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Effects of *in vitro* Drought Stress on Growth, Proline Accumulation and Antioxidant Defense in Sugarcane

Leila Albuquerque Resende de Oliveira[1], Milena Nascimento Cardoso[1], Annie Carolina Araújo de Oliveira[1], Caroline de Araújo Machado[1], Bruno Trindade Cardoso[2], Ana Veruska Cruz da Silva[2] & Ana da Silva Ledo[2]

[1] Programa de Pós-graduação em Agricultura e Biodiversidade, Universidade Federal de Sergipe, Sergipe, Brazil

[2] Embrapa Tabuleiros Costeiros, Aracaju, Sergipe, Brazil

Correspondence: Leila Albuquerque Resende de Oliveira, Programa de Pós-graduação em Agricultura e Biodiversidade, Universidade Federal de Sergipe, Av. Marechal Rondon s/n, São Cristóvão, Sergipe, Brazil. E-mail: leila.a.resende@gmail.com

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Abstract

Drought is the most limiting environmental factor to crop productivity and presents a great variability in the degree of tolerance among and within species, among varieties. The aim of this study was to characterize sugarcane accessions regarding tolerance to water stress during *in vitro* cultivation based on changes in biometric, physiological and biochemical characteristics, within species and among species, to support future breeding programs. Adventitious shoots of five sugarcane accessions: *Saccharum robustum*, *Saccharum spontaneum* and *Saccharum officinarum* species, cultivated in Murashige and Skoog medium supplemented with 2% sucrose and 4 g/l Phytagel were used in five water potentials, 0, -0.3, -0.6, -0.9, -1.2 MPa, induced by mannitol. Survival, length of shoots and roots, number of shoots and roots, biomass, proline content in leaves and activity of antioxidant enzymes were analyzed. There is difference among species, and also, within the same sugarcane species when submitted to *in vitro* drought stress, and *S. officinarum* was shown to be the most tolerant. Proline can be used as a biochemical indicator of response to drought in sugarcane accessions and its accumulation was intensified in *S. robustum* and *S. spontaneum* accessions. Catalase activity remained unchanged with increased drought in sugarcane accessions evaluated.

Keywords: antioxidant enzymes, osmotic adjustment, *Saccharum officinarum*, *Saccharum robustum*, *Saccharum spontaneum*

1. Introduction

Drought stress is one of the environmental factors that causes great damage to the physiological and metabolic processes of plants (Taiz & Zeiger, 2009), and one of the most studied due to its strong impact on crop productivity (Filippou et al., 2014). There is great variability in the degree of drought tolerance among species, and even among varieties of a species. This difference is observed through the value of the ideal water potential for plants and the development stage in which the stress occurs (Inmam-Bamber & Smith, 2005; Pimentel, 2004). In sugarcane, among four distinct growth stages, tillering and rapid growth have been identified as critical for water demand (Ramesh, 2000), causing severe production losses. In these stages, 70-80% of all biomass accumulated during the crop cycle are produced (Oliveira et al., 2010).

Drought tolerance is a mechanism that allows plant to maintain its metabolism, even with reduction of the water potential of tissues (Taiz & Zeiger, 2009), mainly due to the accumulation of compatible solutes and the antioxidant capacity (Verslues et al., 2006). The osmotic adjustment is considered an active mechanism of accumulation of solutes in cells with reduction of the cell water potential without decreasing in turgidity or in cell volume and, consequently, the maintenance of the turgor pressure, which provides the maintenance of the plant vital processes (Basu et al., 2016). When accumulated during water deficit, solutes act to maintain the water balance in the cell, in the protection of enzymes and membranes, and are sources of nitrogen and carbon after rehydration (Ashcroft & Foolad, 2007; Mccree et al., 1984; Yordanov et al., 2003). In addition, plants also activate enzymatic protection mechanisms. These mechanisms include antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidases such as ascorbate peroxidase (APX) (Mittler, 2002), which

are responsible for the elimination of reactive oxygen species (ROS), whose production is reinforced by stress, and characterize a secondary stress, the oxidative stress (Azevedo Neto et al., 2006; Tuteja, 2007).

In order to investigate biochemical and physiological changes in the cultivation of plants under drought, several authors have successfully used the plant tissue culture technique. Many species have already been targeted for studies such as cactus (Balen et al., 2013), wheat (Kacem et al., 2017), potato (Albiski et al., 2012), agave (Puente-Garza et al., 2017), rice (Verma et al., 2013; Wani et al., 2010), banana (Said et al., 2015) and sugarcane (Errabi et al., 2007; Patade et al., 2014, 2012, 2008). Therefore, the aim of this study was to characterize sugarcane accessions tolerant to water stress during *in vitro* cultivation based on changes in biometric, physiological and biochemical characteristics, within and among species, to support future breeding programs.

2. Material and Methods

The experiments were conducted at the Laboratory of Plant Tissue Culture and Laboratory of Plant Ecophysiology of Embrapa Coastal Tablelands, in Aracaju, Sergipe, Brazil.

2.1 Plant Material

As plant material, adventitious shoots were used from: Q45923-*Saccharum robustum*; GH49-*Saccharum robustum*; MIA35301-*Saccharum robustum*; NSL291970-*Saccharum spontaneum*; PI88652-*Saccharum officinarum*, previously established *in vitro*. The accessions were provided by the National Center for Genetic Resources Preservation/ARS/USDA, Fort Collins, CO, USA, which are part of the Active Germoplasm Bank of sugarcane of Embrapa Coastal Tablelands, Aracaju, Sergipe, Brazil.

