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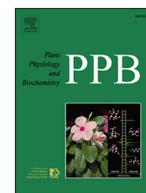

Short communication

Effect of *in vitro* drought stress on phenolic acids, flavonols, saponins, and antioxidant activity in *Agave salmiana*

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A B S T R A C T

This work evaluated the effect of *in vitro* drought stress on morphological characteristics, phenolic compounds, flavonols, saponin content, and antioxidant activity in plantlets of *Agave salmiana*. Drought stress was induced with polyethylene glycol (PEG) at 0, 10, 20 and 30% w/v in Murashige and Skoog solid medium. The determination of specific flavonols and saponins was achieved via HPLC-DAD and HPLC-ELSD, respectively. Compared with the control, plants grown in 30% PEG showed a change in the width of the leaves and a different color, showing less clarity and more darkening ($L = 21.18$, $b = 14.27$) and also had the lowest flavonol content, but the highest total saponin content (tigogenin glycoside, 163 mg of protodioscin equivalents/g dw) and the highest antioxidant activity. Total phenolic compounds did not significantly differ between treatments. *Agave salmiana* plants cultured *in vitro* increased their saponin content and antioxidant activity in response to drought stress induced via PEG.

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1. Introduction

Agave plants are native to Mexico, USA, Central America, and Canary Islands (Martínez-Salvador et al., 2005). In Mexico, this plant is present in 75% of the territory (Nava-Rodríguez, 2014) with a commercial production of 1.6 million Ton in 2012 (Castro-Díaz and Guerrero-Beltrán, 2013). Among the most important agaves are the magueys “pulqueros”, which are represented by the species *Agave americana*, *Agave atrovirens*, *Agave mapisaga*, and *Agave salmiana* (Ortiz-Basurto et al., 2008). *Agave salmiana* is the main species produced and exploited for commercial purposes in central and north of Mexico (Ortiz-Basurto et al., 2008) and it is aimed mostly for the production of beverages such as mezcal and pulque (García-Herrera et al., 2010), but also as a source of bioactive ingredients, such as saponins and phenolic compounds (Santos-Zea et al., 2012). The agavaceae family is an important source of sapogenins with steroidal nature and mainly for saponins, which is the principle in steroidal hormones in laboratory synthesis (Eskander et al., 2010; Nava-Cruz et al., 2015). Compared to other wild *Agave* species, *A. salmiana* has a wide distribution, leaves with a larger surface area, and a 10-fold higher field yield (Pinos-Rodríguez et al., 2009).

Agave salmiana is mostly composed of carbohydrates, which accumulate in the form of fructans, specifically fructose, glucose, xylose, sucrose (Mancilla-Margalli and López, 2006) and oligosaccharides (Ortiz-Basurto et al., 2008). The secondary metabolites found in *Agave salmiana* are phenolic compounds and saponins (Leal-Díaz et al., 2015). The phenolic compounds include flavonols, such as kaempferol and quercetin, in various glycosylated forms (Almaraz-Abarca et al., 2013; Barriada-Bernal et al., 2014) and other flavonoids (Hamissa et al., 2012). Saponins are important secondary metabolites in *Agave* spp (Santos-Zea et al., 2012), and the presence of glycosides of hecogenin, diosgenin, chlorogenin, kammogenin and gentrogenin in the leaves of mature plants has been established (Yokosuka and Mimaki, 2007; Pérez et al., 2014). Saponins have been found useful for many purposes; besides aiding the plant against insect attack, they also exhibit antibacterial, antifungal, anti-cancerogenic, anti-inflamatory, antiobesity, hypocholesterolemic, immunostimulant and antiparasitic properties (Leal-Díaz et al., 2015; Augustin et al., 2011). In pharmaceutical industry, steroideal saponins are important due of its transformation into

Abbreviations: MS, Murashige and Skoog; PEG, Polyethylene-Glycol; ORAC, Oxygen radical absorbance capacity; TP, Total Phenolic; AOX, Antioxidant Capacity; PE, Protodioscin equivalents.

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pharmaceutically valuable derivatives, such as corticosteroids, sexual hormones and steroid diuretics (Santos and Branco, 2014). China, one of the major producers of steroids in the world, uses wild *Dioscorea* species, *Agave americana* L. and *Agave dong* 1, to extract sapogenins (Yanyong and Yue, 2015). Similar saponins have been also reported in the "aguamiel" of *A. salmiana* (Leal-Díaz et al., 2015). From a nutraceutical point of view, several reports have identified high levels of bioactive phytochemicals in *Agave* by/end-products, which have been associated with its antioxidant, anticancer and antidiabetic properties (Hamissa et al., 2012; Ahumada-Santos et al., 2013; Chirinos et al., 2013; Ribeiro et al., 2013; Barriada-Bernal et al., 2014; Zhang et al., 2014). Therefore, *in vitro* culture is a feasible tool for use in examining the production of high levels of secondary metabolites in plants for industrial uses (Zhong et al., 1996; Okršlar et al., 2007) and can be applied to *A. salmina*.

These desert plants are frequently exposed to a wide range of stresses, such as drought, extreme temperatures, salt, oxidative stress and heavy metal toxicity (Jaleel et al., 2009). Drought especially reduces plant growth by affecting various physiological and biochemical processes, such as photosynthesis, respiration, carbohydrates, nutrient metabolism and secondary metabolism (Jaleel et al., 2009). Water deficit has been proven to alter secondary metabolite accumulation (Marchese et al., 2010). Studies of *Agave amaniensis* have reported an increase of saponin content in the presence of heavy metals (Andrijany et al., 1999), while water stress in *in vitro* cultures of *Agave tequilana* drastically modified fructan content (Barreto et al., 2010), suggesting that *Agave* plants do not respond in the same way to water stress under *in vitro* conditions. Recently, *A. salmiana* has been proposed as a model to study the physiological and physicochemical changes involved in drought tolerance due to the ability of the plants to survive under harsh conditions (Peña-Valdivia and Sánchez-Urdaneta (2009)).

