants, and more than 3-fold higher compared to WT plants. Two different glycosylated flavonols were detected in EXW (quercetine) and EXN (kaempferol). Saponins such as hecogenin (0.418-4.321 mgEHe g⁻¹), tigogenin (18.821-31 mgEHe g⁻¹), manogenin (0.288-0.861 mgEHe g⁻¹) and chlorogenin (0.339-2.042 mgEHe g⁻¹), in different glycoside form were detected and quantified. Tigogenin was only found in the plants that pass through *in vitro* process, being more concentrated in IN plants.

In summary, we successfully micropropogated and regenerated *A. salmiana* plants from seeds and they contained different amount of their TP, flavonoids and saponins and AOX capacity compared with WT.

**Keywords:** axillary shoots, micropropagation, tigogenin, hecogenin, kaempferol
INTRODUCTION
The agaves are succulent plants native from Mexico, southwest region of U.S.A., Central America and Canary Islands. Around 75% of the species can be found in Mexico, and 74% of these are endemic (García, 2007; Martínez-Salvador et al. 2005). The Agave species with major revenue produced in Mexico belong to magueys “pulqueros” (Siacon, 2014). This group is represented by the species of Agave americana, Agave atrovirens, Agave mapisaga and Agave salmiana (Cedeño, 1995; García, 2007). Agave salmiana have sexual and asexual reproduction strategies (Arizaga and Ezcurra, 2002). Ramírez-Tobías et al. (2011) determined that 50% of seedlings can be more vigorous than offshoots in A. salmiana, however the availability of seed is limited. The use of micropropagation is an option for the lack of germplasm and has several advantages such as to obtain populations with elite characteristics, stress tolerance, pathogens free, and stable genetic background (Domínguez-Rosales et al. 2008). Many reports have highlighted that Agave species contains phytochemicals with bioactivity (Almaraz-Abarca et al. 2009; Guerra de León et al. 2007; Gutiérrez et al. 2008; Morales-Serna et al. 2010).

The aim of this study was to establish a strategy of micropropagation for A. salmiana, evaluate the impact of this process in the total phenolic compounds and saponins content, antioxidant activity and determine which relation is between the bioactivity and the content of secondary metabolites.

METHODOLOGY
Seeds of A. salmiana were germinated in vitro, establishing an optimal germination protocol for further micropropagation. Three weeks old plantlets were multiplied by axillary shoots method. After remove roots, one single plant was cultured in jars with 20 ml of solid culture medium MS modified with L2 vitamins (Santacruz-Ruvalcaba et al. 1999). After two weeks, 6-benzylaminepurine (BAP) and 2, 4 –Dichrolophenoxyacetic acid (2, 4-D), were added to new MS+L2 solid culture medium (Santacruz-Ruvalcaba et al. 1999). The cultures were transferred to a room with temperature set to 27°C with a photoperiod of 16:8h light:dark. The number of axillary shoots and presence of callus, after 60 days of culture were observed (Santacruz-Ruvalcaba et al. 1999). Once were multiplied, they were put in acclimatization medium and let the plants growth for 30 days. Rooted plants obtained from the previous steps were transferred to field. Irrigation was applied twice per week for the half of plants and the other half was not irrigated to simulate normal field conditions. Total phenolic (TP) concentration was determined using the Folin-Ciocalteu reagent according to the method of Zheng and Wang (2001) and antioxidant (AOX) capacity was determined using the oxygen radical absorbance capacity assay (García-Pérez et al., 2011). Saponins and phenolic compounds were detected and identified using HPLC-MS-TOF and quantified using HPLC-PDA-ELSD. Phenolic compounds were quantified as aglycones of kaempferol, quercetin or myricetin and saponins were quantified as aglycone hecogenin equivalents.

RESULTS AND DISCUSSION
An efficient micropropagation protocol was established to regenerate plants of A. salmiana. Table 1 summarizes the results of axillary shoots generation in A. salmiana. The combination of plant growth regulators to produce the high axillary shoots was 10 mg l⁻¹ of BAP and 0.04 mg l⁻¹ of 2, 4-D, obtaining 14 shoots per explant after 60 days.
Table 1 Number of axillary shoots generated after 60 days by a combination of 2,4-D and by axillary shoots generation technique.

