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ARTICLE

Effect of Water Stress Induced by Polyethylene Glycol on Growth, Proline Accumulation in *Agave americana* L.

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ABSTRACT

The effect of water deficit was determined on both *in vitro* and soil seedling as well as in cells in suspension of *Agave americana* L. In order to do the establishment of cells, the formation of callus was induced; for it two auxins were evaluated: 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-mino-3,5,6-trichloropicolinic acid (picloram) at three concentrations (0.25, 0.5 and 0.75 mg L^{-1}) in three explants (leaf, root and meristems) cultured in MS semi-solid medium. The callogenesis response was related to the type and section of the explant, as well as the regulator used, and a cell suspension was established using 0.5 mg L^{-1} naphthaleneacetic acid (NAA) + 0.5 mg L^{-1} Benzylaminopurine (BAP). Seedlings were exposed to polyethyleneglycol (15% and 30% w/v) with a water potential of −0.87 and −2.67 MPa, respectively, under soil conditions. Water stress was applied through restricted irrigation. Fresh weight, root system growth, and chlorophyll concentration were some of the parameters that were affected by the effect of water deficit on *A. americana* L. Chlorophyll concentration values were significantly decreased by 15 at 30% PEG (19.6 SPAD units) compared to the control treatment. In *in vitro* plants, the highest concentration of proline was found in the roots, being the treatment with 30% polyethylene glycol where the highest concentration of this osmoregulator was obtained (62.5 mg g^{-1} DW). Under restricted irrigation conditions, an increase in proline concentration was observed both in the aerial part (2.2 µg 100 g^{-1} DW) and in the root system (1.8 µg 100 g^{-1} DW). However, the concentrations found were approximately ten times greater, less than those found under *in vitro* conditions. Therefore, the accumulation of proline can be considered an indicator of stress in *Agave Americana* L. growth *in vitro*.

KEYWORDS

Agave americana; water deficit; proline

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1 Introduction

"Stress" in plants can be defined as any external factor that negatively influences the growth, productivity, reproductive capacity, or survival of an organism. Those factors are divided into two categories: the biotic ones, caused by different living organisms such as fungi, bacteria, viruses, and insects, and, the abiotic ones which are caused by environmental factors such as drought, waterlogging, high and low temperatures and salinity, among others [1]. Between the abiotic factors, drought is one of the most important because it negatively affects the growth and development of plants, manifesting its effects from a cell to the entire organism [2]. The most common symptoms that plants present to water deficit or drought stress can be rolled and chlorotic leaves, loss of cell turgor, senescence and, to a lesser extent, necrosis, growth retardation, and plant thinning [3,4].

Exist certain plants that can survive in conditions of severe water stress. The best known among them, are the crassulacean acid metabolism plants (CAM), which are characterized by opening their stomata during night, when temperatures are low and relative humidity is higher, reducing evapotranspiration and increasing water use efficiency [5]. Although CAM-type plant species have great potential in the production of food, fiber, fuel, and fructan polymers (inulin), the area dedicated to their cultivation is still insignificant (0.002%) [6,7]. However, there is little interest for studying this type of plant. Within the CAM plants are those of the *Agave* genus, which are endemic to America since they are distributed from Florida, United States, to the north of South America, including the Caribbean islands. This genus contains approximately 210 species, of which 159 (75%) are present in Mexico, where 129 are endemic [8,9].

One of the main tools for the study of tolerance to abiotic stress in plants is through cell suspension culture since its studies mean a direct uniform population of cells (mainly in liquid form) instead of a multicellular tissue that has a higher degree of organization, which differs according to the location of the tissue or organ [10]. Some studies in *Agave* under *in vitro* or soil conditions have shown that in the presence of water stress induced by PEG, an increase in root length occurs [11] and the suspension of prolonged irrigation does not affect the dry weight of the root and the aerial part [12]. In *Agave potatorum* Zucc plants, cultivated in perlite substrate and fertigated with Hoagland and Aron nutrient solution after five months of suspension of irrigation accumulated more fructans in the head [13]. However, it is not surprising that physiological research is mainly focused on water stress in model plants with the C_3 and C_4 metabolism types. The objective of this study was to evaluate the effect of water deficit on *A. americana* L. on growth and proline accumulation during *in vitro* culture using osmolytes as stress inducer (polyethylene glycol) and grown in soil employing restricted irrigation.

2 Materials and Methods

2.1 Vegetal Material

Agave americana L. shoots was cultivated under aseptic conditions in MS medium (*in vitro* plantlets) for eight months [14]. For their rooting, they were reseeded in MS medium supplemented with 1 mg L^{-1} of indole butyric acid (IBA).