2.2 Growing Conditions and Stress Treatment

Every 30 days, cultures were multiplied in aseptic environment, in test tubes containing 20 ml of the multiplication medium (MM) consisting of salts from the MS medium (Murashige & Skoog, 1962), supplemented with 2% of sucrose, 0.2 mg/l of BAP (6-benzylaminopurine—Sigma-Aldrich) + 0.1 mg/l of kinetin (Sigma-Aldrich), gelified with 3.5 g/l of Phytagel® (Sigma-Aldrich), and pH adjusted to 5.8.

The medium was autoclaved at 121 °C for 20 minutes, at all stages. The cultures were maintained under conditions of (25±2 °C) ambient temperature, photoperiod of 12 hours of light and luminous intensity of 60 μmol m^{-2} s^{-1}.

After three subcultures, shoots with approximately 3 cm long were transferred to test tubes with 20 ml of culture medium consisting of the salts from the MS, supplemented with 3% of sucrose (Synth), and gelified with 4 g/l of Phytagel®, and mannitol (Sigma-Aldrich) added to give final osmotic pressures of 0; -0.3; -0.6; -0.6; -0.9; -1.2 MPa. The tubes were sealed with polyethylene plastic film and transferred to the growth room under the same conditions previously described.

2.3 Growth and Biomass Assessment

After 30 days the variables were measured: survival percentage, number of shoots and roots, length of shoots (cm), length of roots (cm), fresh weight (FW) and dry weight (DW) measured according to Cha-um et al. (2006).

2.4 Proline Estimation

Free proline content was determined on two technical duplicates of each of four biological replicates, according to Bates et al. (1973). Leaf samples (200 mg) were homogenized in aqueous sulfosalicylic acid (Alphatec) (3% w/v; 12 ml). The filtered homogenate (2 ml) was reacted with equal volume each of acid ninhydrin (Sigma-Aldrich) and acetic acid (Neon) at 100 °C for 1 h and the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene (Alphatec) and mixed vigorously with a stirrer for 10-15 s. The chromophore containing toluene was aspirated from the aqueous phase and warmed to room temperature. The absorbance was recorded at 520 nm using toluene as a blank. Proline concentration (μmol/g FW) was determined from a standard curve prepared with L-proline (Sigma-Aldrich).

2.5 Antioxidant Enzyme Assays

2.5.1 Extraction

All the steps in the preparation of the enzyme extract were carried out at 4 °C. The samples (100 mg) were homogenized in 1.5 ml ice cold 50 mM sodium phosphate buffer (pH 7.0) including 0.1 mM ethylene diamine tetra acetic acid-EDTA (Vetec) and polyvinyl pyrrolidone-PVP (Sigma-Aldrich) (1% w/v) in pre chilled mortar and pestle. The homogenate was centrifuged at 14000 g for 20 min at 4 °C. The supernatant was used as a crude enzyme extract for the antioxidante enzyme assays (Peixoto et al., 1999).

2.5.2 Superoxide Dismutase (SOD) Assay

The specific SOD activity was assayed in terms of inhibition of the photochemical reduction of NBT-nitroblue tetrazolium (Sigma-Aldrich) (Beyer & Fridovich 1987). The reaction mixture (3 ml) consisted of 50 mM phosphate buffer (pH 7.8) and 0.1 mM EDTA to which a superoxide generating system containing methionine (Sigma-Aldrich) (14.3 mM), NBT (82.5 µM) and riboflavin (Sigma-Aldrich) (2.2 µM) was added. The reaction was initiated by adding crude enzyme (100 µl). Free radical induced NBT reduction was measured in the reaction medium containing all the ingredients except enzyme. The tubes were kept 30 cm below a light source (4 × 20 W fluorescent tubes) for 6 min in BOD chamber adjusted to 25±2 °C. The reaction was stopped by switching off the light. All the reactants along with 100 µl enzyme extract were incubated in dark as the dark blank. The reduction of NBT was measured by monitoring the change in absorbance at 560 nm. The readings of dark blank were used in calculation of enzyme units. Enzyme activity was expressed as U SOD. 1 U SOD enzyme was defined as the amount of enzyme that brings about 50% inhibition of NBT reduction under the assay conditions.

2.5.3 Catalase (CAT) Assay

CAT activity was measured by following the decomposition of hydrogen peroxide-H_2O_2 as described by Cakmak and Marschner (1992) with some modifications. The activity was measured in a reaction mixture (1 ml) containing 50 mM phosphate buffer (pH 7.0) and H_2O_2 (Merck) (10 mM). The reaction was initiated by adding 50 µl enzyme extract and the activity was determined by monitoring decrease in absorbance at 240 nm (ε = 36 1/mM cm) for 2 min at intervals of 15 s. The slope of the rate assay (ΔA) was used to determine the enzyme activity, which was expressed as as µmol/min g FW.

2.5.4 Ascorbate Peroxidase (APX) Assay

APX activity was determined according to Nakano and Asada (1981). The reaction mixture (a total volume of 2 ml) consisted of 50 mM (pH 7.0) sodium phosphate buffer, 0.1 mM EDTA, 0.25 mM sodium ascorbate (Sigma-Aldrich), 1 mM H_2O_2 and 100 µl enzyme extract. H_2O_2-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm (ε = 2.81/mM cm) for 5 min at intervals of 30 s. APX activity was measured in terms of µmol/min g FW.

2.6 Experimental Designs and Statistical Analysis

A completely randomized design was employed, using a factorial scheme 5 accessions × 5 osmotic potentials, with five repetitions per treatment, containing one shoot per tube per experimental unit.

Data of the variables were submitted to analysis of variance. For the osmotic potentials, the regression test was applied and for accessions, the Scott-Knott test with 5% of significance. The statistical software SAS-9.4 (SAS Institute Inc., 2013) was used for all the analyses.

3. Results

3.1 Growth Assessment

The reduction of the water potential of the medium has significantly affected the *in vitro* growth pattern of sugarcane accessions. There was effect of the interaction between accessions and water potential for the following variables: number and length of sprouts and roots; fresh mass and dry mass. For survival, there was effect of accessions and water potential.

