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Variation of the chemical and biochemical responses to salinity during germination and early growth of seedlings of two populations of *Agave durangensis* Gentry

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Abstract

Agave durangensis (Asparagaceae) supports a mescal industry in Mexico. The primary reproductive strategy of the species is through seeds. The growing demand for agave-based beverages prompts producers to explore new cultivation areas. However, over half of the country's territory comprises arid and semiarid zones, which are significantly impacted by salinity. The objective of the current study was to assess whether salinity induces distinct seed germination potential and varied biochemical and chemical responses in seedlings of 2 populations of *A. durangensis*, potentially leading to different tolerance levels to salinity. Seeds from each population were subjected to four salinity treatments. Germination potential, as well as growth parameters and biochemical and chemical attributes of seedlings, were analyzed. Despite reduced germinability and slower germination speed, seeds from both populations were capable of germinating even under the highest NaCl concentration (100 mM) evaluated. Effects on the growth parameters were recorded; however, the seedlings of both populations survived throughout the experiments, exhibiting increased chlorophyll content and cell viability in most saline treatments. The enzymatic defense mechanism and the accumulation of proline were activated in a salt-dependent manner, a response not observed with phenolic compounds. However, notable accumulation of monomeric anthocyanin occurred under the 2 strongest NaCl concentrations evaluated. Significant interpopulation differences were observed in each type of response, with varied regulation under different NaCl concentrations, some of which were more pronounced than others under specific saline conditions. Seeds from the Durango population were more sensitive to salinity.

Keywords

Maguey cenizo, anthocyanin accumulation, antioxidant enzymes, salt tolerance, seed provenance

Introduction

Soil salinity has been a significant concern in global agriculture throughout human history, impacting more than 800 million ha of arable lands worldwide (1). It particularly restricts crop growth and production in arid and semiarid regions (2), where soil salt content is naturally elevated and precipitation may be insufficient for leaching.

In plants, salt stress induces a variety of responses, encompassing morphological, physiological, biochemical and molecular changes,

especially during seed germination and seedling growth (3).

Salinity induces ionic imbalance, leading to ionic toxicity, osmotic stress and the generation of reactive oxygen species (ROS) (2). The detrimental impact of ROS arises from their ability to oxidize proteins, lipids, membranes and nucleic acids (4). In response to these adverse effects, plants have evolved various mechanisms. One such mechanism involves the accumulation of solutes (or osmolytes), such as proline, which reduces the cytoplasmic osmotic potential, aiding in water absorption and ROS scavenging (5). Other mechanisms include enzymatic and non-enzymatic processes. Enzymatic mechanisms involve increased activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR). Non-enzymatic mechanisms encompass the synthesis and accumulation of specialized metabolites, such as phenolic compounds like flavonoids and tannins, carotenoids and vitamin C (ascorbic acid) (6).

Agave plants, native to Mexico, have served various purposes, including food, medicine, textiles, decoration, fiber production and as raw material for manufacturing distilled alcoholic beverages such as tequila and mescal, as well as fermented beverages. *Agave durangensis* Gentry, an economically significant species, sustains the mescal industry in Durango, Mexico. The primary mode of reproduction for this species is through seeds (7). Currently, the growing demand for agave-based beverages is prompting producers to explore new cultivation areas. However, in Mexico, over half of the territory is covered by arid and semiarid ecosystems, which are particularly susceptible to salinity (8). Therefore, understanding its tolerance to salinity is crucial for maintaining crop production and expanding its cultivation areas.

The response to saline stress in *Agave* species has been minimally explored. It was observed that *Agave deserti* exhibited a reduction in both root and shoot lengths by over 50 mM NaCl (9). Elevated salinity levels led to decreased moisture content and reduced fresh and dry weight in *Agave parryi* Truncata (10). For *Agave sisalana*, dry weight was reported to decrease by 45% at NaCl levels exceeding 150 mM (11). Salinity showed no impact on the growth of *Agave americana* at levels up to 85 mM NaCl; however, at 100 mM NaCl, plant growth was significantly reduced (12). Finally, in hydroponic experiments, changes in biomass accumulation and nutrient levels of young plants of *A. weberi*, *A. parryi* and 2 subspecies of *Agave utahensis* were dependent on salinity (13). Despite valuable contributions, there remains a lack of knowledge regarding the diversity of responses in the majority of the 166 (14) *Agave* species to cope with salt stress. These responses can vary significantly even among species of the same genus, as documented for *Brassica* species (15).

The results from the studies mentioned above for *A. deserti*, *A. parryi*, *A. sisalana*, *A. americana*, *A. weberi* and *A. utahensis* suggest that tolerance to salinity varies depending on the species, development stage, salt concentration and duration of exposure. Rigorous documentation of not only the morphological responses but also the chemical and biochemical responses would enable us to comprehend how species within the genus *Agave* respond to salinity and assess the adaptation potential of these economically important plants to this type of abiotic stress. In the current study, our focus was on comparing the seed germination potential, as well as growth parameters and chemical and biochemical responses of seedlings from two populations of *A. durangensis* under salt stress.

Materials and Methods

We gathered seeds from two distinct natural populations of *A. durangensis* in Durango, Mexico, to examine their germination and early seedling growth under various salinity conditions. The seed collection took place in July 2022.

Plant material and culture conditions

Seeds of *Agave durangensis* were collected from Nombre de Dios (population *D*: 23° 42’ 21.8” N, 104° 12’ 39.12” W; 1988 m altitude) and El Mezquital (population *M*: 23° 45’ 48” N, 104° 31’ 14” W; 2376 m altitude). A sample of 100 seeds from each population was deposited in the Germplasm Collection of the Interdisciplinary Research Center for the Integral Regional Development, unit Durango of the National Polytechnic Institute (Mexico) (AG-20221 and AG-20222 were the register numbers for *D* and *M,* respectively). The seeds underwent surface-sterilized for 10 min in a 1 % (v/v) sodium hypochlorite solution and were then rinsed with distilled water. After sterilization, 60 seeds were germinated on a cotton layer placed in 140 mm sterile Petri dishes (experimental lot). The seeds were moistened with 30 mL distilled water as a control (0.01 dS/m electrical conductivity) or saline water solution at 25, 50, 75 or 100 mM NaCl, corresponding to 3.2, 5.6, 8.0 and 9.7 dS/m electrical conductivity, respectively. Each saline treatment was conducted for 3 independent experimental lots. The Petri dishes were labeled and incubated at 28˚C/20˚C (day/night temperatures) with a daily photoperiod of 12 hrs.