2.2 Callogenesis Induction

Two synthetic auxins were evaluated: 2,4-Dichlorophenoxyacetic acid (2,4-D) and 4-mino-3,5,6-Trichloropicolinic acid (picloram) at different concentrations (0.25, 0.5, and 0.75 mg L^{-1}) in three different explants [leaf, root, and apical meristems (undifferentiated cells responsible for the apical growth located in the center of the stem) in MS semisolid medium]. For the leaf and the meristem three different sections of the explant (base, middle and apical) were used with the thin cell layer (TCL) technique proposed by Monja-Mio et al. [15]. The response variables were the percentage of callus formation, type, and coloration.

2.3 Induction of Cell Suspension of Agave americana L.

For callus growth in a liquid medium, two strategies were used. The first strategy consisted of growing the selected callus in the same callus induction treatment, and the second strategy consisted of using combinations of hormones: the first treatment was using the combination of 2,4-D + 6-Benzylaminopurine (BAP) (0.5 + 9 mg L^{-1}) [16], and the second treatment was using the combination of naphthaleneacetic acid (NAA) + BAP (0.5 + 0.5 mg L^{-1}) [17]. Cell viability was determined using the Trypan blue technique [18].

2.4 Water Stress in Cell Suspensions of Agave americana L.

To determine the effect of water stress on the cell suspension, three treatments with four replicates each were used: polyethylene glycol 8000 (PEG) (0%, 15%, 30% w/v) in MS liquid medium added with NAA + BAP (0.5 + 0.5 mg L^{-1}). The experimental unit consisted of a 500 mL flask with a volume of 50 mL of liquid medium + 1 g of fresh weight (FW) of the cell suspension, which was kept for 30 days.

2.5 Water Stress Induced with PEG in Vitro Seedlings of Agave americana L.

Eight-month-old seedlings cultivated *in vitro* were used, which were transplanted into a semi-solid MS medium with different concentrations of PEG (15% and 30% w/v) with 10 repetitions each so that the experimental unit consisted of a flask with a seedling. The seedlings were exposed to stress for 60 days under controlled conditions of 18 h light/6 h darkness and at 25°C. The water potential in the medium was measured using a Dewpoint PotentiaMeter model WP4.

2.6 Water Stress by Suspension of Irrigation in Seedlings Agave americana L.

Plants from *in vitro* culture were planted in 307 mL expanded polystyrene cups with a mixture of agrolite: peat moss (1:3) at 25°C and with a photoperiod of 16 h light/8 h darkness and one irrigation every third day for 90 days. The water deficit consisted of stress through irrigation once a week and as a control one every third day. The volume of water used was 30 mL, calculated with the field capacity of the substrate.

2.7 Chlorophyll Content

For the chlorophyll content, the Minolta Spad 502 plus meter was used. The method of Arnon [19] was used to determine the chlorophyll content (a, b, and total). The concentration of chlorophyll a, b and total was determined using the following formulas proposed by Inskeep and Bloom [20].

$$\text{Chl a} = 12.7 \times \text{A664} - 2.79 \times \text{A647}\left(\mu g\ \mu L^{-1}\right)$$

$$\text{Chl b} = 20.7 \times \text{A647} - 4.62 \times \text{A664}\left(\mu g\ \mu L^{-1}\right)$$

$$\text{Chl total} = 19.9 \times \text{A647} - 8.8 \times \text{A664}\left(\mu g\ \mu L^{-1}\right)$$

where: (A664) and (A647) represent the absorbance values read at 664 and 647 nm wavelength wave, respectively. Chl a: chlorophyll a; Chl b: chlorophyll b; Chl total: total chlorophyll.

2.8 Determination of Proline Content

To determine the proline content, the protocol described by Bates et al. [21] was used and its concentration was determined from a standard curve as follows:

$$\mu\text{mol proline } g^{-1}\text{of dry weight} =$$

$$[(\mu g \text{ proline} \cdot \text{mL de toluene})/(115.5\ \mu g\ mol^{-1})]/[\text{dry weight in grams of sample}/5]$$

2.9 Experimental Design

A 3 × 2 × 3 factorial design (three types of explants, two hormones, and three hormone concentrations) was performed with a comparison of Tukey means (P-value ≤ 0.05) for callus induction. To see the effect of water deficit during *in vitro* conditions, a completely randomized design was carried out, four repetitions were used for cells in suspension and 10 repetitions for seedlings. For growing in soil *ex vitro* conditions, 12 replicates were used using an Analysis of Variance (ANOVA) and a Tukey mean comparison test (P-value ≤ 0.05). In all cases, the Statgraphics Centurión XV program was used.

3 Results

3.1 Callogenesis Induction

All three types of explants presented different responses to the concentrations of the regulators evaluated (Fig. 1). In leaf explants, no morphogenic response was observed (Fig. 1A). Regarding the meristem (undifferentiated cells responsible for the apical growth of plants), all treatments presented callus formation (Figs. 1B and 1C).

