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Research article

Effects of saline elicitors on saponin production in *Agave salmiana* plants grown *in vitro*

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ABSTRACT

Agave plants are natives of Mexico and have an important role in the functional food industry. *Agave salmiana* grows in dry and desert soils, which are high in salt content; however, little is known about its response to saline conditions. In this study, *A. salmiana* plants grown *in vitro* were exposed to 0.1, 0.5, and 1.0 mM of salt elicitors, including $AlCl_3$, NaCl, and $CoCl_2$, and saponin synthesis and morphological characteristics were examined. Saponins were identified and quantified in ethanolic extracts using HPLC-ELSD. Root length and number, leaf length and number, and plant fresh weight were evaluated to determine the phenological condition of the plant. The presence of salts at various concentrations did not affect the physiological characteristics of the plant. Moreover, 0.5 mM NaCl induced a higher production of total saponin. Chlorogenin glycoside 1 (CG1) and hecogenin glycoside 1 (HG1) content remained unchanged across treatments. By contrast, CG2 and HG2 concentrations tended to decrease in response to increased concentrations of $AlCl_3$, NaCl, or $CoCl_2$. *In vitro* salt elicitors could be a feasible tool in the synthesis of specific saponins, without compromising on plant biomass. Our findings can be used in further generation of low saponin agave plants in field for the improvement of fermentation yield.

1. Introduction

Agave species are succulents native to Mexico, the southwest region of USA, Central America, and the Canary Islands; approximately 75% of known species are found in Mexico, of which 74% are endemic (Martínez-Salvador et al., 2005). In Mexico, "agaves pulqueros'' represent an industry of more than $42 million USD (SIAP, 2019) and include *A. americana*, *A. atrovirens*, *A. mapisaga*, and *A. salmiana* (Puente-Garza et al., 2017a), which are being explored as a commercial source of alcohol and nutraceuticals, such as phenolic compounds, policosanols, and saponins (Santos-Zea et al., 2012).

In the arid and semi-arid regions of the world, soil salinity is a primary constraint (Razaq, 2018). In Mexico, one problem associated with climate is desertification, which affects 22.17% of the national territory (SEMARNAT, 2017). The most abundant type of soil in Mexico includes leptosol (covers 28.3% of national territory), followed by regosol (13.7%), phaeozems (11.7%), and calcisol (10.4%) (SEMARNAT, 2017). Calcisol is found in the arid zones of Mexico, including Chihuahua, Aguascalientes, Baja California, Coahuila, Durango, Nuevo León, Sonora, and Zacatecas (SEMARNAT, 2017). Under these conditions, salts and minerals can regulate the production of secondary plant metabolites, such as saponins, by up- or down-regulating signaling pathways (Lozano et al., 2017), thereby benefiting the plant by participating in mechanisms of plant defense and environmental interactions (Lozano et al., 2017; Szakiel et al., 2010). Although soil salinity is potentially damaging to the plant, it can increase the production of or induce *de novo* synthesis of secondary metabolites in plants cultured *in vitro* (Espinosa-Leal et al., 2018).

Furthermore, although these soils can be harsh for plant development, the presence of some non-essential salts at low concentrations (0.1–0.5 mM) can improve plant growth (Wa Lwalaba et al., 2017; Sathyseelan and Karthika, 2019). Cobalt is present in acidic or calcareous soils and can interfere with chlorophyll biosynthesis, causing the formation of reactive oxygen species; however, it can be used to raise crop yield (Wa Lwalaba et al., 2017). Studies on *in vitro* cultures have shown that at a low concentration, Co increases the quality of tissues (Al-Mayahi, 2013). Similarly, the uptake of Al induces oxidative burst by the induction of NADPH oxidase in the cell walls (Achary et al., 2012); however, at low concentrations, Al can improve plant yield and development (Sathyaseelan and Karthika, 2019). By contrast, in some *Agave*

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spp., such as *A. utahensis*, high soil concentration of NaCl can cause a deficiency of nutrient ions, including Ca^{2+}, K^{+}, and Mg^{2+}, because of uptake competition (Bergsten et al., 2016). However, NaCl acts as an elicitor for saponin production (Haghigi et al., 2012).