Germination

The germinated seeds were tallied daily from day 1 to day 12. The % of germination (G) and germination speed (S), were calculated (Eqns. 1 and 2, respectively).

$$G(\%)=[n/N]*100 \qquad \text{Eqn. 1}$$

where n is the number of germinated seeds at day 12 and N is the total number of seeds

$$S=[\text{germinated seeds at day 1} / 1] + \ldots + [\text{germinated seeds at day n} / n] \qquad \text{Eqn. 2}$$

where n is the number of days after sowing

Seedling growth parameters

Twenty 12-day-old seedlings (bearing the first primary leaf) were randomly selected from each treatment. Length (L), fresh weight (FW) and dry weight (DW) of each individual were recorded. The relative water content (RWC) of

seedlings was determined using a method previously described (16). After recording FW, leaves were immersed in distilled water inside a closed Petri dish for 4 h, and then the turgor weight (TW) of each sample was recorded. Leaf samples were then placed in a preheated oven at 70˚C for 24 h to obtain DW. Subsequently, RWC was calculated (Eqn. 3).

$$RWC(\%)=[(FW/DW)/(TW/DW)]*100 \quad \text{Eqn. 3}$$

Biochemical responses

Antioxidant enzymes

Enzyme extracts and estimation of soluble protein content (SPC) were conducted from 12-day-old seedlings, following a previously established method (17). SPC was determined using a standard curve of bovine serum albumin (BSA), recording the absorbance at 595 nm (slope: 0.723, intercept: – 0.449, r = 0.996). SPC values were expressed as milligrams of BSA equivalents per gram of fresh weight (mg EBSA/g FW).

The activities of APX, SOD and CAT were measured according to previously described methods (18). APX activity was estimated using the value 2.8/mM/cm as the extinction coefficient of reduced ascorbate and the enzyme activity was expressed as millimoles of ascorbate per minute per gram fresh weight (mmol AsC/min/g FW). SOD activity was estimated by its ability to inhibit the photochemical reduction of nitroblue tetrazolium salt (NBT). One unit of SOD was defined as the amount of enzyme activity that inhibited the photoreduction of NBT to blue formazan by 50 % and the SOD activity of each extract was expressed as units of SOD per gram of protein (U SOD/g Prot). The activity of CAT was expressed as CAT units per gram of protein (U CAT/g Prot).

Proline (Pro)

The Pro content was determined according to a previous method (16) using a proline standard curve, and the absorbance was recorded at 520 nm (slope: 0.954, intercept: 0.055, r = 0.998). Pro content was expressed as milligrams per gram of fresh weight (mg/g FW).

Ascorbic acid (AsC)

The AsC contents were determined following a prior method (19), utilizing a standard curve of AsC and recording the absorbance at 760 nm (slope: 1.689, intercept: 0.070, r = 0.996). AsC content was expressed as micrograms of AsC equivalents per gram of dry tissue (µg AsCE/g DT).

Photosynthetic pigments

The seedlings (0.1 g) were ground in a pre-chilled mortar with 8 mL of acetone 80 % (v/v). The mixture was filtered and the volume was adjusted to 10 mL with cold acetone. Total chlorophyll (TChl) and total carotenoid (TC) concentrations were calculated (17).

Chemical responses

Phenolic extracts

Phenolic extracts of 12-day-old seedlings were prepared (19). Aliquots were taken to determine the content of the following phenolic compounds and antioxidant activities.

Total phenolics (TP)

TP was determined according to a previously described method (19) using a standard curve of gallic acid (GA) and the absorbance was recorded at 765 nm (slope: 4.919, intercept: 0.088, r = 0.998). Concentrations were expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g DE).

Total flavonoids (TF)

TF was determined following a previous method (20) using a standard curve of quercetin (Q), and the absorbance was recorded at 765 nm (slope: 16.59, intercept: 0.079, r = 0.998). Concentrations were expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g DE).

Condensed tannins (TCT)

TCT was determined according to Herald et al. (21) from a standard curve of epicatechin (E), registering the absorbance at 500 nm (slope: 0.8, intercept: 0.015, r = 0.998). Concentrations were expressed as milligrams of epicatechin equivalents per gram of dry extract (mg QE/g DE).

Monomeric anthocyanins (TMA)

The TMA contents were determined using the previously described differential pH method (22), with reference to the molecular weight (449.2) and molar absorption (26,900) of cyanidin-3-O-glucoside. The results were expressed as milligrams of cyanidin-3-O-glucoside equivalents per gram of dry extract (mg C3gE/g DE).

Antioxidant capacity

The scavenging capability of seedling phenolic extracts against DPPH (2,2-diphenyl-1-picrylhydrazyl) was determined (22). The activity was quantified as the amount of antioxidant required to reduce the initial DPPH concentration by 50% (CE_{50}), expressed in milligrams per milliliter (mg/mL). Additionally, the antioxidant capacity of the samples was assessed through the iron-reducing power (FRAP) assay (23). The absorbance at 700 nm was recorded for each sample, where higher absorbance values indicated greater reducing power.

Cell injury (CI)

CI resulting from various concentrations of salt in *A. durangensis* seedlings was determined using the triphenyl tetrazolium chloride (TTC) method (24). In this procedure, a solution of TTC (0.8%, w/v) in 0.05 M phosphate buffer (pH 7.4) was employed to suspend 0.1 g samples of control and treated seedlings, followed by a 16-hour incubation in the dark at 25 °C. Subsequently, the seedlings were rinsed 3-5 times with distilled water and treated with 3 mL of 95% ethanol. The reduced formazan was extracted in a boiling water bath for 5 minutes. After cooling, the final volume was adjusted to 3 mL with 95% ethanol and the absorbance was recorded at 530 nm.

Data analysis

All assays were conducted with three independent pools. Data were assessed for significant differences using One-Way ANOVA. Two-way ANOVA was employed to examine

significant differences between the 2 populations under analysis. Mean separation was carried out using Tukey's test, implemented in GraphPad Prism 8.0. Correlations between the parameters were assessed using the Pearson correlation coefficient ($p \leq 0.05$). The contribution of each chemical and biochemical attribute to the differentiation of populations was evaluated through Principal Component Analysis (PCA). Chemical and biochemical relationships among plants from different treatments were determined separately through cluster analyses. PCA and cluster analysis were performed using Past 4.07b.

Results

Germination

Seeds from both populations of *A. durangensis* exhibited the ability to germinate under low, moderate and high salinity conditions. However, a salt-dependent decrease in both germination (G) and seedling survival (S) was observed, with the reduction in germination being non-significant in both populations (Table 1). The two-way ANOVA (Table 2) revealed significant interpopulation differences in both germination and seedling survival in response to varying salinity treatments. The declines in germination and seedling survival with increasing salt concentration were more pronounced in population D compared to population M.

Growth patterns

Salinity stress led to a salt-dependent reduction in leaf length (L) and fresh weight (FW) in both populations of *A. durangensis* (Table 3). The smallest L value (0.97 cm) and the lowest FW value (0.23 g) were observed in population M at 100 mM NaCl. Population D exhibited an unclear salt-dependent decrease in dry weight (DW), contrasting with the observations in population M. The salt-dependent reduction in relative water content (RWC) was not clearly evident in either population. Two-way ANOVA (Table 2) indicated significant differences in RWC, L, FW, and DW between populations D and M in response to increasing NaCl concentrations. Only for L, a non-significant interaction between population and salt condition was observed (Table 2).