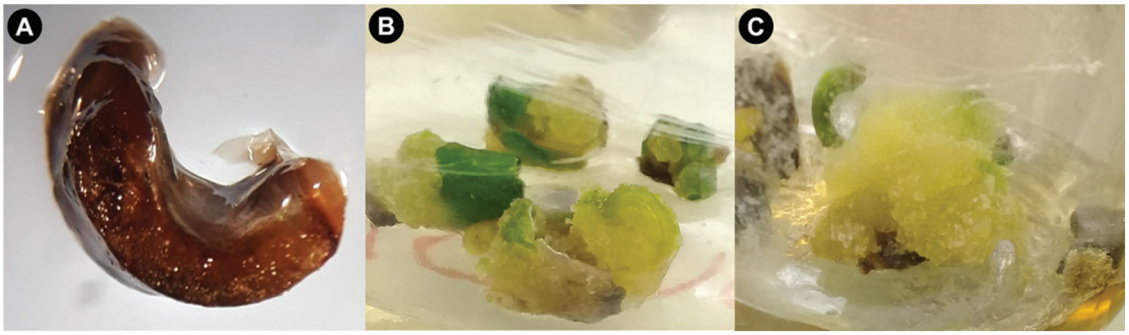

Figure 1: Response of *Agave americana* L. explants to various auxin concentrations in the culture medium. (A) Leaf oxidation. (B) Callus in meristem culture in MS + 2,4-D (0.75 mg L^{-1}), and (C) Callus in meristem culture in MS + picloram (0.5 mg L^{-1}) at 60 days of induction

During the evaluation of the influence of the sections of the meristem as an explant, the results showed that when using the base of the meristem, the best treatment was with 2,4-D at 0.5 and 0.75 mg L^{-1}. For the middle part of the meristem, picloram showed better results, presenting callus formation percentages of 70% and 50% at 0.5 and 0.75 mg L^{-1}, respectively (Table 1). According to the Pareto diagram (Fig. 2), it was determined that the factors that exert a significant effect ($P < 0.05$) were the explant section (Factor C) and the combination of auxin-explant section (Factors AC) for callus production from the meristem.

Table 2 reports the characteristics of the callus obtained from the sections of the meristem evaluated. Friable callus was found in all treatments, which made them suitable for the culture of cells in suspension since they maintained a constant growth. In addition, they showed a greater capacity to disintegrate.

3.2 Induction of Cell Suspension of Agave americana L.

For the induction of the cell suspension, only the callus originating from the meristem was used (Fig. 3A). As mentioned above, the first strategy used was to grow the callus in the same concentrations as those of the induction, which were, the concentrations of 0.5 and 0.75 mg L^{-1} for both auxins (2,4-D and picloram). At these concentrations somatic embryos were presented after four weeks in the liquid medium, while at the concentration of 0.25 mg L^{-1} there was no embryogenic response or biomass increase. The second strategy used for induction, the treatment with the combination of 2,4-D + BAP (0.5 + 9 mg L^{-1}) did not present biomass growth; however, the combination of 2,4-D + BAP

(0.5 + 0.5 mg L^{-1}) (Fig. 3B) showed viable cells (Fig. 3D) coupled with biomass generation without cell regeneration even after six weeks of subcultures, although callus darkening was also present (Fig. 3C).

Table 1: Callus-induction from different sections of meristematic tissues

Synthetic auxin [mg L^{-1}]	% Callus formation frequency explant sections		
	Basal	Middle	Apical
2,4-D			
0.25	75 b	0 a	0 a
0.50	80 a	0 a	0 a
0.75	80 a	0 a	0 a
Picloram			
0.25	35 b	0 c	40 a
0.50	0 c	70 a	30 b
0.75	50 a	50 b	0 a

Note: *Values with different letters are significantly different ($P \leq 0.05$). Each value is the mean; n = three replicates.

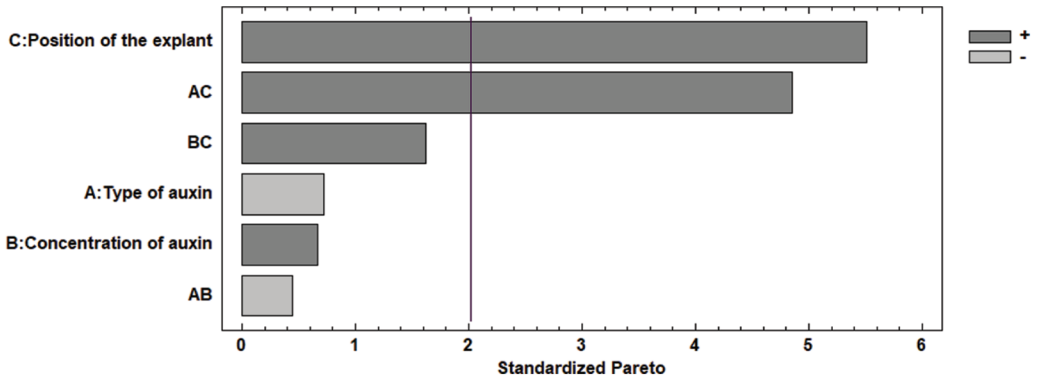

Figure 2: Pareto chart of the standardized effects of different types of auxin, the auxin concentration, and the section of the explant on callus formation. *P*-value ≤ 0.05 represents statistically significant