There was a linear reduction in survival as water potential became more negative (Figure 1).

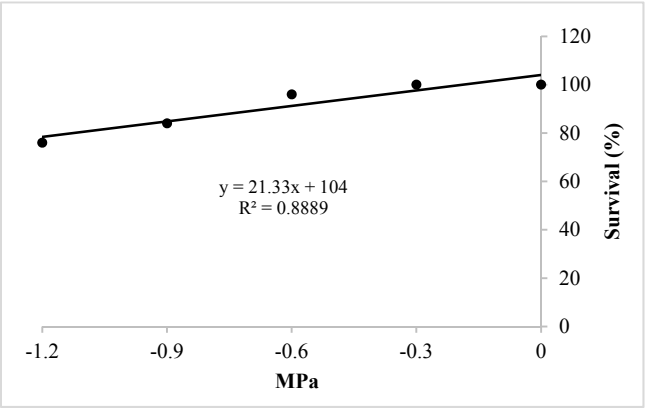

Figure 1. Effect of drought stress on the percentage of survival of sugarcane accessions at 30 days of *in vitro* cultivation

S. robustum-Q45923 accession presented lower survival rate (72%), differing from the others that reached from 88 to 100% survival rate (Table 1).

Table 1. Survival (%) of sugarcane accessions, at 30 days of *in vitro* cultivation under drought stress

Accessions	Survival (%)
S. robustum (Q45923)	72 b
S. robustum (GH49)	96 a
S. robustum (MIA35301)	100 a
S. spontaneum (NSL291970)	100 a
S. officinarum (PI88652)	88 a
CV (%)	10.75

Note. Means followed by the same letter in the column do not differ from each other by the Scott-Knott test ($p \leq 0.05$).

Increasing mannitol concentration in the culture medium promoted different shoots emission responses (Figure 2). In *S. robustum*-GH49, the lowest emission of adventitious shoots (1.09 shoots/explant) was reached at potential of -1.06 MPa, and for *S. officinarum*-PI88652, -0.86 MPa with mean of 0.44 shoots/explant. On the other hand, shoot formation was linearly negative in *S. robustum*-Q45923 and MIA35301 and *S. spontaneum*-NSL291970, with decreasing water potential (Table 2). In the absence of water stress, *S. officinarum*-PI88652 accession presented higher mean (11.0 shoots/explant), and in the two more severe treatments, there was not difference among accessions.

For shoot length, there was a linear response with significant reduction in *S. robustum*-MIA3530, and quadratic in the other accessions with potential reduction. The lowest lengths were observed at -1.01 MPa for *S. robustum*-Q45923 with 0.019 cm, -0.93 MPa for *S. robustum*-GH49 with 2.37 cm, -0.93 MPa for *S. officinarum*-PI88652 with 2.76 cm, and -1.02 MPa for *S. spontaneum*-NSL291970 with 3.50 cm, the latter having the highest mean growth in all potentials studied (Table 2).

Regarding the number of roots, there was a linear negative response for *S. robustum*-Q45923 and quadratic for the other accessions. The highest root induction occurred at -0.006 MPa, with average of 4.83 roots for *S. robustum*-MIA3530. The lowest root emission occurred at -0.93 MPa, with 1.0 root for *S. robustum*-GH49, -1.08 MPa with 0.02 root for *S. officinarum*-PI88652, and -0.96 MPa with 1.8 roots for *S. spontaneum*-NSL291970, and the latter was highlighted due to its higher root production (12.8) in absence of stress. In the most severe treatment, -1.2 MPa, only *S. spontaneum*-NSL291970 emitted root, but not differentiating from the absence of root of the other accessions. With increased stress, *S. robustum*-MIA3530 and *S. spontaneum*-NSL291970 did not present difference (Table 2).

Considering root length, there was a linear reduction with the increase of water stress in *S. robustum*-Q45923 and GH49. For this variable, *S. robustum*-MIA3530 presented higher mean value (2.58 cm) at -0.19 MPa. For *S.*

spontaneum-NSL291970 and *S. officinarum*-PI88652, the lowest lengths, 0.56 and 0.01 cm, were observed at -0.49 and -1.11 MPa respectively. In the control treatment, *S. robustum*-GH49 and MIA35301 presented the lowest lengths, and in the two more severe treatments, there was not difference among accessions (Table 2).

Table 2. Number of shoots, length of shoots, number of roots, and length of roots on sugarcane accessions, at 30 days of *in vitro* cultivation under drought stress