In vitro platforms have been suggested as suitable tools for the biosynthesis of secondary metabolites (Espinosa-Leal et al., 2018). Induction of *in vitro* drought stress in *A. salmiana* was shown to increase the accumulation of phenolic compounds and saponins (Puente-Garza et al., 2017b). The use of different salts as abiotic elicitors in *in vitro* cultures and plants, been shown to increase the secondary metabolite content in plants, including *Hordeum vulgare* (Ali and Abbas, 2003), *Cassia acutifolia* (Nazif et al., 2000), *Datura metel* (Ajungla et al., 2009), *Grevillea* spp. (Kennedy and De Filippis, 1999), *Solanum* spp. (Daneshmand et al., 2010), and *Digitalis lanata* (Ohlsson and Berglund, 1989), indicating that secondary metabolite content can be increased by using salts. In *Agave* spp., saponins are the best studied and have been shown to have physiological, immunological, and pharmacological properties, in addition to having health benefits due to their anticancer, anti-inflammatory, and antifungal activities (Santos-Zea et al., 2012; Leal-Díaz et al., 2015). *In vitro* studies have reported that Co improves the synthesis of saponins in the callus of *Agave amaniensis* (Andrijany et al., 1999), and PEG acts as an elicitor to improve saponin content and bioactivity in *A. salmiana* (Puente-Garza et al., 2017b). *A. salmiana* has a high potential as a source of high value products, such as saponins, in the presence of saline elicitors found in the soil and has been scarcely explored.

Therefore, we used an *in vitro* model of agave plants, where the plants were exposed to different concentrations of salt elicitors ($AlCl_3$, NaCl, and $CoCl_2$), and assessed their response in terms of saponin content and phenotypic characteristics.

2. Materials and methods

2.1. Plant material and media preparation

Four-month-old *A. salmiana* plants (n = 50) grown from seed *in vitro* were used. Briefly, the seeds were surface disinfected using a solution of 50% (v/v) commercial bleach (Cloralex®, 5.25% w/w, Monterrey, Mexico) for 15 min. Then, the seeds were submerged in 96% ethanol for 2 min and washed with distilled water. The seed were cultivated in jars containing 20 mL 1/10 MS solid culture media. The cultures were transferred to an environmental chamber set at 27 °C, with a photoperiod of 12:12 h (light:dark) (Puente-Garza et al., 2015). Light intensity was set to 6600 lux. After 12 wk, the plants were transferred to jars containing 20 mL of fresh solid MS + L2 media supplemented with 0.3% sucrose. The roots were removed before transplantation and one plant was cultivated per jar, maintained in the chamber at 27 °C with a photoperiod of 16:8 h (light:dark). After 3 wk, the plants were transferred to MS + L2 and different salt concentrations. NaCl, $AlCl_3$, and $CoCl_2$ were used as elicitors at 0.1, 0.5, and 1 mM. The cultures were evaluated for 90 d in the chamber at 27 °C with a photoperiod of 16:8 h (light:dark).

2.2. Physical characteristics of the plants

After 90 d of *in vitro* treatment, the plants were removed from the culture and washed. Root length and number, leaf length and number, and plant fresh weight were recorded, according to Puente-Garza et al. (2017b).

2.3. Saponin extraction and quantification

Saponins were extracted as describes Puente-Garza et al. (2017a). Briefly, the plants were stored at −80 °C overnight and lyophilized. The dried plants were pooled (10 plants per treatment) and ground using a laboratory mixer mill (MM 400, Retsh, Verder Scientific, Germany). Then, 100 mg powder and 1 mL methanol/water (80/20, v/v) solution were agitated (Vortemp 1550, Labnet Int. Inc. Edison, NJ) for 2 h at 150 rpm and 30 °C. The extract was centrifuged at 3000 rpm for 5 min. The recovered supernatant was dried, resuspended in 1 mL of methanol/-water (50/50, v/v) and stored at −20 °C for further analysis. At least three extracts were prepared from each pooled sample. The extracts were analyzed using HPLC (Agilent Technologies, 1200 series, Santa Clara, CA) coupled to an evaporative light scattering detector (ELSD), according to Puente-Garza et al. (2017a). Briefly, a gradient elution program consisting of phase A (water with 0.1% formic acid) and phase B (acetonitrile with 0.1% formic acid) at a flow rate of 0.8 $mL*min^{-1}$ was used. The gradient was set and run as follows: 82% phase A was maintained for 15 min, decreased to 25% in 10 min, and maintained for 5 min, before being reduced to 0% in 10 min, followed by 100% phase B for 10 min. Detection via ELSD was accomplished using nitrogen as the drying gas, 3.4 bar pressure, and 45 °C tube temperature. Saponins were quantified as protodioscin equivalents (PE) (Sigma-Aldrich, St. Louis, MO). The identity of saponins were corroborated employing the same conditions in HPLC-MS-TOF, as reported by Puente-Garza et al. (2017a).