Table 2: Callus-forming morphology in regenerated callus from different explant sections of meristem

Synthetic auxin	[mg L^{-1}]	Type of callus	Callus color (according to section of explant)		
			Basal	Middle	Apical
2,4-D	0.25	Friable	Cream	Cream	Cream
	0.50	Friable	Cream	Cream/green	Cream/green
	0.75	Friable	Cream	Cream/green	Cream/green
Picloram	0.25	Friable	Cream	Cream	Cream
	0.50	Friable	Cream	Cream	Cream/green
	0.75	Friable	Cream	Cream	Cream

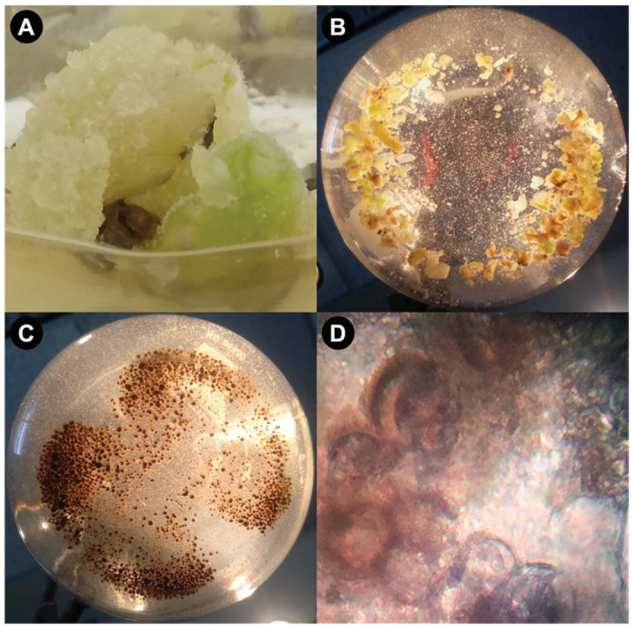

Figure 3: Cell suspension cultures from meristematic callus in liquid MS medium with 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP. (A) Callus induction from meristematic callus (0.75 mg L^{-1} 2,4-D). (B) Establishment of cell suspension in a liquid medium. (C) Oxidized callus in liquid medium after 15 d. (D) Cell not stained by trypan blue dye after six weeks

3.3 Effect of Polyethylene Glycol (PEG) on Cells Suspension of Agave americana L.

Polyethylene glycol had an effect on the fresh weight of *Agave* cells at both concentrations evaluated (15% and 30%), observing a significant decrease of 35% and 79%, respectively, compared to the control (Table 3).

Table 3: Fresh weight of cell suspension in liquid medium added with PEG after 30 d

% PEG	Fresh weight (g)
0	0.90 ± 0.03 a
15	0.59 ± 0.03 b
30	0.19 ± 0.03 c
LSD	0.13

Note: *Values with different letters are significantly different ($P \leq 0.05$). Each value is the mean ± standard error of n = three replicates. LSD: least significant difference.

3.4 Effect of Polyethylene Glycol (PEG) on in Vitro Seedlings of Agave americana L.

The water potential values showed that the treatments were found in a range of light (−0.5 to −0.8 MPa) to severe stress (>−1.5 MPa) (Table 4).

Different responses were observed in *A. americana* L. seedlings that were exposed to PEG treatments (Fig. 4). A significant reduction of 62% and 75% was observed in the Fresh Weight (FW) of the aerial part and the Dry Weight (DW) of the root system respect to the control. In this last parameter, a reduction was observed at the formation of roots in the treated seedlings with different concentrations of PEG evaluated

with respect to the control treatment (Table 5). The number of roots formed was significantly reduced up to 77% in the presence of PEG. However, no significant effect was observed on the root length as well as the DW of the aerial part and FW of the root system as the PEG concentration increased.