Water Potential (MPa)	Accessions				
	S. robustum (Q45923)	*S. robustum* (GH49)	*S. robustum* (MIA35301)	*S. spontaneum* (NSL291970)	*S. officinarum* (PI88652)
Number of shoots					
0	4.60 c	9.00 b	4.20 c	3.80 c	11.00 a
-0.3	3.80 b	5.60 a	3.20 b	1.60 c	2.40 c
-0.6	1.25 b	1.80 b	2.80 a	3.00 a	1.60 b
-0.9	1.00 a	2.00 a	1.80 a	2.20 a	2.25 a
-1.2	1.00 a	1.00 a	1.00 a	2.00 a	1.00 a
Equation y =	3.3333x + 4.3300	$6.9841x^2 + 14.9143x + 9.0571$	2.60x + 4.16	1.00x + 3.12	$12.8175x^2 + 22.0976x + 9.9871$
R^2	0.8321	0.9695	0.9877	0.2915	0.8613
CV (%)	12.29				
Length of shoots (cm)					
0	10.50 b	9.20 b	8.10 c	18.60 a	8.00 c
-0.3	5.40 b	5.40 b	6.70 b	8.80 a	4.66 b
-0.6	1.12 c	2.20 c	3.20 b	6.20 a	3.44 b
-0.9	0.50 b	3.60 a	1.10 b	5.10 a	3.12 a
-1.2	0.20 b	2.37 a	0.44 b	3.20 a	3.00 a
Equation y =	$10.8333x^2 + 21.3666x + 10.5550$	$7.7381x^2 + 14.4357x + 9.0379$	6.9733x + 8.0920	$13.7302x^2 + 27.9762x + 17.7514$	$5.8214x^2 + 10.8307x + 7.7999$
R^2	0.9933	0.9152	0.956	0.9553	0.9785
CV (%)	1154				
Number of roots					
0	6.40 d	9.80 b	5.20 d	12.80 a	8.40 c
-0.3	4.60 a	4.40 a	3.60 b	2.60 b	5.00 a
-0.6	2.50 b	1.60 b	4.60 a	4.40 a	1.00 b
-0.9	1.00 b	0.80 b	2.20 a	3.80 a	0.75 b
-1.2	0.0 a	0.0 a	0.0 a	1.20 a	0.0 a
Equation y =	5.4667x + 6.18	$9.8889x^2 + 18.4000x + 9.5600$	$-3.6508x^2 - 0.4476x + 4.8228$	$10.1587x^2 + 19.5238x + 11.1886$	$7.1825x^2 + 15.636x + 8.5329$
R^2	0.9845	0.9889	0.8908	0.7256	0.9794
CV (%)	19.53				
Length of roots (cm)					
0	5.00 a	2.70 b	2.30 b	5.30 a	4.20 a
-0.3	4.10 a	1.40 c	2.90 b	2.10 c	2.60 b
-0.6	1.75a	0.60 b	2.20 a	1.80 a	0.50 b
-0.9	1.00 a	0.30 a	0.80 a	1.20 a	0.4 a
-1.2	0.0 a	0.0 a	0.0 a	0.30 a	0.0 a
Equation y =	4.3667x + 4.9900	2.1667x + 2.3000	$-2.7778x^2 - 1.1000x + 2.4800$	$3.4127x^2 + 7.7286x + 4.9343$	$3.4921x^2 + 7.7238x + 4.2886$
R^2	0.9648	0.8989	0.9358	0.9186	0.9743
CV (%)	17.48				

Note. Means followed by the same letter in the line do not differ by Scott-Knott test ($p \leq 0.05$).

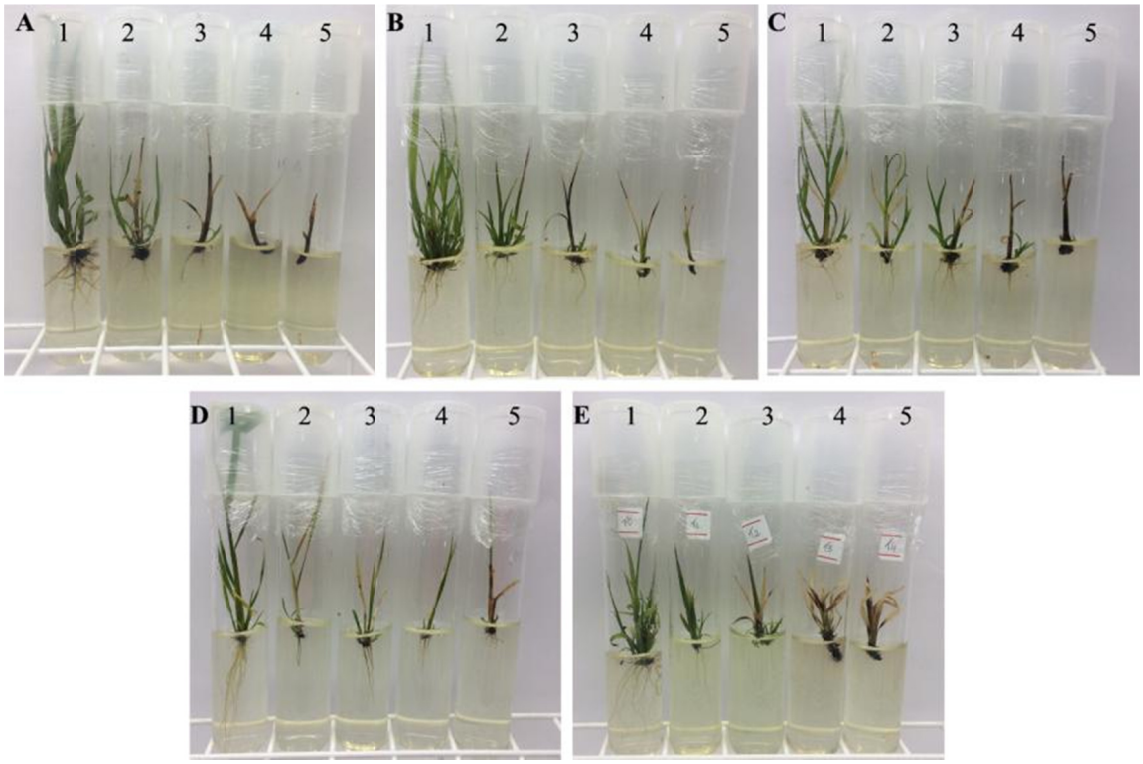

Figure 2. Sugarcane shoots at 30 days of *in vitro* cultivation in different water potentials. A: *S. robustum* (Q45923), B: *S. robustum* (GH49), C: *S. robustum* (MIA35301), D: *S. spontaneum* (NSL291970), E: *S. officinarum* (PI88652). 1: 0 MPa; 2: -0.3 MPa; 3: -0.6 MPa; 4: -0.9 MPa; 5: -1.2 MPa

3.2 Biomass

In relation to the fresh weight of shoots, for *S. robustum*-MIA35301, there was a linear reduction with the increase of drought stress (Table 3). For *S. robustum*-Q45923 and GH49, the lowest fresh weight, 0.13 g and 0.04 g, were reached at potentials -0.9 and -1.02 Mpa, respectively. In *S. spontaneum*-NSL291970, the lowest fresh weight, 0.05 g, was obtained at -0.81 MPa, whereas in *S. officinarum*-PI88652, the potential of -0.75 MPa was the one that caused the highest decrease in weight, generating 0.12 g. There was not difference among accessions at -1.2 MPa.