2.4. Statistical analysis

The physical characteristics of the plants and saponin content were subjected to analysis of variance, PCA, and Pearson's correlation test, using the Minitab 19 Statistical Software. Differences among means were compared using the Tukey test at $p < 0.05$.

3. Results

3.1. Physical characteristics of agave plants

The number and length of leaves and roots and plant fresh weight in the presence of different salts are shown in Table 1. No statistically significant difference was found in the number of roots and leaves and plant fresh weight in the presence of 0.1, 0.5, and 1.0 mM of various salts. However, $CoCl_2$ was toxic to the plant because it caused plant death at high concentrations (Table 1).

The presence of different salts at various concentrations did not affect the physiological characteristics of the plants, with the exception of 0.1 mM and 0.5 mM $CoCl_2$, which negatively impacted root length (12.9 and 9.37 cm, respectively) compared with the longest root with

Table 1
Physical characteristics of *Agave salmiana* after 90 d of *in vitro* saline elicitor treatment, using various salts at different concentrations.

Salt	Content (mM)	Roots (#)	Leaves (#)	Roots length (cm)	Leaves length (cm)	Fresh weight (g)
$AlCl_3$	0.1	2.85 ± 1.06 a[a]	5.71 ± 0.76 a	22.18 ± 4.36 a	2.94 ± 0.53 ab	0.72 ± 0.29 a
	0.5	3.70 ± 1.41 a	5.20 ± 0.632 a	16.05 ± 6.65 ab	3.02 ± 0.62 ab	0.95 ± 0.40 a
	1.0	3.66 ± 1.22 a	5.55 ± 0.527 a	15.97 ± 6.58 ab	3.08 ± 0.46 ab	1.11 ± 0.56 a
NaCl	0.1	3.11 ± 0.78 a	4.77 ± 0.44 a	18.27 ± 2.99 ab	3.09 ± 0.67 ab	0.99 ± 0.49 a
	0.5	3.55 ± 1.42 a	5.22 ± 1.09 a	17.92 ± 4.28 ab	3.35 ± 0.74 ab	0.80 ± 0.46 a
	1.0	4.22 ± 1.56 a	5.66 ± 1.11 a	19.32 ± 5.83 ab	3.32 ± 0.86 ab	1.18 ± 0.68 a
$CoCl_2$	0.1	4.33 ± 1.32 a	5.55 ± 1.13 a	12.90 ± 6.30 b	3.55 ± 0.66 a	0.85 ± 0.36 a
	0.5	4.00 ± 1.00 a	4.66 ± 0.57 a	9.37 ± 4.19 b	1.99 ± 0.45 b	0.46 ± 0.13 a
	1.0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Control	0.0	4.33 ± 2.08 a	5.00 ± 0.00 a	14.40 ± 3.02 ab	3.13 ± 0.71 ab	1.06 ± 0.26 a

[a] Different letters denote statistically significant differences at $p < 0.05$.

0.1 mM $AlCl_3$ (22.17 cm). All plants in 1.0 mM $CoCl_2$ died and were not quantified for further characterization.

The longest roots were observed in the presence of 0.1 mM $AlCl_3$. Treatment with 0.5 mM $CoCl_2$ exhibited the lowest root and leaf lengths. In general, an increment in salt concentration caused a decrement in root length.

By contrast, an increment in NaCl concentration caused an increment in the number of roots, whereas the presence of $CoCl_2$ caused a decrease in the number of leaves. However, compared to the control, the number of roots remained the same, whereas root length tended to increase.