Biochemical responses

The impact of salt stress on SPC, APX, SOD, CAT activities and Pro levels in *A. durangensis* seedlings is illustrated in Fig. 1A-E. SPC in population D exhibited a salt concentration-dependent increase ($r = 0.98$, $p < 0.01$), while in population M, the salt-dependent increase was not clearly evident ($r = 0.20$, $p < 0.01$). APX activity showed a direct correlation with salt concentration in population D ($r = 0.79$, $p < 0.01$), whereas in population M, the direct correlation was not distinctly observed ($r = 0.60$, $p < 0.01$). Both populations exhibited a salt-dependent increase in SOD ($r = 0.96$ for D and $r = 0.93$ for M, $p < 0.01$), CAT ($r = 0.62$ for D and $r = 0.97$ for M, $p < 0.01$), and Pro content ($r = 0.92$ for D and $r = 0.99$ for M, $p < 0.01$).

The salt-induced accumulation of Ascorbic Acid (AsC) exhibited distinct patterns in the two populations under analysis (Fig. 1F). In population D, the increase in AsC was dependent on salt concentration. Conversely, in population M, AsC accumulated at higher levels than the control, but this effect was only notable under the 25 and 50 mM NaCl treatments (16.75 and 12.75 mgEAsC/gDT respectively).

In the seedlings of population D, all 5 saline treatments induced the accumulation of TChl compared to the control. However, for the seedlings of population M, only the moderate (50 mM NaCl) and high salt (100 mM NaCl) concentrations induced the accumulation of TChl (Fig. 2A). In both populations, the highest total chlorophyll content was observed under the 50 mM NaCl treatment, although the absolute values differed (Fig. 2B).

Table 1. Effect of salinity on percentage of germination (G) and speed of germination (S) of seeds from two populations of *Agave durangensis*

NaCl	G		S	
	D	***M***	***D***	***M***
Control	82.18 ±6.82^{a}	91.04±6.33^{a}	37.00±4.33^{a}	34.42±0.08^{a}
25	76.86 ±5.40^{a}	90.10±8.18^{a}	34.42±0.08^{a}	34.72±1.90^{a}
50	79.65±4.25^{a}	91.06±0.79^{a}	27.83±1.08^{b}	33.89±2.52^{a}
75	70.77±5.48^{a}	93.95±0.96^{a}	21.89±1.73^{c}	33.67±1.61^{a}
100	71.53±5.10^{a}	82.98±3.49^{a}	21.61±1.05^{c}	27.44±0.67^{b}

Table 2. Results of two-way ANOVA (F values) of the effect of population and concentration of NaCl on the percentage of germination (%), speed of germination (S), relative water content (RWC), length (L), fresh weight (FW) and dry weight (DW) in two populations of *Agave durangensis*

Source of variation	G	S	RWC	L	FW	DW
Interaction	1.89 $_{NS}$	8.606***	1.655*	1.890$_{NS}$	6.67**	4.42*
Population	3.18*	38.87****	10.24***	3.189*	37.55****	11.44***
NaCl (mM)	37.47*	46.65****	3.589**	37.47**	16.78*	9.28*

**** $p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p > 0.01$, NS non-significant.

Table 3. Effect of salinity on water content (RWC), length (L), fresh weight (FW) and dry weight (DW) of seedlings of two populations of *Agave durangensis*

NaCl (mM)	RWC (%)		L (cm)		FW (g)		DW (g)	
	D	***M***	***D***	***M***	***D***	***M***	***D***	***M***
Control	96.17±1.29^{a}	85.69±4.49^{a}	5.07±1.02^{a}	4.25±0.78^{a}	3.25±0.22^{a}	1.66±0.44^{a}	0.12±0.04^{a}	0.22±0.03^{a}
25	95.14±1.88^{a}	86.44±1.58^{a}	3.39±1.50^{b}	2.50±1.00^{b}	2.11±0.83^{b}	1.11±0.39^{a}	0.09±0.008^{a}	0.14±0.04^{a}
50	81.72±4.44^{b}	82.90±1.89^{a}	2.42±1.15^{c}	2.19±0.80^{c}	0.95±0.20^{c}	1.00±0.13^{a}	0.16±0.01^{a}	0.16±0.006^{a}
75	75.89±9.15^{c}	77.97±1.77^{a}	1.58±0.89^{d}	1.17±0.56^{d}	0.49±0.16^{d}	0.56±0.13^{b}	0.10±0.03^{a}	0.12±0.01^{b}
100	79.17±0.96^{b}	69.90±14.82^{b}	1.18±0.54^{e}	0.97±0.43^{e}	0.44±.011^{d}	0.23±0.06^{c}	0.09±0.02^{a}	0.06±0.02^{c}