The dry weight production of shoots decreased in *S. robustum*-Q45923 and GH49, *S. spontaneum*-NSL291970 and *S. officinarum*-PI88652 accessions in response to water deficit, with reductions of 36%, 80%, 42% and 22%, respectively. In the absence of drought stress, there was not difference among accessions and for the most severe treatment, *S. robustum*-GH49 accession obtained lower weight.

For roots, there was a linear negative reduction in the weight of accessions with increased drought stress. In the absence of stress, *S. spontaneum*-NSL291970 presented higher dry weight (0.0040 g), and *S. robustum*-Q45923, *S. spontaneum*-NSL291970 and *S. officinarum*-PI88652 presented higher fresh weight, 0.0416 g, 0.0503 g and 0.0406 g respectively. In relation to the most severe water potentials, there was not difference among accessions.

Table 3. Biomass (fresh and dry weight) of sugarcane accessions, at 30 days of *in vitro* cultivation under drought stress

Water Potential (MPa)	Accessions				
	S. robustum (Q45923)	*S. robustum* (GH49)	*S. robustum* (MIA35301)	*S. spontaneum* (NSL291970)	*S. officinarum* (PI88652)
Fresh weight of shoots (g)					
0	0.3711 b	0.4576 a	0.3709 b	0.3677 b	0.4583 a
-0.3	0.2720 a	0.2355 a	0.2721 a	0.0922 b	0.1466 b
-0.6	0.1511 a	0.1054 a	0.1750 a	0.1386 a	0.1267 a
-0.9	0.1366 b	0.0605 b	0.2124 a	0.1043 b	0.2106 a
-1.2	0,1442 a	0.0442 a	0.1284 a	0.1149 a	0.1858 a
Equation y =	$0.2539x^2 + 0.5010x + 0.3786$	$0.3943x^2 + 0.8071x + 0.4519$	$0.1816x + 0.3407$	$0.3903x^2 + 0.6328x + 0.3325$	$0.5377x^2 + 0.8056x + 0.4186$
R^2	0.9804	0.9963	0.8411	0.7812	0.7769
CV (%)	4.74				
Dry weight of shoots (g)					
0	0.0454 a	0.0506 a	0.0342 a	0.0421 a	0.0441 a
-0.3	0.0378 a	0.0348 a	0.0358 a	0.0172 b	0.0275 b
-0.6	0.0145 a	0.0186 a	0.0288 a	0.0239 a	0.0207 a
-0.9	0.0344 a	0.0122 b	0.0355 a	0.0206 b	0.0340 a
-1.2	0.0289 a	0.0100 b	0.0233 a	0.0245 a	0.0343 a
Equation y =	$0.0376x^2 + 0.0572x + 0.0462$	$0.0295x^2 + 0.0699x + 0.0512$	ns	$0.0379x^2 + 0.0560x + 0.0388$	$0.0426x^2 + 0.0555x + 0.0424$
R^2	0.5456	0.9951	ns	0.7066	0.7369
CV (%)	0.92				
Fresh weight of roots (g)					
0	0.0416 a	0.0285 b	0.0186 b	0.0503 a	0.0406 a
-0.3	0.0283 a	0.0045 b	0.0082 b	0.0053 b	0.0013 b
-0.6	0.0050 a	0.0021 a	0.0052 a	0.0040 a	0.0011 a
-0.9	0.0007 a	0.0002 a	0.0020 a	0.0035 a	0.0000 a
-1.2	0.0000 a	0.0000 a	0.0000 a	0.0010 a	0.0000 a
Equation y =	$0.0368x + 0.0372$	$0.0204x + 0.0193$	$0.0145x + 0.0155$	$0.0334x + 0.0328$	$0.0275x + 0.0251$
R^2	0.8685	0.6387	0.8839	0.5703	0.5307
CV (%)	0.82				
Dry weight of roots (g)					
0	0.0029 b	0.0015 c	0.0014 c	0.0040 a	0.0023 c
-0.3	0.0026 a	0.0002 b	0.0005 b	0.0009 b	0.0009 b
-0.6	0.0008 a	0.0001 a	0.0003 a	0.0006 a	0.0000 a
-0.9	0.0000 a	0.0000 a	0.0001 a	0.0002 a	0.0000 a
-1.2	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
Equation y =	$0.0028x + 0.0029$	$0.0011x + 0.0010$	$0.0011x + 0.0011$	$0.0029x + 0.0028$	$0.0018x + 0.0017$
R^2	0.8941	0.5991	0.7891	0.7126	0.7355
CV (%)	0.07				

Note. Means followed by the same letter in the line do not differ by Scott-Knott test ($p \leq 0.05$). ns = not significant.

3.3 Proline

There was effect of the interaction between accessions and water potentials for the proline content (Table 4). Proline accumulation was induced in all accessions with increasing mannitol concentration, except for *S. officinarum*-PI88652, in which there was accumulation only up to -0.56 MPa in an attempt to supply the need for osmotic adjustment. After this potential, lower osmolyte synthesis occurred, demonstrating plant recovery and possible water deficit tolerance of this accession. The maximum proline content was detected in *S. robustum*-Q45923 accession (28.07 µmol/g) at -1.2 MPa, but not differing from *S. robustum*-GH49 (21.10 µmol/g) and *S. spontaneum*-NSL291970 (24.45 µmol/g) for this potential. In the control treatment, there was no difference among accessions. For the other treatments, *S. robustum*-Q45923 showed the highest averages.