Leaf length had the highest and lowest value in the presence of $CoCl_2$ (3.55 at 0.1 mM and 1.99 at 0.5 mM $CoCl_2$, respectively). $AlCl_3$ had a tendency to increase leaf length, whereas NaCl increased leaf length at 0.5 mM concentration. By contrast, the number of leaves decreased with an increase in $CoCl_2$ concentration, increased with an increase in NaCl concentration, and was maximum at the lowest concentration of $AlCl_3$.

3.2. Saponin quantification

The total content of saponins in each treatment is summarized in Fig. 1. The global tendency of total saponins was to decrease, whereas that of salt concentration was to increase ($r = -0.405$, $p = 0.026$), with the exception of NaCl at 0.5 mM.

Four saponins were identified and quantified (Fig. 2 and Fig. 3). The most abundant saponins included chlorogenin glycoside (CG2) and hecogenin glycoside (HG2). Plants in 1.0 mM $CoCl_2$ died. Chlorogenin glycoside 1 (CG1) and hecogenin glycoside 1 (HG1) content did not change across salt type and concentration, with the exception of 1.0 mM NaCl, where these saponins were not detected (Fig. 3).

An increment in $AlCl_3$ between 0.1 mM and 1.0 mM caused a decrease in CG2 and HG2 content (Fig. 3). This response was similar in tendency when compared with $CoCl_2$, where an increment in salt concentration caused a decrease in CG2 and HG2 content. In these cases and the control, CG2 was always present at a higher concentration than HG2.

By contrast, NaCl had a different effect on CG2 and HG2 content. At 0.1 mM concentration, NaCl behaved similar to other salts in controlling the saponin pattern; at 0.5 mM NaCl, HG2 content was higher than CG2 content. Notably, when the concentration of NaCl was increased, the pattern of saponins was restored (CG2 > HG2), and CG1 and HG1 could not be detected.

To determine a predictive model, principal components analysis was performed. Based on Principal component analysis (PCA) (Fig. 4). There was no atypical data in the analysis. The first two components (Principal Component 1, PC1, and Principal Component 2, PC2), are related to the two main responses of plants at different salt type and contents. PC1, has high loading in fresh weight (0.473), CG1 (−0.426) and HG1 (−0.427). Number of leaves (0.367) and root length (0.345) loadings, suggest that biomass of plant increases. These variables are related with the presence of nutrients and antinutrients in the medium, so we can conclude that the first component was related to the nutritional properties to the plant given by the medium; while the plants were vigorous and healthy in appearance, only fresh weight increased while the presence of CG1 and HG1 decreased. The high and medium content (1.0 and 0.5 M, respectively) of NaCl and $AlCl_3$, had a positive effect, while all $CoCl_2$ treatments had a negative effect on *A. salmiana* development (Fig. 4).

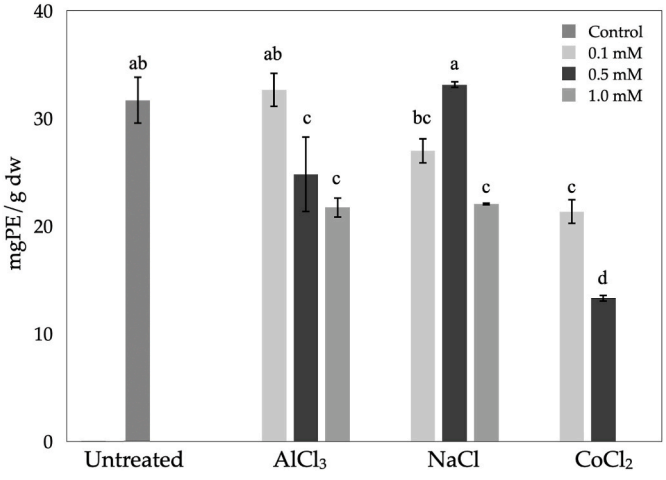

Fig. 1. Total saponin content of *Agave salmiana* plants after 90 d of *in vitro* saline stress, using various salts at different concentrations. Different letters denote statistically significant differences at $p < 0.05$.

The PC2 most relevant loadings were calculated for HG2 (0.475), CG2 (0.524) and total saponins (0.668). These loadings were positive, suggesting that some salts and content, can work as elicitors of saponin biosynthesis. The 0.5 M NaCl and $AlCl_3$ 0.1 M treatments, presented a positive effect, while $CoCl_2$ treatments had a negative effect.