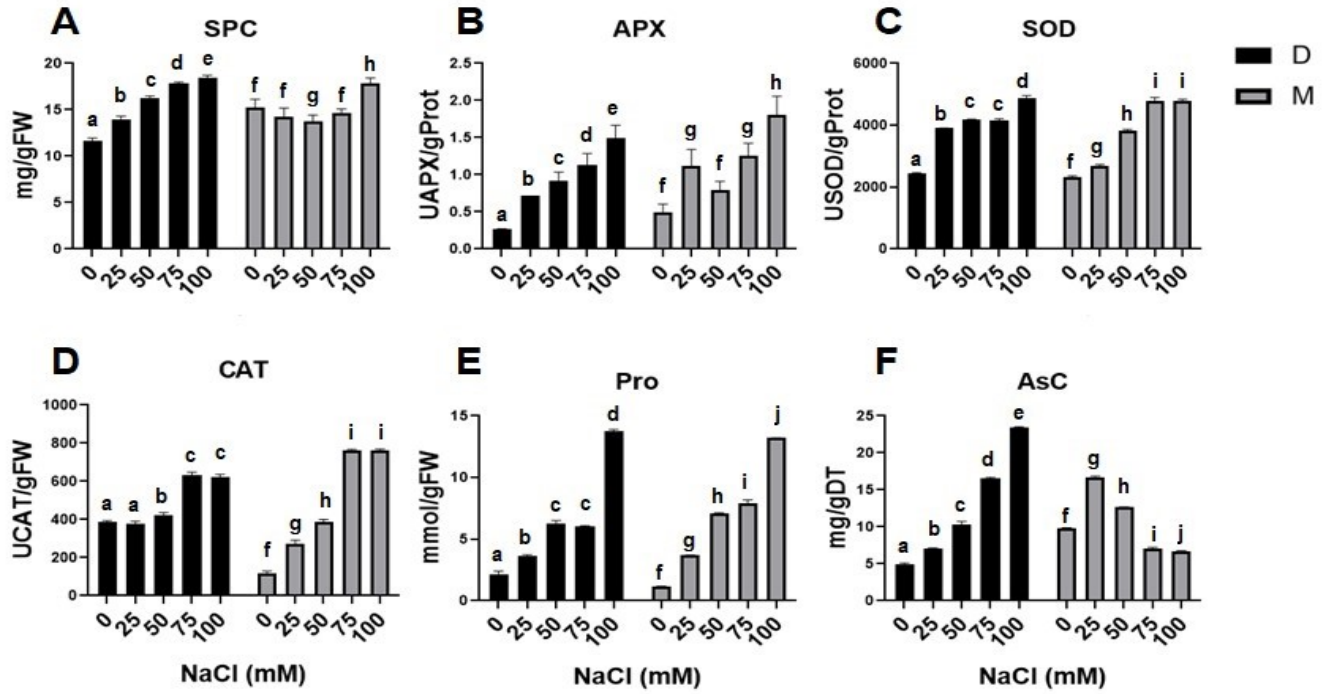

Fig. 1. Effect of salt stress on A: Soluble proteins content (SPC), B: Ascorbate peroxidase (APX), C: Superoxide dismutase (SOD), D: Catalase (CAT), E: Proline (Pro), and F: Ascorbic acid (AsC) in seedlings of *Agave durangensis* from two populations of Durango, Mexico (D: Population Durango, M: Population El Mezquital). The values represent the mean of three independent replicates ± SE ($p < 0.05$).

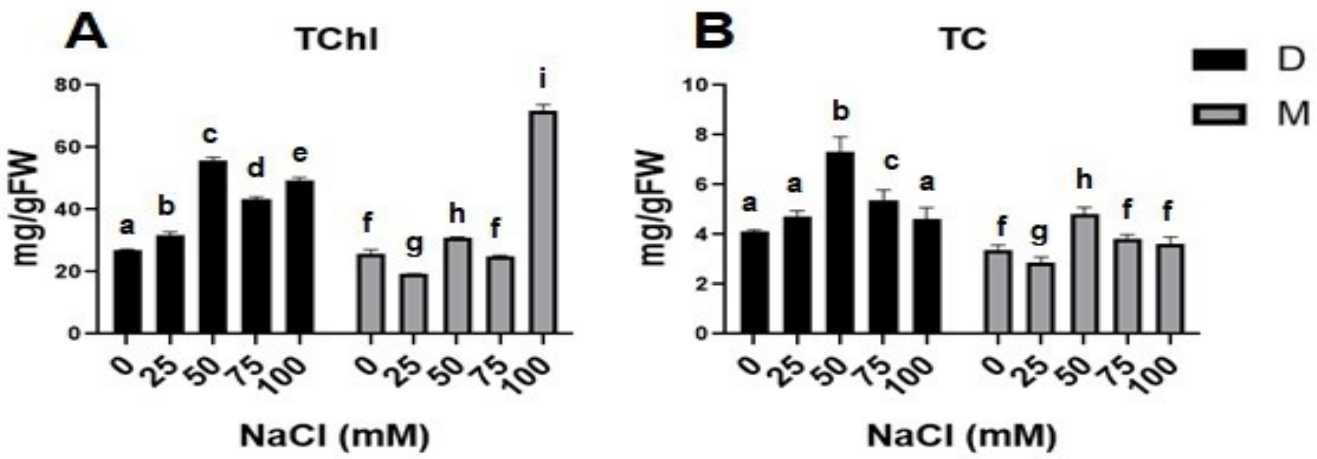

Fig. 2. Effect of salt stress on A: Total chlorophyll (TChl) and B: Total carotenoids (TC) of seedlings of two populations (D: Population Durango and M: Population El Mezquital) of *Agave durangensis*. The values represent the mean and standard deviation of three independent replicates ($p < 0.05$).

According to the two-way ANOVA, *A. durangensis* seedlings exhibited significant differences not only in response to various salinity treatments but also between populations (Table 4). The interaction population* NaCl suggested that populations D and M demonstrated distinct response patterns to increased salt stress for SPC, APX, CAT, SOD, TChl, TC, AsC, and Pro.

Chemical responses

The non-enzymatic antioxidant contents of TP, TF, TCT and TMA in *A. durangensis* seedlings are depicted in Fig. 3A -D. The concentrations of TP, TF, TCT and TMA for both populations were not dependent on salt levels. Notably, the 25 mM NaCl treatment was the only one inducing a higher TP level (1.13 times) compared to the control, but only in population D. Conversely, in population M, a salt-dependent reduction of TP levels was observed. A significant difference in TF content was evident in unstressed seedlings between populations, with those from population M accumulating about 2.81 times as much as seedlings from population D. In population M, TF decreased with increasing NaCl concentrations. TCT induction was observed only in population D under low salinity (25 mM NaCl), while in population M, TCT decreased with increasing NaCl concentrations. In contrast to other phenolic compounds, TMA was induced under the 2 strongest saline treatments (75 and 100 mM NaCl) in both populations (2.77 and 2.27 times more compared to control respectively, in D; whereas 1.40 and 1.37 times more compared to control respectively, in M).

The DPPH* scavenging capacity and FRAP of phenolic extracts from the two populations of *A. durangensis* were illustrated in Fig. 4A-B. Interpopulation variations are evident under both unstressed and salt-stressed conditions, with unstressed seedlings of population M exhibiting the highest antioxidant capacity. Both DPPH* scavenging capacity and FRAP were not dependent on salt levels in the seedlings of both populations. The results revealed that the highest DPPH*