Table 4: Water potential of semi-solid medium MS supplemented with PEG

PEG [%]	Water potential [MPa]
0	−0.47
15	−0.87
30	−2.67

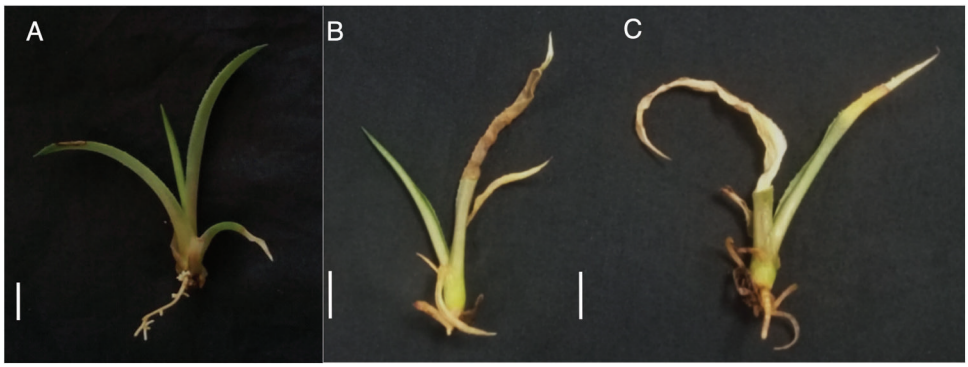

Figure 4: Effect of Polyethylene glycol (PEG) on *Agave americana* L. seedlings after 60 d. (A) Control; (B) 15% PEG; (C) 30% PEG. Bar = 1 cm

Table 5: Effect of Polyethylene glycol (PEG) on the growth parameters of *Agave americana* L. after 60 d

% PEG	Root system			Root		Aerial part	
	Plants with roots (%)	Number of roots per plants	Length (cm)	Weight (g)			
				Fresh	Dry	Fresh	Dry
0	80	2.1 ± 0.32 a	0.61 ± 0.22 a	0.006 ± 0.00 a	0.008 ± 0.00 a	0.9 ± 0.08 a	0.05 ± 0.00 a
15	40	0.5 ± 0.32 b	0.54 ± 0.22 a	0.003 ± 0.00 a	0.0012 ± 0.00 b	0.52 ± 0.08 b	0.05 ± 0.00 a
30	60	0.8 ± 0.32 b	0.36 ± 0.22 a	0.004 ± 0.00 a	0.0018 ± 0.00 b	0.34 ± 0.08 b	0.05 ± 0.00 a
LSD		1.14	0.78	0.007	0.006	0.28	0.02

Note: *Values with different letters present a significant difference ($P \leq 0.05$). ±Data are the mean standard error of n = three replicates. LSD: least significant difference.

3.5 Water Deficit in Soil Grown Plants of Agave americana L.

Agave americana L. plants under water deficit conditions (Fig. 5) showed a decrease in FW, and DW (40% and 30%, respectively) in the aerial part in comparison to the control (Table 6). This same response was presented in the root system, observing a decrease in FW, DW and length (75%, 60%, and 20%, respectively) when plants were exposed to water stress.

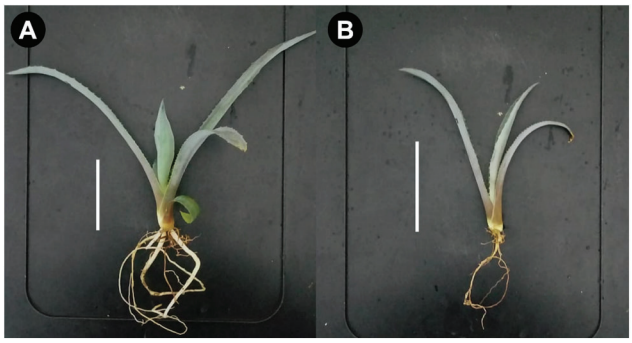

Figure 5: Effect of irrigation on *Agave americana* L. after 60 d. (A) Constant irrigation (B) Restricted irrigation. Bar = 1 cm

Table 6: Effect of restricted irrigation on morphological characteristics of *Agave americana* L. after 60 d

Type of irrigation	Aerial part			Root system		
	Weight (g)		Height (cm)	Weight (g)		Length (cm)
	Fresh	Dry		Fresh	Dry	
Constant	20.68 ± 0.95 a	1.1 ± 0.08 a	15.69 ± 0.46 a	1.32 ± 0.11 a	0.19 ± 0.01 a	26.78 ± 1.8 a
Restricted	12.97 ± 0.95 b	0.75 ± 0.08 b	16.25 ± 0.46 a	0.34 ± 0.11 b	0.08 ± 0.01 b	20.97 ± 1.8 b
LSD	2.79	0.24	1.37	0.32	0.04	5.5

Note: n = 12, *Values with different letters present a significant difference ($P \leq 0.05$), ± standard error. LSD: least significant difference.

3.6 Chlorophyll Content

Chlorophyll content values were significantly decreased by 15% at 30% PEG (19.6 SPAD units) compared to the control treatment (29.3 SPAD units) (Table 7). When grown under soil conditions, plants did not present a significant difference ($P \leq 0.05$) between treatments (Table 8). However, the concentrations of chlorophyll a, b, and total showed an increase of 45%, 60%, and 50%, respectively, compared to control when soil-grown plant grew under water deficit conditions.