Our results reveal that in *A. salmiana* saponin biosynthesis does not necessarily lead to good development of plants *in vitro*. Further studies are needed to develop a strategy for improving production of secondary metabolites and plant biomass.

4. Discussion

4.1. Physical characteristics

We did not observe changes in the physical characteristics of plants in the presence of different salts at various concentrations. Our findings suggest that salt type and concentration were not stressful for the plant, as suggested by Brouwer et al. (1985), where soil salinity was achieved by using more than 3 $g*L^{-1}$ NaCl. However, the toxicity effect observed in 1.0 mM $CoCl_2$, match with the reported response with concentrations over 0.5 mM (Grover and Purves, 1976).

Although Al inhibits cell division and elongation and root growth, low concentration (0.1–0.5 mM) of non-essential minerals can influence plant growth and development (Sathyaseelan and Karthika, 2019). Low concentrations of Co were reported to promote several growth processes, including stem and coleoptile elongation, leaf disc expansion, and bud development, by inhibiting ethylene biosynthesis (Grover and Purves, 1976). *In vitro* studies with Co have revealed that high-quality granular callus can be obtained with $CoCl_2$ (Al-Mayahi, 2013), suggesting the use of this salt in improving the growth of *in vitro* cultures.

Compared with the results of hydric stress studies on *A. salmiana in vitro* using PEG 8000 (Puente-Garza et al., 2017b), root length and leaf length and number increased in the presence of salts in our study. However, the longest roots were observed at low salt content ($AlCl_3$ 0.1 mM, 22.18 cm) in other studies, suggesting that rooting might have a positive response to these salts, which positively affect the growth and development of many plants (Sathyaseelan and Karthika, 2019; Grover and Purves, 1976). Thus, it is possible to establish a feasible strategy *in vitro* without compromising on biomass.

4.2. Saponin content

All detected saponins were reported by Puente-Garza et al. (2017a) (Fig. A1). Chlorogenin glycoside 1 (CG1) is a 5 sugar units saponin; chlorogenin glycoside 2 (CG2) is a 4 sugar units saponin; hecogenin glycoside 1 (HG1) is a 5 sugar units saponin and hecogenin glycoside 2 (HG2) is a 4 sugar units saponin. In *in vitro* stress studies with *A. salmiana* (Puente-Garza et al., 2017b), the increase in saponin content was directly proportional to an increment in drought stress, reaching the highest content at the highest PEG8000 concentration in the medium. These results are contrary to those in our study, where an increment in the concentration of metal ions showed a decrease in saponin content, with the highest saponin content observed at lower concentrations of metal ions.

The decrease in saponin content in the presence of high $AlCl_3$

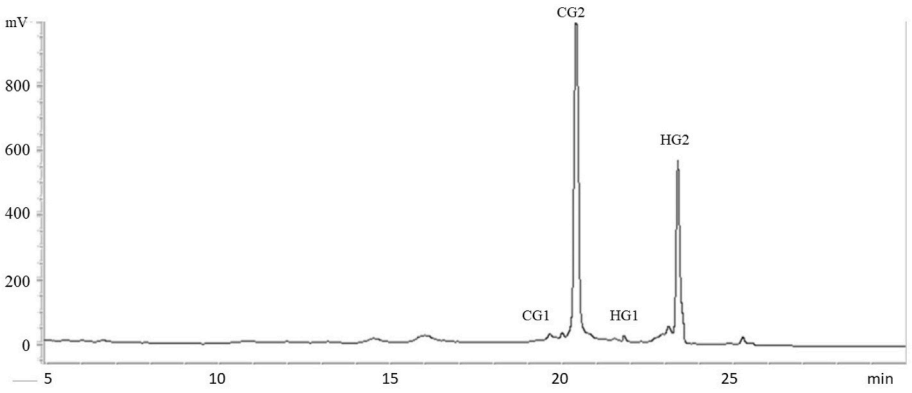

Fig. 2. Chromatogram obtained by Evaporative Light Scattering Detector (ELSD) of saponins quantified in plants from 0.1 M $AlCl_3$. CG1-2, chlorogenin glycosides 1 and 2; HG1-2, hecogenin glycosides 1 and 2.