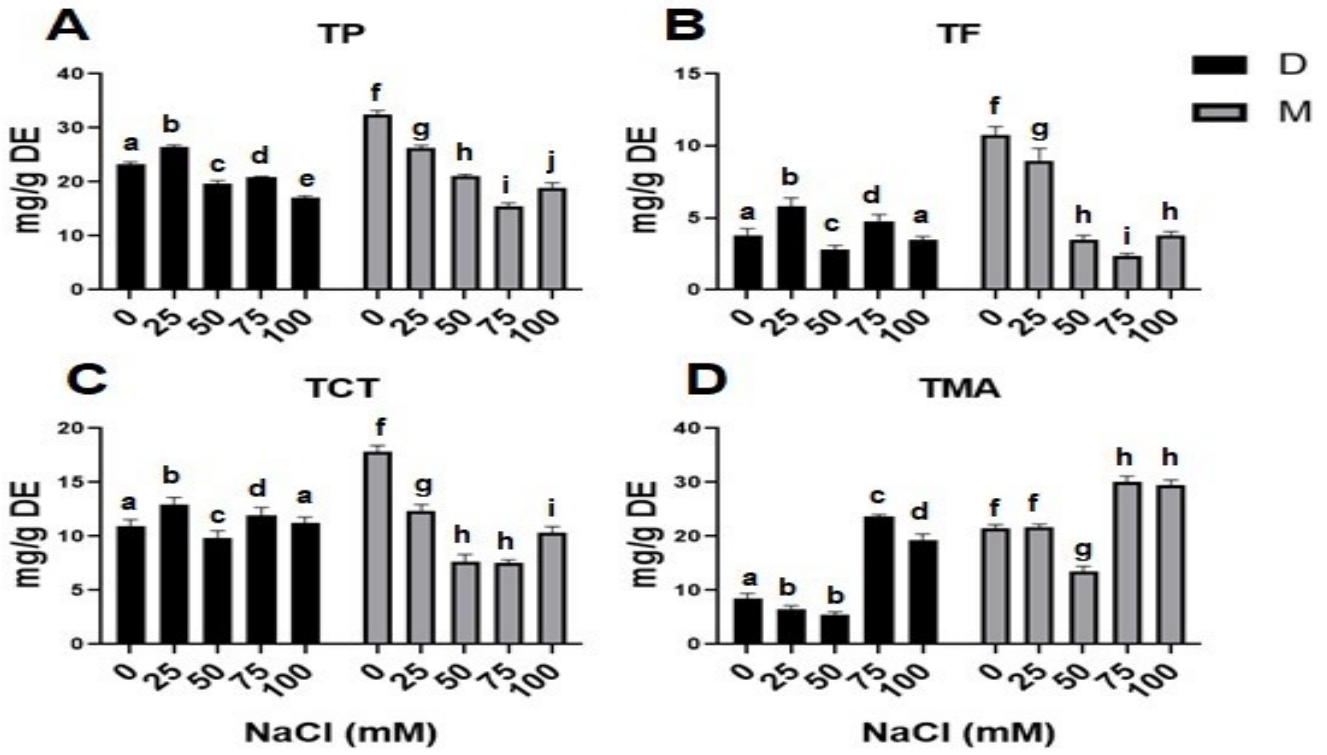

Fig. 3. Effect of different NaCl concentrations on A: Total phenolics (TP), B: Total flavonoids (TF), C: Condensed tannins (TCT), and D: Monomeric anthocyanins (TMA) in seedlings of *Agave durangensis* from two populations (D: Population Durango, M: Population El Mezquital). All the values are the mean of three independent replicates ± SD ($p < 0.05$).

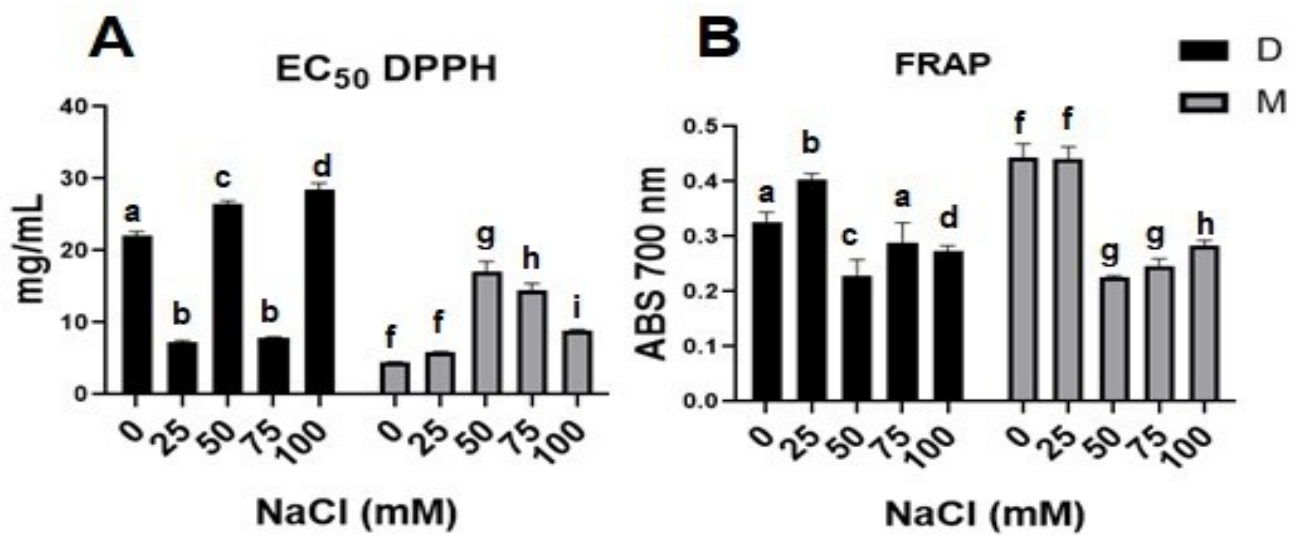

Fig. 4. Effect of NaCl stress on A: DPPH (2,2-diphenyl-1-picryl-hydrazyl) scavenging capacity and B: Iron reducing power (FRAP) in seedlings of *Agave durangensis* from two wild populations (D: Population Durango, M: Population El Mezquital).

scavenging capacity was induced at 25 and 75 mM NaCl (IC50 = 7.33 mg/mL, IC50 = 7.98 mg/mL respectively) for population D but not for population M. Low salt stress (25 mM NaCl) was the only condition inducing an increase in FRAP in the seedlings from population D.

According to the two-way ANOVA, *A. durangensis* seedlings exhibited significant differences not only in response to various salinity treatments but also between populations (Table 4). The interaction "population *NaCl concentration" indicated that populations D and M demonstrated distinct response patterns to increased salt stress for TP, TF, TCT and TMA, as well as the DPPH scavenging capacity affected by salt stress. For TCT and FRAP, the different response patterns observed in D and M to the increase in salt concentrations were not statistically significant (Table 4).

Cell injury (CI)

The TTC reduction ability was utilized to assess cellular damage caused by salinity in *A. durangensis* seedlings (Fig. 5). Under the lowest salinity condition assessed (25 mM NaCl), TTC reduction ability decreased by 22.10% compared to control seedlings in population D, whereas it increased by 71.50% compared to control seedlings in population M. Exposure to 50 mM NaCl led to a decline in TTC reduction activity in seedlings of both populations (29.07% in D and 8.83% in M, compared to the respective controls). At 75 mM NaCl, seedlings of both populations were able to recover TTC reduction ability, which was

Table 4. Results of two-way ANOVA (F values) on effect of population and concentration of NaCl on soluble protein content (SPC), ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), total phenolics (TP), total flavonoids (TF), condensed tannins (TCT), monomeric anthocyanins (TMA), total chlorophyll (TChl), total carotenoids (TC), ascorbic acid (AsC), proline (Pro), DPPH scavenging capacity (DPPH), iron reducing power (FRAP), and cell injury (CI) in two populations of *Agave durangensis*

Source of variation	Interaction	Population	NaCl (mM)
SPC	70-98****	12.02**	139.6***
APX	3.49*	14.23***	71.89****
CAT	976.2****	54.68****	1912****
SOD	222.8****	129.2****	1888****
TP	390.0****	130.3****	1243****
TF	249.6****	307.3****	366.6****
TCT	232.1****	2.93 $_{NS}$	258.9****
TMA	717.5****	5663****	639.0****
TChl	817.7****	574.7****	2024****
TC	9.00****	214.7****	69.07****
AsC	5973****	830.00****	1500****
L-Pro	159.8****	38.45****	9824****
DPPH	412.6****	1159****	494.3****
FRAP	18.8****	0.6014$_{NS}$	21.68****
CI	99.00****	331.7****	202.2****

**** p < 0.0001, ***p < 0.001, **p < 0.01, *p > 0.01, NS non-significant.

higher than that displayed by the respective control seedlings. However, at the strongest NaCl concentration, TTC reduction ability tended to decrease in seedlings of population D (Fig. 5)

According to the two-way ANOVA, *A. durangensis* seedlings exhibited significant differences not only in response to various salinity treatments but also between populations (Table 4). The interaction "population* NaCl concentration" suggested that populations D and M displayed distinct response patterns to increased salt stress for CI (Table 4).