<table>
<thead>
<tr>
<th>BAP (mg l⁻¹)</th>
<th>2, 4 - D</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.66</td>
<td>0.25</td>
<td>0.00</td>
<td>1.66</td>
</tr>
<tr>
<td>0.5</td>
<td>3.00</td>
<td>2.75</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>1.0</td>
<td>1.33</td>
<td>2.66</td>
<td>2.33</td>
<td>0.33</td>
</tr>
<tr>
<td>5.0</td>
<td>2.33</td>
<td>6.00</td>
<td>10.33</td>
<td>0.00</td>
</tr>
<tr>
<td>10.0</td>
<td>3.50</td>
<td>2.00</td>
<td>7.33</td>
<td>14.00</td>
</tr>
</tbody>
</table>

* BAP: 6-Benzylaminopurine. 2-4-D: 2, 4 – dychlorophenoxyacetic acid

This protocol allows us to regenerate whole plants from wild type genotypes in 16 weeks using the germinated young plantlets. This protocol was 40% more efficient in term of time and 25% more efficient in term of plants/explant than the proposed by Chen et al. (2014) for A. Americana.

Table 2 Phenolic compound and saponin content. Phenolic compounds are quantified as aglycones of the corresponding flavonol (mg/g dw) and saponins in Hecogenin equivalents (mg EHe/g dw) NQ = no quantifiable metabolite.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EXW</th>
<th>EXN</th>
<th>IN</th>
<th>WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaempferol 1</td>
<td>0.290</td>
<td>0.384</td>
<td>0.163</td>
<td>0.147</td>
</tr>
<tr>
<td>Kaempferol 2</td>
<td>0.000</td>
<td>0.132</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Quercetin 1</td>
<td>0.117</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Manogenin 1</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>NQ</td>
</tr>
<tr>
<td>Manogenin 2</td>
<td>0.861</td>
<td>NQ</td>
<td>0.474</td>
<td>0.288</td>
</tr>
<tr>
<td>Chlorogenin 1</td>
<td>1.104</td>
<td>2.042</td>
<td>0.678</td>
<td>0.339</td>
</tr>
<tr>
<td>Chlorogenin 2</td>
<td>0.970</td>
<td>1.707</td>
<td>1.101</td>
<td>0.595</td>
</tr>
<tr>
<td>Gentrogenin 1</td>
<td>0.000</td>
<td>1.190</td>
<td>1.437</td>
<td>0.891</td>
</tr>
<tr>
<td>Tigogenin 1</td>
<td>18.821</td>
<td>22.625</td>
<td>31.007</td>
<td>0.000</td>
</tr>
<tr>
<td>Hecogenin 1</td>
<td>3.785</td>
<td>4.231</td>
<td>3.168</td>
<td>0.418</td>
</tr>
<tr>
<td>Hecogenin 2</td>
<td>5.227</td>
<td>3.561</td>
<td>0.968</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Free phenolic compounds were detected in all extracts of A. salmiana. WT plants show higher content of phenolic, with 13.06 mgEGA/g dw and EXW had the lower concentration with 9.02 mgEGA/g dw. All the extracts show antioxidant activity. The IN plants shows the higher antioxidant activity (369.84 µmolTE/g dw). Between WT (130.39 µmolTE/g dw), EXW (143.38 µmolTE/g dw) and EXN (184.13 µmolTE/g dw) samples, there was not significant statistically difference. The differences could be because the presence of different molecules in response of the environment from which the plants came from (Almaráz-Abarca et al., 2013). It could be identified a total of three different flavonols and 8 different saponins in the extract (Table 2). The number of flavonols identified coincide with A.
americana and A. sisalana, which had reports from 2 to 4 different flavonols, but mismatch with other species, such as A. victoria-reginae and A. striata, which number of identified flavonols ascends to 11 and 14 (Almaráz-Abarca et al., 2013). Tigogenin was the saponin more abundant compared with the other saponins. Hecogenin glycoside 2 was not found in WT. The tigogenin glycoside 1 and hecogenin glycoside 2, were a kind of marker to identify the plantlets that were generated in in vitro and the wild-type. The tigogenin generated the higher concentration in the IN samples, confirming the response of the secondary metabolites to an environment of micropropagation with high moisture (Barreto et al., 2010).

CONCLUSION

Agave salmiana was micropropagated by axillary shoots from seeds. The process of micropropagation generated 14 buds per explant, with an efficiency of 87.5% in 16 weeks. The micropropagation causes changes in the antioxidant activity of the extracts but not had the same effect in the phenolic compound composition. The saponins hecogenin and tigogenin appeared only in plants that went through the micropropagation process. The tigogenin content was much more higher compared to other saponins, presenting the highest content in the IN. This suggest that the process of micropropagation could be a platform for the generation of steroidal compounds in agave, avoiding to wait long periods of time to generate biomass for industry like pharmaceutical, that employs this kind of compounds as precursors.

ACKNOWLEDGEMENTS

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REFERENCES