Table 4. Free proline content (µmol/g FW) on sugarcane accessions, at 30 days of *in vitro* cultivation under drought stress

Water Potential (Mpa)	Accessions				
	S. robustum (Q45923)	*S. robustum* (GH49)	*S. robustum* (MIA35301)	*S. spontaneum* (NSL291970)	*S. officinarum* (PI88652)
0	1.9482 aB	2.6421 aC	3.4355 aB	2.3145 aD	1.9437 aB
-0.3	2.9253 cB	16.5837 aA	6.278 cB	10.771 bC	7.7151 cA
-0.6	5.3459 bB	12.3126 aB	5.5376 bB	9.4547 aC	6.2025 bA
-0.9	8.2974 bB	12.3255 aB	5.9051 bB	16.1684 aB	2.9909 bB
-1.2	28.0704 aA	21.1017 aA	12.3783 bA	24.4512 aA	3.4127 cB
Equation y =	$30.256x^2 + 17.102x + 3.2402$	$-10.887x + 6.4609$	$-5.8376x + 3.2044$	$-16.557x + 2.6978$	$-9.8398x^2 – 11.212x + 3.039$
R^2	0.9399	0.5714	0.6846	0.9064	0.4866
CV (%)	12.42				

Note. Means followed by the same lowercase letter in the line and upper case in the column do not differ by Scott-Knott test ($p \leq 0.05$).

3.4 Antioxidant Enzymes

In relation to the activity of antioxidant enzymes, there was effect between accessions and water potentials (Table 5). SOD activity was induced with increasing stress in *S. spontaneum*-NSL291970 and from -0.57 MPa in *S. robustum*-MIA35301, in the rest of accessions, activity was not observed. At the highest stress level, the highest mean enzymatic activity was reached by *S. robustum*-GH49 (1.3804 µmol/min g).

For APX, there was higher enzyme activity only for *S. robustum*-GH49 and MIA35301 with increased stress, demonstrating that in this last accession, H_2O_2 produced by the SOD activity was catalyzed by the action of the enzyme. In the control treatment, higher averages were observed in *S. robustum*-Q45923 (4.6273 µmol/min g) and *S. officinarum*-PI88652 (4.7063 µmol/min g). Under conditions of -1.2 MPa, *S. robustum*-GH49 and *S. officinarum*-PI88652 presented higher SOD activity, 5.2889 and 5.8542 µmol/min g, respectively, evidencing that there is a difference among species and within the same sugarcane species.

Regarding CAT, there was not significant increase in the activity of this enzyme for any of the accessions studied. This may be the result of a much higher affinity for peroxidases than for catalase for $H_2O_2^-$ *S. robustum*-Q45923 stood out presenting the highest average at the highest water stress levels.

Table 5. Activity of antioxidant enzymes on sugarcane accessions, at 30 days of *in vitro* cultivation under drought stress

Water Potential (Mpa)	Accessions				
	S. robustum (Q45923)	*S. robustum* (GH-49)	*S. robustum* (MIA35301)	*S. spontaneum* (NSL291970)	*S. officinarum* (PI88652)
SOD (U SOD)					
0	1.9050 aA	1.3725 bA	0.8983 cB	0.8464 cB	0.9612 cA
-0,3	1.2455 aC	1.4187 aA	1.2850 aA	0.8840 bB	0.9044 bA
-0,6	1.4473 aB	1.4112 aA	0.9430 bB	0.8671 bB	0.9071 bA
-0,9	1.5004 aB	1.1503 bB	1.1150 bA	1.1885 bA	0.9548 bA
-1,2	1.1393 bC	1.3804 aA	0.9473 bB	1.1105 bA	1.0239 bA
Equation y =	0.4255x + 1.7028	ns	$-0.4722x^2 - 0.5426x + 0.4671$	-0.2776x + 0.8128	ns
R^2	0.4684	ns	0.2488	0.6912	ns
CV (%)	3.69				
CAT (µmol/min g FW)					
0	0.5334 aA	0.3383 bA	0.5212 aA	0.5705 aA	0.3497 bA
-0,3	0.4737 aA	0.3433 bA	0.5089 aA	0.5628 aA	0.3769 bA
-0,6	0.5246 aA	0.2816 cA	0.2870 cB	0.5703 aA	0.3905 bA
-0,9	0.5273 aA	0.2469 cA	0.4461 aA	0.3594 bB	0.3634 bA
-1,2	0.5897 aA	0.2647 cA	0.2913 cB	0.4802 bA	0.2506 cB
Equation y =	ns	0.0812x + 0.3437	0.1742x + 0.5154	0.1280x + 0.5854	$-0.2545x^2 + 0.2348x + 0.3428$
R^2	ns	0.7788	0.5187	0.4384	0.9573
CV (%)	3.50				
APX (µmol/min g FW)					
0	4.6273 aA	1.9388 bC	1.0041 bB	2.0117 bA	4.7063 aA
-0,3	1.6093 cB	4.2901 bB	3.4457 bA	2.1163 cA	5.6414 aA
-0,6	2.9613 bB	5.1731 aA	1.3157 cB	0.9869 cA	5.7105 aA
-0,9	2.8748 cB	4.0883 bB	1.9881 cB	1.7749 cA	5.3253 aA
-1,2	2.3300 cB	5.2889 aA	3.2795 bA	1.8472 cA	5.8542 aA
Equation y =	$2.7841x^2 + 4.4505x + 4.0474$	$-3.3881x^2 - 6.2318x + 2.2463$	-1.0310x + 1.5880	ns	ns
R^2	0.3993	0.7614	0.1923	ns	ns
CV (%)	8.68				

Note. Means followed by the same lowercase letter in the line and upper case in the column do not differ by Scott-Knott test ($p \leq 0.05$). ns = not significant.