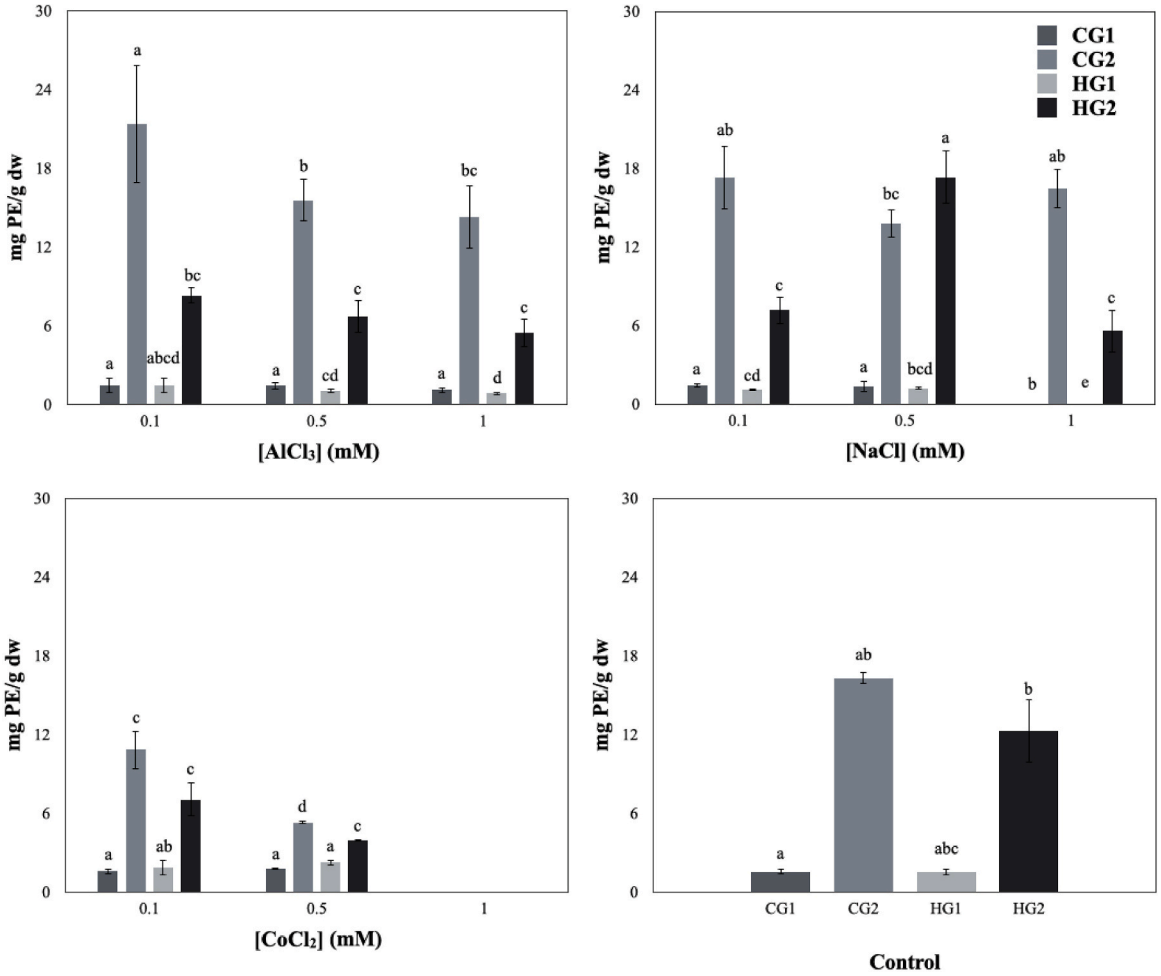

Fig. 3. Saponin content in plants grown *in vitro* after 90 d of culture in media containing salt. mg PE/g dw: protodioscin equivalents per gram dry weight; CG1-2: chlorogenin glycoside 1 and 2; HG1-2: hecogenin glycoside 1 and 2 (n = 3).

concentration can be explained by the increased activity of NADPH oxidase in the presence of Al^{3+} (Achary et al., 2012). Increased activation of NADPH oxidase leads to the overconsumption of NADPH, required for squalene synthesis by the steroid synthesis pathway, which is also important for saponin synthesis (Radisky and Poulter, 2000). Al^{3+} can be found in acidic and calcareous soils, which can be a problem at lower pH values because Al^{3+} has a higher toxicity to plants at a lower pH compared with Al ($Al(OH)^{4-}$) at high pH (Sathyaseelan and Karthika, 2019). These toxic effects include inhibiting cell division and elongation, and subsequently, root growth (Sathyaseelan and Karthika, 2019).