High-molecular-weight solutes, such as polyethylene glycol (PEG), have been used to induce water deficit in plants in *in vitro* tissue culture (Verslues et al., 2006). The use of stress in *in vitro* plant cell cultures has been widely used to induce the *de novo* synthesis of compounds (Ramakrishna and Ravishankar, 2011), such as phenols (Sánchez-Rodríguez et al., 2011). Therefore, stress induced *in vitro* will help in understanding the mechanism of action and the synthesis and regulation of key phytochemicals, such as phenols and saponins. Due to the fact that young *Agave* plants have high levels of bioactive compounds and are a better source of secondary metabolites than mature plants (Francis et al., 2002; Leal-Díaz et al., 2015), it is possible that young *in vitro Agave salmiana* plants could be a good source of metabolites with antioxidant activity, such as phenolic compounds and saponins. Antioxidant activity has been reported for different *Agave* plants, being found mostly in their leaves (Santos-Zea et al., 2012). In the present study, *A. salmiana* plants were exposed to severe drought conditions using high doses (>10%) of PEG because previous studies (Barreto et al., 2010) have demonstrated that low PEG concentrations do not modify the morphological, physiological and biochemical characteristics of these plants.

The aim of this work is to evaluate the differential response to *in vitro* drought stress on the part of 7-week-old *A. salmiana* plantlets in terms of their morphological plant characteristics; phenolic compound, flavonol and saponin content and antioxidant activity.

2. Materials and methods

2.1. Plant material

Agave salmiana seeds and plants were provided by the Agmel SA de CV Company. Seeds were collected in October 2014 from a natural population in Ejido, Puebla, Saltillo, Coahulia, Mexico (25°24′54″N; 101°18′11″O; 1442 masl). Seeds were taken from dehiscent fruits and germinated in the first two months after collection.

2.2. In vitro plants

In vitro A. salmiana plants were established by growing seeds under sterile conditions and established at the Plant-Insect Molecular Interaction Laboratory. The seeds were surface-disinfected by soaking them in distilled water containing 1.5% (v/v) of commercial liquid soap and 200 μL of Tween 20® (Sigma-Aldrich, St. Louis, MO) for 2 min. They were then washed with distilled water for 5 min and placed in a solution of 50% (v/v) commercial bleach (Cloralex®, 5.25% w/w, Monterrey, NL) for 15 min. The seeds were then submerged in 96% ethanol for 2 min and washed with distilled water. Mechanical scarification was performed by removing the area close to the seed micropyle using a scalpel. A batch of 100 seeds was cultivated in glass jars with 20 mL of Murashige and Skoog (MS) 2.2 g/L, pH 5.8, at a density of five seeds per jar. The cultures were transferred to an environmental chamber (Sheldon Manufacturing, Inc., Cornelius, OR) set to 27 °C and a photoperiod of 12:12 h light:dark (6600 lux).

2.3. Stress induction

The stress medium consisted of MS 4.4 g/L, pH 5.8, 30 g/L sucrose and L2 vitamins (Puente-Garza et al., 2015) supplemented with various concentrations of polyethylene-glycol, PEG (PEG8000, Sigma Aldrich®, St Louis, MO). Four treatments were established: 0% PEG or control (CTL), 10% PEG (P10), 20% PEG (P20) and 30% PEG (P30) (Barreto et al., 2010). *In vitro* seven week-old plantlets were selected to be transplanted into the stress medium according to their vigorousness and healthy appearance. Roots from the selected plants were trimmed before being transplanted to the stress medium according with Kelly (2009). Two plantlets per jar were transferred, with five jars per treatment. Then, the *in vitro* cultures were placed in an environmental chamber (Sheldon Manufacturing, Inc., Cornelius, OR) set to 27 °C and a photoperiod of 12:12 h light:dark (6600 lux) for 60 days (Barreto et al., 2010).

2.4. Phenotypic evaluation and sample preparation

After 60 days, the *in vitro* plants were removed from the environmental chamber and evaluated in terms of the phenotypic characteristics of each treatment. The plants were separated by hand from the culture media, and the number of leaves per plant, the length and width of the leaves per plant and the number of plants with roots were evaluated and recorded (Peña-Valdivia and Sánchez-Urdaneta, 2009). Color parameters was performed by image software analysis (Image J, Maryland, USA) and color parameters L^*, a^* and b^* were taken. The plants from each treatment were collected separately, stored at −80 °C overnight and then lyophilized. The dried plant tissue was ground using a mixer ball mill (MM 400; Retsch/Verder Scientific, Col. Germany) and stored at −20 °C for further phytochemical analyses.

2.5. Phytochemical extraction

The dried plant tissue (100 mg) was homogenized and extracted using 1 mL of methanol-water 80:20 (v/v) solution. The extract was placed in a shaking incubator (Vortemp 1550, Labnet Int. Inc., Edison, NJ) for 2 h at 150 rpm and 30 °C. Then, the extract was centrifuged at 3000 rpm for 5 min. The supernatant was separated, vacuum dried, re-suspended in 1 mL methanol-water 50:50 (v/v),

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