PCA and cluster analyses

PC1 and PC2 of the PCA, based on all the evaluated responses (Fig. 6A), collectively accounted for 99.9% of the total variance (98.8% and 1.1% respectively). PC1 was primarily associated with SOD activity, while PC2 was predominantly linked to CAT activity. Seedlings under moderate and strong salt treatments from both D and M populations were distinguished from unstressed seedlings of both populations and seedlings of M under low salt treatment by their elevated SOD activity. Unstressed seedlings of D and seedlings treated with 75 mM NaCl from this population, along with seedlings of M under low and strong salt treatments, were separated from all others by their high CAT activity.

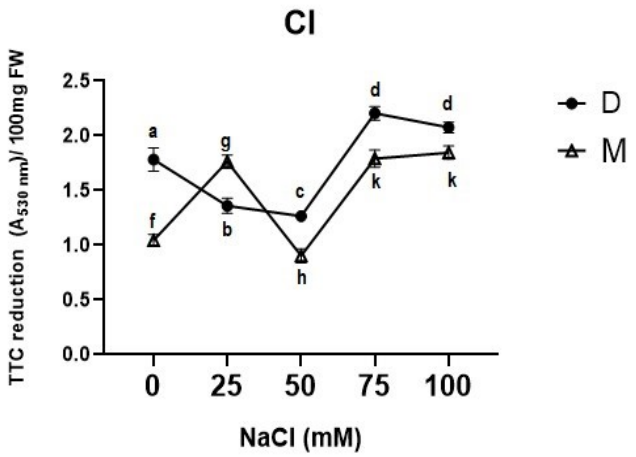

Fig. 5. Effect of salt stress on cellular injury (CI) in seedlings of two populations of *Agave durangensis* (D: Population Durango, M: Population El Mezquital). All the values represent the mean of three independent replicates. TTC: Triphenyl tetrazolium chloride.

The similarity in responses among *A. durangensis* seedlings from various saline treatments was evaluated through cluster analyses (Fig. 6B). These analyses indicated that unstressed seedlings from both populations of *A. durangensis* and the seedlings of population M under low salt concentration exhibited similar responses. Samples from these treatments formed a single group, while samples from other treatments formed a distinct and more heterogeneous group.

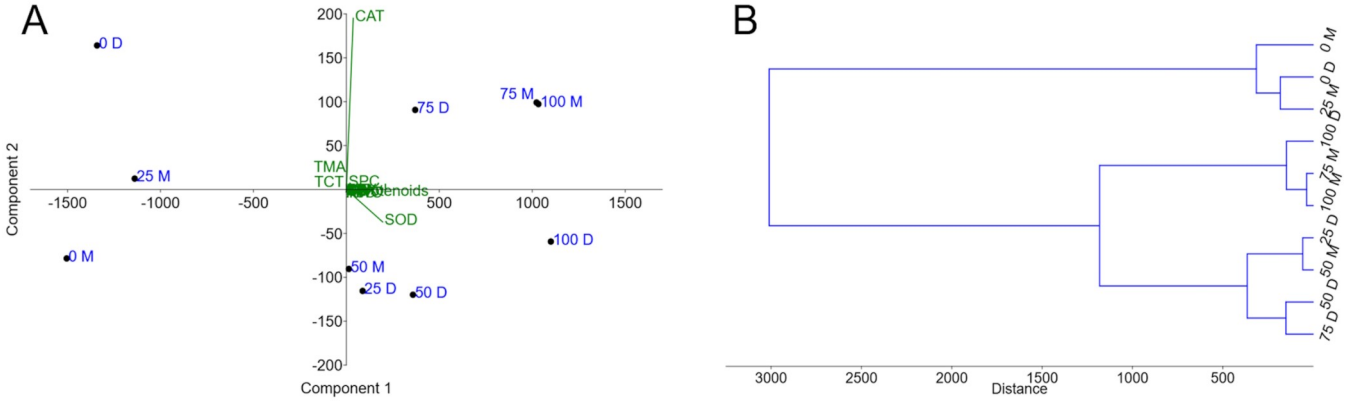

Fig. 6. Results of the principal component analysis (A) and cluster analysis (B), comparing the chemical and biochemical responses (acronyms as described in Table 5) of seedlings from two populations (D: Population Durango, M: Population El Mezquital) of *Agave durangensis* to different NaCl concentration (0-100 mM).

Discussion

Seed germination is a crucial stage in the plant life cycle with significant agronomic implications, as it determines plant establishment and crop yield (25). Moreover, the survival of natural plant populations relies to a large extent on the germination potential of their seeds (26).

It has been established that saline stress can negatively correlate with germination and seed vigor (27), depending on genotype, salinity intensity and the duration of exposure (28). Our results indicate a significant difference in the germination potential of seeds between the two analyzed populations of *A. durangensis*, under both non-stress and stress conditions, with seeds from population M displaying a higher germination potential. The findings also suggest that, despite having different germination potentials, both populations can germinate across a wide range of saline conditions, albeit at a reduced speed compared to non-stress conditions. The demonstrated capacity of *A. durangensis* seeds to germinate under diverse salinity conditions implies the possibility of extending its cultivation area. This is particularly significant since, at present, both its natural geographical distribution and cultivation are limited to a small area in northern-central Mexico (7). However, further studies, encompassing salt tolerance in other stages of development, should be conducted to validate this proposition.

Salt stress can reduce germination speed due to various effects, including: a) reduction of osmotic potential, leading to decreased water and nutrient intake; b) alteration of cell division and expansion; c) ion toxicity, causing chlorophyll damage due to magnesium deficiency resulting from sodium over accumulation (29) and e) alteration of enzymatic activity, leading to hormonal imbalance (30), among others. The delay in the germination of *A. durangensis* seeds was directly correlated with salt concentration and reduction of water content ($r = 0.80$ for D and $r = 0.85$ for M, $p < 0.05$), which is consistent with findings reported for other species, such as wheat (*Triticum aestivum* L.) (31).