A low concentration of NaCl (0.1%) has been related with an increase of 1.15 times in the content of saponins in ginseng (Naik and Al-Khayri, 2016); a higher concentration was related to a decreased biomass (Jeong and Park, 2006). Higher concentrations of NaCl change the osmotic potential due to reduced water content, which causes an increase in the activity of peroxidases and indoleacetic acid oxidase. Peroxidases are involved in the degradation of secondary metabolites (Ajungla et al.,

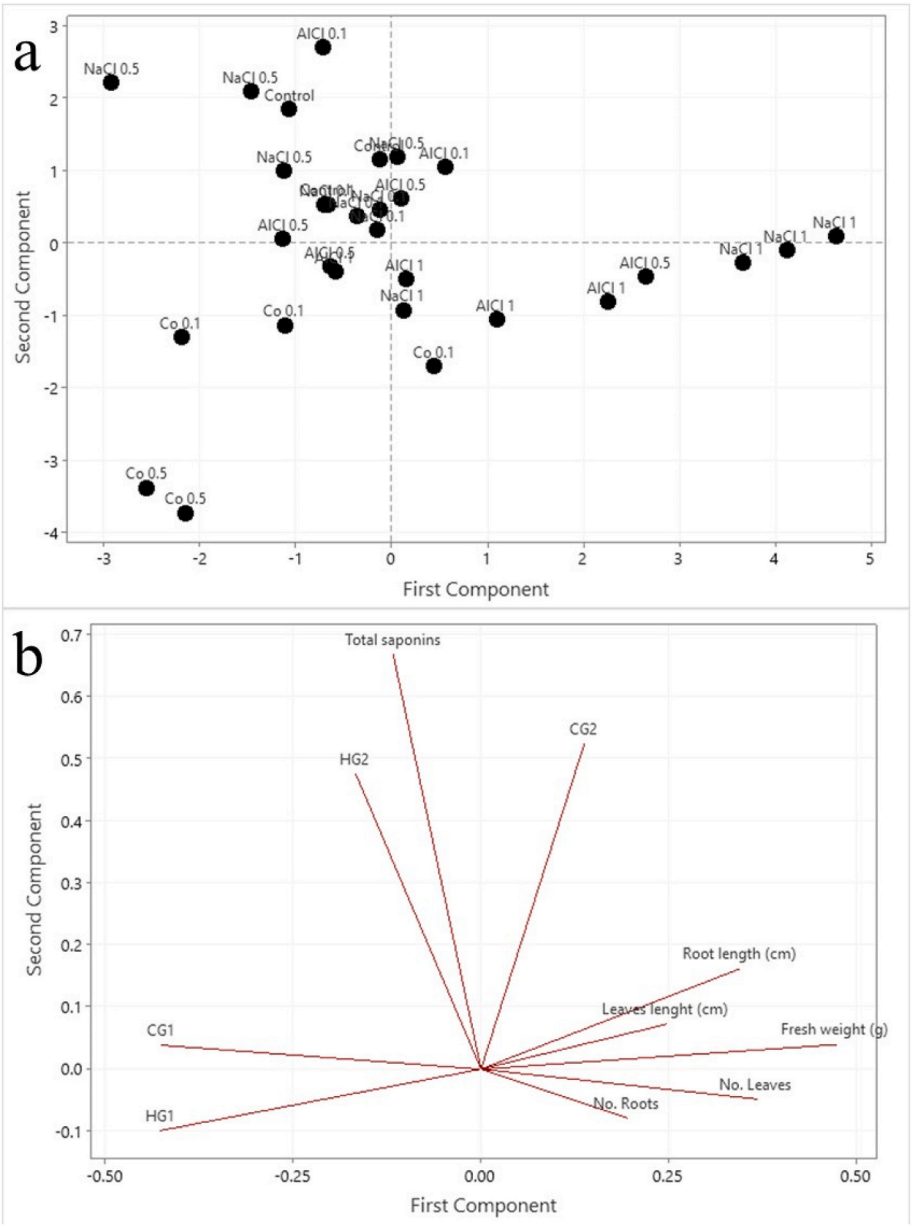

Fig. 4. Principal component analysis (PCA) for evaluating the physiological characteristics and saponin content of *Agave salmiana* samples after cultivation for 90 d in medium containing saline elicitors. The first component describes the response to growth enhancers for phenological traits and the second component describes a response of the elicitor for saponin content. (a) Score plots of physiological characteristics and saponin content. (b) Loading plots of physiological characteristics and saponin content.