Table 7: Chlorophyll content in *Agave americana* L. plants grown *in vitro* in presence of polyethyleneglycol

PEG (%)	SPAD units	Chlorophyll a (µg g^{-1} FW)	Chlorophyll b (µg g^{-1} FW)	Chlorophyll total (µg g^{-1} FW)
Control	29.3 ± 2 a	52.18 ± 8.7 a	20.83 ± 4.4 a	80.31 ± 14.3 a
15	20.9 ± 4 ab	69.6 ± 8.7 a	33.3 ± 4.4 a	113.25 ± 14.3 a
30	19.6 ± 2 b	45.1 ± 8.7 a	24.11 ± 4.4 a	76.29 ± 14.3 a
LSD	8.5	32.1	16.3	52.9

Note: n = 12 (chlorophyll meter); n = 5 (Chlorophyll a, b, total), *Values with different letters present a significant difference ($P \leq 0.05$), ± standard error. LSD: least significant difference.

3.7 Proline Accumulation in Agave americana L. Seedlings Exposed to Water Stress

In vitro seedlings showed a significant increase in proline accumulation in both the aerial (60%) and root parts when exposed to 30% PEG (Figs. 6A and 6B) compared to the control treatment. In the *in vitro* grown plants, the proline accumulation was much higher in the roots than in the aerial parts (Figs. 6A and 6B). Similarly, in plants grown in soil, the proline accumulation increased 50% in leaves (Fig. 6C) and 100% in roots (Fig. 6D) under restricted irrigation conditions.

Table 8: Chlorophyll content in plants grown in the soil under water stress conditions

Type of irrigation	SPAD units	Chlorophyll a (μg g^{-1} FW)	Chlorophyll b (μg g^{-1} FW)	Chlorophyll total (μg g^{-1} FW)
Constant	78.03 ± 1.12 a	61.93 ± 11.2 b	27.3 ± 7.8 b	98.1 ± 20 b
Restricted	78.09 ± 1.12 a	114.65 ± 11.2 a	66.2 ± 7.8 a	199.1 ± 20 a
LSD	3.1	36.74	25.69	65.32

Note: n = 12 (chlorophyll meter); n = 5 (Chlorophyll a, b, total), *Values with different letters are significantly different (*P*-value ≤ 0.05). ±Values are the mean standard error of n = 3. LSD: least significant difference.