4. Discussion

4.1 Growth Assessment

The signaling pathway of any abiotic stress involves certain key steps such as signal perception, transduction, responsiveness, combined with activation of physiological, and metabolic reactions (Pérez-Clemente et al., 2013; Liu et al., 2014). In this process, plant cells first perceive stress stimulus through sensors or receptors localized mostly at the cell membrane. Then, the receiver signaling molecules activate the intracellular ones through second messengers like, inositol phosphate, cyclic nucleotides, ROS, nitric oxide, sugars and calcium ions. Subsequently, these second messengers initiate the corresponding signaling pathways to transduce the signals which modulate the expression of stress responsive genes, regulating various physiological and metabolic processes (Bhargava & Sawant, 2013; Joshi et al., 2016).

Under condition of water deficiency, plants present a series of morphophysiological changes as part of strategies to reduce the deleterious effects of low water availability, thus constituting drought tolerance mechanisms (Kramer, 1980). Inman-Bamber and Smith (2005) report genotypic variation in sugarcane regarding tolerance to water deficit, which is expressed in different organizational levels ranging from cellular responses to morphological changes (Passioura, 1997).

Water deficiency affects sugarcane yield, reducing the size of plants, and some variables such as tiller production; shoot diameter, number and height; leaf area and finally the individual weight of shoots (Rao et al., 2005; Robertson et al., 1999). According to Silva et al. (2008), the variation in plant height is indicative of tolerance or susceptibility of the genus *Saccharum* to water deficiency.

Mannitol-induced drought also caused a marked decrease in growth attributes such as shoot length and leaf area in seven sugarcane cultivars, with reduction level varying among the different genotypes (Cha-Um et al., 2012). Munawarti et al. (2014) worked with *S. spontaneum* and attributed the smaller reduction in the height of BOT-53 and BOT-54 accessions to the drought tolerance.

Variations in the soil water availability promote differences in the development of the plant root system, affecting nutrient absorption and the deep root system is characteristic of drought tolerant cultivars. Thus, plants submitted to drought condition present alterations that can prevent and tolerate water loss and in these alterations, the root system is the first region of the plant to detect and signal to the other cells and organs the effects of drought stress (Taiz & Zeiger, 2009).

In the same way, Cha-Um et al. (2012) verified that root length also decreased in all seven cultivars under osmotic stress.

4.2 Biomass

The reduction of fresh and dry weight of roots and shoots is explained by the fact that there is a decrease in their emission and length as the medium potential is reduced. Even using morphological strategies to avoid the negative effects of water deficiency, sugarcane plants present significant decreases in phytomass production, reflecting physiological changes caused by low water availability, such as reduction of photosynthetic activity (Singels et al., 2005). This sensitivity is due to the reduction of cell division due to the impact of lack of water on the cell expansion rate as a result of loss of turgor (Taiz & Zeiger, 2009).

A 35% reduction in phytomass in young sugarcane plants was verified due to water restriction in a period of high evaporative demand (Inman-Bamber, 2004). Cha-Um and Kirdmanee (2008) working with *S. officinarum* observed a decrease in fresh and dry weight of seedlings submitted to water deficit under extreme dry conditions using 300 and 400 mM of mannitol. Medeiros et al. (2013) working with two sugarcane varieties, RB867515 and RB962962, observed that only variety RB867515 decreased the dry weight of shoots and roots in response to drought.

4.3 Proline

Proline is one of the most important organic osmolytes that accumulates in various plant species in response to environmental stresses such as drought, salinity, UV radiation, heavy metals and extreme temperatures (Verbruggen & Hermans, 2008).

The increased proline concentration in stressed plants can be an adaptation to overcome the stress condition, constantly providing energy for survival and growth, by acting on the osmotic adjustment, aiding in the control of the entry and exit of water in cells, cytoplasm and vacuoles (Jaleel et al., 2007). In addition, a number of other functions are attributed to this amino acid such as: antioxidant in the elimination of reactive oxygen species (ROS) (Molinari et al., 2007; Tuteja, 2007), plasma membrane protection and macromolecule integrity (Vanrensburg et al., 1993; Silveira et al., 2003) and as a source of carbon and nitrogen (Gupta & Huang, 2014).

In sugarcane, a positive connection between proline increase and drought stress tolerance was observed by Abbas et al. (2014) when evaluating thirteen varieties under drought stress induced by polyethylene glycol in greenhouse, distinguishing the most tolerant genotypes. Similar results were also found by Cha-Um and Kirdmanee (2008) in *S. officinarum* in the presence of mannitol, and by Patade et al. (2012) in callus cultures of *S. officinarum*-Co86032 in the presence of 20% polyethylene glycol. However, some authors support the hypothesis that proline accumulation is a symptom of stress rather than an adaptive property (Rampino et al., 2006; Sanchez-Rodriguez et al., 2010). Cia et al. (2012) reported a broad controversy on the protective properties of plant-soluble proline increase under drought stress, in which tolerant varieties showed less proline accumulation compared to sensitive plants after 10 days of water stress in greenhouse. Errabi et al. (2007) also found similar results in callus cultures of *Saccharum* sp.

4.4 Antioxidant Enzyme

Reactive oxygen species (ROS) are generated in plant cells by normal cell metabolism or due to unfavorable environmental conditions such as drought, salinity, heavy metals, herbicides, nutritional deficiency or radiation. ROS are highly cytotoxic and can react with various metabolites causing oxidative damage to enzymes, proteins,

lipids and nucleic acids. ROS production is controlled by various enzymes of antioxidant defense systems (Karuppanapandian et al., 2011). As a metalloenzyme, SOD is the first enzyme in detoxification processes, transforming O_2^- into H_2O_2 (Sen, 2012). The produced H_2O_2 can be converted into H_2O and O_2 by CAT or, only into H_2O by the action of APX (Noctor & Foyer, 1998).