To the best of our knowledge, no studies have been conducted to determine the germination potential of salt-stressed seeds in the genus *Agave*. It was reported germination (G) values between 99% and 100% and seedling emergence (S) values between 44 and 47.6 seeds/day for non-stressed seeds of 3 natural populations of *A. durangensis* (32). The values reported were higher than those found in the current study for the 2 populations analyzed within the same species (32). The discrepancy between the previous results and those of the present study for G and S of the control group could be attributed to differences in the experimental conditions used in each study (32). However, the documented high Interpopulation genetic variability for *A. durangensis* (7) may have contributed significantly. This genetic variability in the species could also have influenced the observed Interpopulation variations in relative water content (RWC), leaf length (L), fresh weight (FW) and dry weight (DW). Our findings align with those previously reported, indicating salt-dependent reductions in root and shoot growth for seedlings of *Agave deserti* (9). Our results are also in line with those of Schuch and Kelly (10), who reported salt-dependent reductions in fresh weight, dry weight and moisture content of *Agave parryi* Truncate, although these authors did not provide information on the age of the plants analyzed.

The relative water content (RWC) decreased in response to NaCl treatments for the two populations of *A. durangensis* analyzed. However, the smaller percentage decrease in RWC in population M compared to population D suggests that seedlings from population D were more susceptible to saline stress than those from population M. RWC is considered an accurate and simple parameter to confirm salt stress in plants, as it indicates a loss of turgor resulting from limited water availability for cell growth processes (16). Despite reduced growth parameters, seedlings of *A. durangensis* under any salt treatment exhibited good survival, suggesting significant adaptation potential to salinity. A salt-dependent decrease in RWC has been observed in other plant species, such as *Juglans microcarpa* L. (33).

Accurately recording changes in germination parameters in salinized seeds is crucial to support the development of conservation, improvement and crop expansion programs for economically important plant species, such as *A. durangensis*.

Agaves are xerophytic plants adapted to thrive in hostile environments. However, there is limited information about their biochemical and chemical responses to saline stress, making it challenging to compare our results with those reported for other species within the genus. Enhanced activation of antioxidant enzymes leading to reactive oxygen species (ROS) removal can improve tolerance to salt stress. Superoxide dismutase (SOD), the initial enzyme in the antioxidant system, transforms highly reactive hydroxyl radicals and superoxide $(O_2^{\bullet})^-$ into hydrogen peroxide (H_2O_2), thereby reducing oxidative damage to DNA, proteins and membranes. Catalase (CAT) subsequently catalyzes the degradation or reduction of H_2O_2 to H_2O and O_2, completing the detoxification process initiated by SOD (18). The ascorbate-glutathione cycle participates in ROS scavenging, with increased ascorbate peroxidase (APX) content, reducing H_2O_2 to O_2 and water (6).

Our results indicated that all salt treatments stimulated the activities of SOD, CAT and APX in seedlings from the 2 populations of *A. durangensis* analyzed, reaching the highest activity at 100 mM NaCl. These findings align with a previous report (34), indicating the highest activity of antioxidant enzymes in seedlings of *Asparagus officinalis* L. at high NaCl levels (120 mM). The similarity in the response of antioxidant enzymes between *A. durangensis* and *A. officinalis* (both belonging to the same family, Asparagaceae) suggests a common strategy against salinity in the family. The results imply that *A. durangensis* possesses an effective enzymatic defense mechanism for scavenging ROS and coping with salt stress under low, moderate and strong NaCl concentrations,

particularly the latter. Our results also suggest a concomitant role for these three enzymes under increased salt concentration for *A. durangensis* seedlings. Contrastingly, different results have been reported for other plant species; for example, *Physalis ixocarpa* (Husk tomato) exhibited a complementary role for CAT and SOD under increased salt concentration (28), while neither CAT nor SOD responded to increases in salt concentration in *Pisum sativum* L. (pea) (35). It has been reported that plants with high levels of antioxidant enzymes express better resistance to ROS (25). Thus, based on the current results, *A. durangensis* seedlings demonstrate good resistance to ROS.

Plants accumulate a diverse array of organic solutes, known as osmolytes, to anticipate external osmotic changes and cope with salt stress. Examples of these osmolytes include proteins and proline (1, 33). Our results indicated that soluble protein content (SPC) in seedlings from population D was salt-dependent, whereas in seedlings from population M, SPC increased only under 100 mM NaCl. This suggests that, for seedlings from population D, SPC serves as an important osmotic regulator under low, moderate and strong saline conditions, while for seedlings from population M, SPC is relevant only under strong saline conditions. The role of SPC as an osmotic regulator was also evident in other plants, such as safflower (*Carthamus tinctorius* L.) (17).

The proline (Pro) content for both analyzed populations of *A. durangensis* significantly increased under salt stress (Fig. 1E). The accumulation of Pro is a crucial adaptive response in some plant species to salt stress, contributing to cellular osmotic adjustment and the physiological maintenance of homeostasis (28). Proline serves as a multifunctional compound, protecting enzymes, stabilizing their structures and acting as a reactive oxygen species (ROS) scavenger (3). According to our results, the strong correlation found between salt concentration and Pro accumulation ($r = 0.92$ for D and $r = 0.99$ for M, $p < 0.01$) suggests that this amino acid is a key biochemical mechanism in *A. durangensis* seedlings against low, moderate and high saline stress. By reducing their osmotic potential, plants can avoid dehydration caused by saline stress, thereby improving the transportation, accumulation, and compartmentalization of organic solutes (17). A similar crucial role of proline has been reported for other plant species, such as *Physalis ixocarpa* (28).

Another indicator of salinity tolerance is ascorbate (AsC). This compound serves as an effective water-soluble antioxidant capable of donating electrons (25) in enzymatic processes, primarily as a substrate for ascorbate peroxidase (APX) and non-enzymatic reactions, directly involved in the removal of reactive oxygen species (ROS) (36). Additionally, AsC plays a role in plant photoprotection, the biosynthesis of flavonoids and anthocyanins and the biosynthesis of plant hormones, making it a multifunctional metabolite that contributes to restoring the homeostatic state of stressed plants (37).

The NaCl-dependent increase in AsC observed for population D suggests that this compound is a significant protector, capable of alleviating the negative effects of ROS overproduction induced by NaCl increments, possibly acting in association with APX, as both APX activity and AsC content showed NaCl-dependent trends (Figs. 1B and 1F). However, for seedlings from population M, the protective role played by AsC was only observed under low salt stress (25 mM NaCl), a condition in which AsC might have been directly involved in the removal of ROS. A NaCl-dependent increase in AsC has been reported for other plants, such as *Moringa oleifera* Lam. (38).