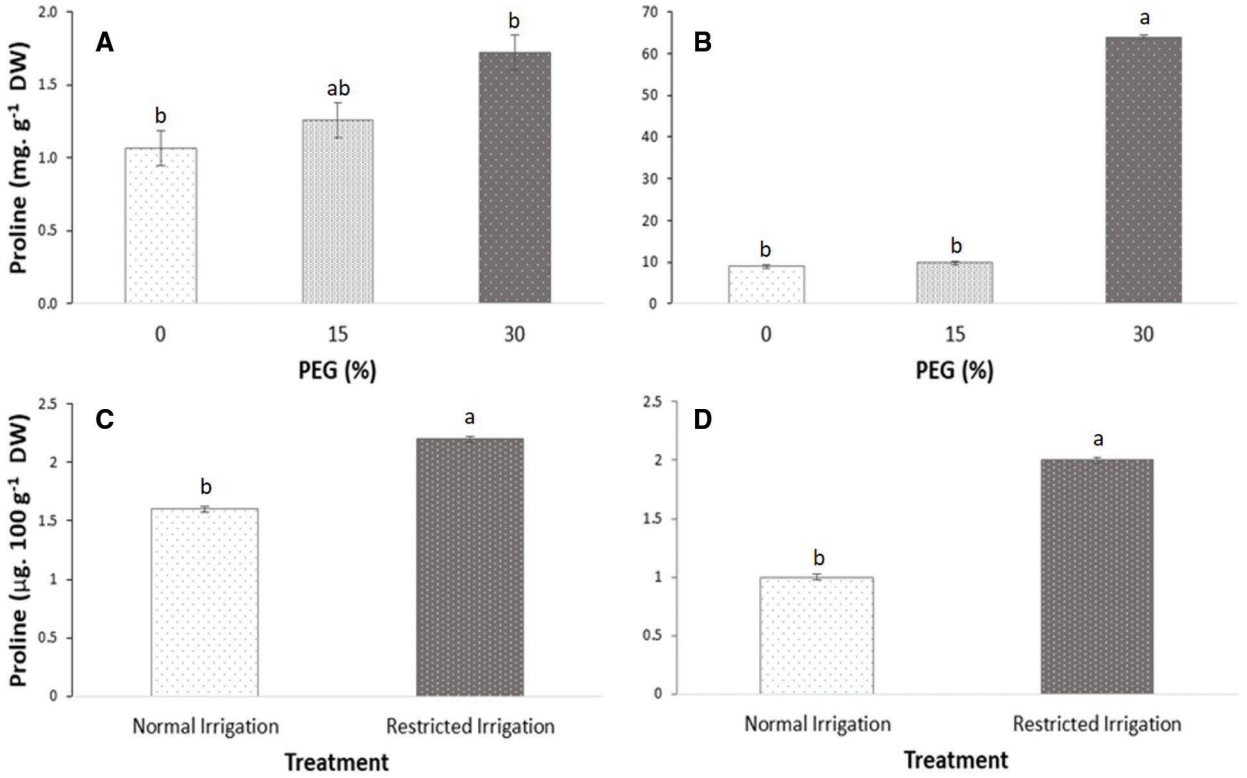

Figure 6: Proline concentration. Plants grown *in vitro* with PEG (A) Leaf and (B) Root; plants grown in soil (C) Leaf and (D) Root. n = 5. Values with different letters are significantly different (*P*-value ≤ 0.05)

4 Discussion

It was only possible to induce calluses from meristem explants with both 2,4-D and Picloram, this response could not be obtained in leaf explants, perhaps due to the oxidation, phenolization and necrosis that occurred, which suggests the presence of phenols in the leaves of *A. americana* L. [22,23]. Phenols are produced by reactive oxygen species (ROS), during the explants cutting stress. These molecules damage lipids, proteins, enzymes and DNA [24] causing the cell to be unable to react with phytohormones, reducing the induction and callus formation. Regarding embryogenesis in the root, the only work with Agave where root tissues were used for a morphogenic process is that of Portillo et al. [25]. They found a low formation of embryos (7.25) using 3 mg L^{-1} of 2,4-D in *Agave tequilana*. Therefore, this type of explant can be considered feasible for the use of embryogenesis in this genus.

The meristem are undifferentiated cells responsible for the apical growth of plants. Due to their cell division capacity, they are considered a good source of explants. Our results are in the range reported in other works of *A. Americana*, regarding the percentage of callus where the meristem was used [20,26], finding percentages ranging from 80% to 100%. However, the callus obtained in those works was of the organogenic type, which is not desirable to generate a cell suspension.

There are few reports on the response of the meristem section and its effect on callus generation. One of them is that of Monja-Mio et al. [15] who evaluated different sections of stems of *Agave fourcroydes* using the thin cell sheet technique. They found the best results in the apical part, due to the amount of meristematic tissue present in it. However, the studies reported are based specifically on the study of the meristematic zone of the apical part of the meristem in *Arabidopsis thaliana*. This is because these cells give rise to the generation of organs and cell division processes.

Alternatively, it has been observed that auxins affect the dedifferentiation and redifferentiation of tissues in different ways, depending on the studied *Agave* species. Cancino-García et al. [27] evaluated the effect of two auxins (indole acetic acid "IAA" and 2,4-D) on the growth of three different species of Agave (*A. angustifolia*, *A. fourcroydes* and *A. tequilana*). They found that in the absence of auxins *A. angustifolia* developed roots while in the presence of 0.5 µM IAA and 2,4-D, these plants developed shoots and calluses, respectively. The other two species only generated roots in the absence of auxin. In the same study, where 32 ARF (Auxin Response Factor) genes related to the growth of lateral roots, embryogenesis, leaf expansion, and fruit development, the results showed different expression patterns in the three *Agave* species in the presence and absence of auxins. Consequently, it was concluded that *A. angustifolia* has a different mechanism for the callus initiation process compared to *A. fourcroydes* and *A. tequilana*. The coloration of the callus is what allows to elucidate whether it is an organogenic or non-organogenic callus. Studies have been carried out in this regard in cocoa [28], coffee [29,30], rice [31] and banana [32]. In the case of *Agave*, no literature indicates the type of response that can be generated concerning the coloration of the calluses in the species.

Cell suspension cultures have been developed in species such as A. *tequila* [33] and *A. amaniensis* [34]. The results in this study would be the first for *A. americana* L. The use of cells in suspension to analyze abiotic stress responses is based on the fact that *in vitro* cultured cells behave similarly to intact plant cells subjected to water deficit conditions [35]. The addition of PEG to the medium produces osmotic stress [36] and decreases the water potential [37–39] which negatively affects growth. Studies have reported a lower growth rate in the presence of PEG in date palm [40], cane [41], tomato [42], wheat [43], and soybean [44].

In our study, the use of PEG in *A. americana* L. shoot cultivated under aseptic conditions (*in vitro* plants) showed a similar response to that of root length. Anyhow, there was a tendency to decreased root growth as reported by Puente-Garza et al. [11]. These authors reported that *in vitro* seedlings of *Agave salmiana* subjected to stress with PEG for 60 days showed a significant decrease in root length compared to control plants (2.63 cm) concerning treatments with PEG at 10%, 20% and 30% (0.6, 0.3 and 0.1 cm respectively). In *A. americana* plants the number of roots formed was significantly reduced in the presence of PEG at 15% and 30%, but the length was not affected. The presence of PEG in a liquid medium could cause a flooding stress, which could explain why the plants did not generate the same number of roots as the control did.