2009) and indoleacetic acid oxidase lowers auxin levels, causing growth retardation (Ali and Abbas, 2003).

The presence of Co^{2+} in soil, hinders the fixation of CO_2 in CAM plants (Palit et al., 1994), such *A. salmiana*, suggesting why plants grown *in vitro* died at higher concentrations of $CoCl_2$. However, the presence of low levels of Co in soil improves drought resistance, increases expression of secondary metabolites, such as pigments, and increases the height, leaf area, and fresh and dry weight of plants (Akeel and Jahan, 2020).

Squalene synthase, involved in the biosynthesis of saponins, is dependent on Mg^{+} and NADPH (Lograsso et al., 1993). Due to the interaction of Co and NaCl with Mg (Bergsten et al., 2016; Andrijany et al., 1999), the activity of this enzyme could be affected. This change in activity can affect the pattern of saponins in the presence of NaCl, due to pathway deviation, which correlates with plant maturity (Marker and López, 1947). Both affected molecules (CG2 and HG2) are precursors of more simple molecules, such as tigogenin, and stress studies in *A. salmiana* have shown similar patterns of changes (Puente-Garza et al., 2017b).

It is possible to observe a differentiation in the synthesis of saponins in terms of type of molecule, which is regulated by the presence of different salt types and concentrations, without affecting the physiological properties of *A. salmiana* plants grown *in vitro*. These results can suggest a feasible strategy for low saponin agaves in field, which are desirable in spirit drinks industry, since the presence of saponins in process could inhibit organisms in fermentation (Zamora-Pedraza et al., 2010) and may cause contact dermatitis in agave managers (Urbina et al., 2018).

Using PCA (Fig. 4), the responses can be categorized into two groups, explained by PC1 and PC2: those that have an effect over the phenotypic characteristics (PC1) and those that affect secondary metabolite content (PC2). The positive displacement over PC1 can be explained by the presence of NaCl and $AlCl_3$, which at low concentration can help in the development and growth of plants (Sathyaseelan and Karthika, 2019; Grover and Purves, 1976); in the other hand, the negative displacement over PC1, which is caused by the presence of $CoCl_2$, is related to the negative effect reported for this mineral (Palit et al., 1994). While NaCl and $AlCl_3$ can work as an enhancer of growth, $CoCl_2$ inhibits the development of plants.

For PC2, the presence of 0.5 M NaCl caused a positive displacement, explained by the biosynthesis of saponins (HG2, CG2 and total saponins). The positive elicitor response is explained by the interaction of NaCl with NADPH and its role on the saponin pathway (Bergsten et al., 2016).

Thus, at specific concentrations, salt elicitors present a feasible tool for the generation of specific saponins in *in vitro* environments. Also, these salts can be employed for growth and development of plants, without compromising plant biomass, as suggested for other tissues employed for saponin synthesis (Andrijany et al., 1999). These results could be used to develop strategies for saponin production in an *in vitro* system of *A. salmiana*.

5. Conclusion

Neither salt content nor type affected the phenological characteristics of *A. salmiana in vitro*. $CoCl_2$ was toxic to the plants because the plants did not survive at higher concentrations of Co. An increase in salt content in the medium caused a decrease in the saponin content of the plants, suggesting a feasible tool for the generation of low saponin, useful in spirit drinks industry. NaCl showed a different response in saponins pattern compared with the control and other treatments. Further studies on salts interactions and synergies, to establish a strategy for generating specific saponins in *A. salmiana in vitro*, are warranted.

Author contributions statement

Conceptualization, CAPG and SGL; methodology, CAPG; formal analysis, CAPG, CAEL; investigation, CAPG; writing-original draft preparation, CAPG; writing-review and editing, CAPG, CAEL and SGL; supervision, SGL. All authors have read and agreed to the published version of the manuscript

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2021.03.017.

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