Photosynthesis is one of the most critical physiological processes in plants, highly sensitive to salinity. Sodium over-accumulation can rapidly lead to a decrease in the concentrations of other essential minerals, such as K, Ca and Mg, with Mg being crucial for chlorophyll synthesis (30). Reduced chlorophyll content has been observed in salt-sensitive cultivars of various species, including pumpkin (39); however, salt-tolerant species may exhibit increased or unchanged chlorophyll and carotenoid levels under salinity conditions (1). Therefore, the observed general increase in total chlorophyll (TChl) in salt-stressed seedlings compared to control seedlings for both populations, D and M, suggests that *A. durangensis* is a salt-tolerant species in the analyzed growth stage (Fig. 2A).

Carotenoids are specialized metabolites that have been reported to play a protective role in plants against salinity (2). These compounds are significant due to their multiple valuable bioactivities that contribute to human health (40). Carotenoids possess lipophilic antioxidant properties, as the conjugated double bonds in their structure allow them to quench singlet oxygen (6). In plants, carotenoids play essential roles in photosynthesis and photo protection processes (41). However, based on the data in Fig. 2B, their role as protectors against salinity stress for *A. durangensis* appears to be significant only under moderate saline conditions (50 mM NaCl).

Phenolic compounds, including flavonoids, tannins and phenolic acids, constitute a diverse group of specialized metabolites widely distributed in the plant kingdom, with a broad spectrum of biological activities, among which antioxidant activity is particularly important. For the 2 analyzed populations of *A. durangensis*, the levels of total phenols (TP), total flavonoids (TF), total condensed tannins (TCT) and total monomeric anthocyanins (TMA) were not dependent on salt concentrations. However, under the 25 mM NaCl treatment, the levels of TP, TF and TCT were found to be higher in seedlings of population D, but not in those of population M, compared to control seedlings. This suggests that these phenolics may represent a strategy to cope with low salt stress. Due to their recognized antioxidant capacity, the accumulation of phenolic compounds is considered an important plant strategy against salinity in several plant species, such as rice (*Oryza sativa* L.) (42).Despite the high correlation observed between the DPPH scavenging capacity and FRAP with the content of TP, TF and TCT, a minor

importance of these compounds as a strategy to cope with salinity conditions was revealed for *A. durangensis* seedlings. Similar results were reported for other plant species, such as Husk tomato (*Physalis ixocarpa* L.) (28). The variation in responses found in different plant species may be the consequence of the particular genetically controlled responses developed by each species, even in related ones. Species-specific responses in the genus *Brassica* (Brassicaceae) have been identified (15).

Anthocyanins were the only phenolic compounds that played a significant role as protectors under the highest NaCl concentrations evaluated (75 and 100 mM) for the two analyzed populations of *A. durangnesis*. Some authors have reported that reactive oxygen species (ROS) generated by abiotic stress activate transcription factors involved in anthocyanin biosynthesis (43). Due to their crucial antioxidant activity, anthocyanins protect plant cells from damage caused by ROS, aiding plants in adapting to stress conditions. Furthermore, the current results revealed that these saline conditions are suitable for *A. durangensis* seedlings to accumulate high levels of total monomeric anthocyanins (TMA). The richness in TMA reached under strong salt stress could be useful in medicinal, pharmaceutical and cosmetic industries. The bioactivities of these compounds include antioxidant, antidiabetic, anticancer, anti-inflammatory, antimicrobial and anti-obesity effects, as well as the prevention of cardiovascular diseases (44).

Monitoring changes in TTC-reduction activity serves as a valuable tool for predicting salinity tolerance (45). The colorless 2,3,5-triphenyl tetrazolium chloride (TTC), a redox indicator, turns red when reduced to 1,3,5-triphenyl formazan (TPF) by cells (46). The efficiency of this conversion provides an index of mitochondrial dehydrogenase activity, the rate of tissue respiration and consequently, cell viability (47). According to the results in Fig. 5, a low NaCl concentration (25 mM) can improve cellular viability in certain genotypes of *A. durangensis*. This type of response has been reported for a macroscopic marine alga (*Ulva fasciata*) (48). The most damaging concentration of NaCl was 50 mM for seedlings of D, and it was the only harmful concentration for seedlings of M (Fig. 5). However, seedlings of both populations displayed impressive resilience when exposed to stronger salinity conditions, under which they were able to increase mitochondrial activity. The recovery of cell viability under extreme salinity conditions has been reported for some plant species, but typically with the assistance of certain stimulants, as observed in tomatoes (47).The current results suggest that there are genotypes of *A. durangensis* that increase the activity of mitochondrial dehydrogenases under low and strong NaCl exposure, while being susceptible under moderate NaCl concentration. The link between salt tolerance and the respiratory response to salinity is complex; respiratory rate per se might not be a determinant key of salt tolerance. However, optimal deployment of mitochondrial mechanisms is associated with plant salt tolerance (49), emphasizing the importance of evaluating these mechanisms. The recovery of cell viability at high salt concentrations was associated with Pro content, the activities of CAT, APX and SOD and TMA content, suggesting that all these responses supported the recovery of cell viability in the 2 populations of *A. durangensis*.

Significant Interpopulation differences were observed in all responses to saline stress, except for TCT and FRAP (Table 4). These differences between the two analyzed populations of *A. durangensis* align with the high genetic variability reported for this agave (8), which may confer a high degree of phenotypic plasticity to the species. Each response was regulated differently under a specific NaCl concentration, with some responses being more significant than others as protectors against salinity under particular saline conditions. Our results may account for the observation that salt stress induces variable effects, either removing or adding methylation levels to different salt-responsive genes. These effects can be species-specific or genotype-specific, leading to differential enhancement or reduction in the expression of salt-responsive genes (50).

The PCA results underscored the significance of SOD and CAT activities in distinguishing between seedlings subjected to various saline treatments. Cluster analysis results suggested that collectively, all the evaluated responses of unstressed and salt-stressed *A. durangensis* seedlings hold considerable potential for differentiation among plants cultivated under different salt treatments. This discriminative potential carries agronomic implications and may contribute to the development of a fingerprinting approach for the comprehensive utilization of the species.

Conclusion

Agave durangensis exhibits intricate defense mechanisms against salt stress, with variations observed among genotypes and under different levels of salt stress. Despite a reduced germination potential and seedling growth, *A. durangensis* seedlings demonstrate survival capabilities even under strong salt concentrations. The primary biochemical defenses, including the activities of APX, SOD and CAT, along with Pro content, play crucial roles in combating low, moderate and strong salt stress. However, TMA emerges as a significant contributor to cell viability recovery under strong NaCl concentrations. Given the variation in responses with increasing NaCl concentrations, documenting specific responses becomes imperative for understanding the adaptation potential of economically important plants to salinity. The significant adaptation potential observed in *Agave durangensis* seedlings from the analyzed populations bodes well for cultivation in saline soils of semiarid regions.

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Authors' contributions

NAA conceived and designed the study, participated in the collection of planting material, performed the statistical analysis and analyzed and interpreted data. GGH, JAAR, RTR and EADA participated in the collection of plant material and carried out the total chemical and biochemical determinations. All authors made a critical revision, providing intellectual content. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None.

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