The decrease in biomass has been seen in other CAM-type plants such as *Aloe vera*, Silva et al. [45] mentioned that, in this type of plant, the most important amount of biomass is presented mainly in the leaves. The loss of biomass may be related rather to dehydration of the seedlings caused by the high concentrations of PEG in the medium. This causes an increase in the water potential (more negative) in the culture medium compared to the internal potential of the plant so there is an outflow of water from

the plant to the medium to maintain an osmotic adjustment. Therefore, the biomass production of the aerial part depends on the availability of water in the environment [46]. This is because *A. americana* is not so dependent on stomatal closure as a measure of tolerance to water deficit, since it can use other mechanisms such as: the accumulation of osmolytes such as proline and fructans, that provide membrane stability and osmotic adjustment. In the presence of PEG, no significant differences were found in the different photosynthetic pigments using the acetone method. Otherwise with the chlorophyllometer, the highest concentrations of PEG were found to have the lowest values of chlorophyll. The decrease of pigments only present at the highest concentrations could be whereas at these concentrations the *Agave americana* L. plants may be under water stress. This could induce the production and accumulation of ROS, which induce lipid peroxidation in membranes and that ultimately, leads to chlorophyll degradation and loss of photosynthetic activity [47,48].

The response to the decrease in biomass in *A. americana* L. plants under conditions of water deficit due to decreased irrigation was similar to that found *in vitro* using PEG. Langlé-Argüello et al. [13] demonstrated a decrease in the number, thickness, and FW of the leaves in *Agave potatorum* Zucc. after 5 months of drought. On the other hand, Bergsten et al. [49] found a decrease in root FW, height and total FW in *Agave weberi* plants with water deficit after 80 days. Ramirez-Tobias et al. [12] showed that the DW of the root and leaf of *A. americana* L. is not affected under conditions of water deficit (−3.5 MPa) even after 14 months under greenhouse conditions in plants obtained from seeds. This evidence that the response to water deficit in the soil through root growth to increase water absorption seems unnecessary; conserving the water stored in its leaves and stems are a more efficient response. However, in *Agave* species such as *A. angustifolia* subsp. *tequilana*, *A. asperrima*, *A. lechuguilla*, and *A. striata* increased root biomass (−3.5 MPa) demonstrated the heterogeneity between *Agave* species.

Furthermore, it was observed that after 60 days *A. americana* plants with water deficit did not decrease the concentration of photosynthetic activity. Ramírez-Tobias et al. [50] evaluated two species of *Agave* (*A. striata* and *A. salmiana*) under water deficit, finding a reduction of chlorophyll-a compared to the control of one third and two thirds for *A. striata* and *A. Salmiana*, respectively, this demonstrates the heterogeneity of the species studied.

Altogether, few works are based on the effect of water stress in *Agave*. Peña-Valdivia et al. [51] found a significant increase in proline accumulation from 1.6 to 2.1 mmol mg^{-1} DW in the roots of *Agave salmiana* when they were under stress conditions (−2.5 MPa). Langlé-Argüello et al. [13] also studied the effect of water deficit and the physiological and morphological characteristics, and the generation of proline in roots. They evaluated the effect of restricted irrigation (−3.5 MPa) in eight species of Agave, having an average of 0.52 µmol 100 mg^{-1} of DW under frequent irrigation (−0.7 MPa). The only exception was on *A. americana* where there was 0.31 µmol 100 mg^{-1}, under conditions of humidity restriction (−3.5 MPa) the proline content increased to an average of 1 µmol 100 mg^{-1}. Riaz et al. [52] evaluated the morphological characteristics and proline accumulation in *Agave sisalana* leaves (from 1.23 to 4.26 µmol proline g^{-1} FW) when the plants were under restricted irrigation (2% irrigation). Delatorre-Herrera et al. [53] evaluated the effect of restricted irrigation on *Aloe vera*, finding a significant increase in proline accumulation in plants at 25% irrigation (0.8 mg g^{-1} DW) compared to the control (0.4 mg g^{-1} DW).

5 Conclusion

Water deficit both *in vitro* and grown in soil decreased the growth of the aerial part, as well as the root system in *A. americana* L. plants. Proline was an indicator of stress in both conditions.

The highest concentration of this osmolyte was found in the root system, mainly on *in vitro* plants. Therefore, the increase in proline constitutes a protective response in *Agave americana* L. favoring cellular homeostasis under stress conditions, although this accumulation will depend on the species, the physiological age of the plant and the time it was subjected to stress.

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