In this study, it was demonstrated that the enzymatic activity was activated only in some accessions with increased water stress, evidencing that possibly induced stress was not enough to produce ROS, or that these were controlled by the action of the non-enzymatic antioxidant defense system, by the action of proline for example.

If the osmotic stress is very intense or persists for a long time, a decrease in the antioxidant enzymatic activity can also be observed, as already observed in sugarcane callus cultures (Patade et al., 2012), in *Sesuvium portulacastrum* (Lokhande et al., 2010a, 2010b), and in *Kentucky bluegrass* plants (Wang & Huang, 2004).

The activity of SOD, APX and CAT under drought stress induced with tissue culture techniques has been reported in a wide range of plant species. Balen et al. (2013) compared enzymatic activities of cactus tissues exposed to NaCl and mannitol and reported that the response of APX activities was greater than the CAT activity. Chai et al. (2005) worked with banana genotypes and observed that higher APX and SOD activities were associated with greater protection against oxidative damage induced by water stress. Shehab et al. (2010) analyzed rice plants under water stress and concluded that the two-fold increase in enzyme activity is a strong indication that drought treatment actually led to oxidative stress. Torabi and Niknam (2011) showed different trends in the activity of antioxidant enzymes in two *Salicornia* species under salt and drought stresses, evidencing increased activity in one of the species analyzed.

Mechanisms of protection to water stress were observed in sugarcane accessions studied, indicating that plant-level efforts were needed to cope with stress. Osmotic adjustment played an important role in water retention as well as the growth reduction and antioxidant activity, serving as the basis for the selection of more tolerant accessions for future breeding programs.

5. Conclusions

There are differences among species, and also, within the same sugarcane species when submitted to *in vitro* water stress.

S. officinarum presents better results, demonstrating to be more tolerant to water stress.

Proline can be used as a biochemical indicator of response to water stress in sugarcane accessions.

Proline accumulation is intensified in *S. robustum* and *S. spontaneum* accessions.

CAT activity remains unchange with increased water stress in the accessions evaluated.

References

Abbas, S. R., Ahmad, S. D., Sabir, S. M., & Shah, A. H. (2014). Detection of drought tolerant sugarcane genotypes (*Saccharum officinarum*) using lipid peroxidation, antioxidant activity, glycine-betaine and proline contents. *Journal of Soil Science and Plant Nutrition, 14*(1), 233-243. https://doi.org/10.4067/S0718-95162014005000019

Albiski, F., Najla, S., Sanoubar, R., Alkabani, N., & Murshed, R. (2012). *In vitro* screening of potato lines for drought tolerance. *Physiology and Molecular Biology of Plants, 18*(4), 215-321. https://doi.org/10.1007/s12298-012-0127-5

Ashraf, M., & Foolad, M. R. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany, 59*, 206-216. https://doi.org/10.1016/j.envexpbot.2005.12.006

Azevedo Neto, A. D., Prisco, J. T., Enéas-Filho, J., De Abreu, C. E. B., & Gomes-Filho, E. (2006). Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environmental and Experimental Botany, 56*, 87-94. https://doi.org/10.1016/j.envexpbot.2005.01.008

Balen, B., Tkalec, M., Rogie, T., Simac, M., Stefanie, P. P., Roncevie, S., … Krsnik-Rasol, M. (2013). Effects of iso-osmotic NaCl and mannitol on growth, proline content, and antioxidant defense in *Mammillaria gracilis* Pfeiff. *in vitro*-grown cultures. *In vitro Cellular and Developmental Biology-Plant, 49*, 421-432. https://doi.org/10.1007/s11627-013-9523-y

Basu, S., Ramegowda, V., Kumar, A., & Pereira, A. (2016). Plant adaptation to drought stress. *F1000Research, 5*(F1000 Faculty Rev.), 1554. https://doi.org/10.12688/f1000research.7678.1

Bates, L. S., Waldren, R. P., & Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant Soil, 39*, 205-207. https://doi.org/10.1007/BF00018060

Beyer, W. F., & Fridovich, I. (1987). Assaying for superoxide dismutase activity: Some large consequences of minor changes in condition. *Analytical Biochemistry, 161*, 559-566. https://doi.org/10.1016/0003-2697(87)90489-1

Bhargava, S., & Sawant, K. (2013). Drought stress adaptation: Metabolic adjustment and regulation of gene expression. *Plant Breeding, 132*, 21-32. https://doi.org/10.1111/pbr.12004

Cakmak, I., & Marschner, H. (1992). Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascrobate peroxidase, and glutathione reductase in bean leaves. *Plant Physiology, 98*, 1222-1227. https://doi.org/10.1104/pp.98.4.1222

Chai, T. T., Fadzillah, N. M., Kusnan, M., & Mahmood, M. (2005). Water stress-induced oxidative damage and antioxidant responses in micropropagated banana plantlets. *Biologia Plantarum, 49*(1), 153-156. https://doi.org/10.1007/s00000-005-3156-9

Cha-Um, S., & Kirdmanee, C. (2008). Effect of osmotic stress on proline accumulation, photosynthetic abilities and growth of sugarcane plantlets (*Saccharum officinarum* L.). *Pakistan Journal of Botany, 40*(6), 2541-2552.

Cha-Um, S., Supaibulwatana, K., & Kirdmanee, C. (2006). Water relation, photosynthetic ability, and growth of Thai Jasmine rice (*Oryza sativa* L. ssp. *indica* cv. KDML 105) to salt stress by application of exogenous glycinebetaine and choline. *Journal of Agronomy and Crop Science, 192*, 25-36. https://doi.org/10.1111/j.1439-037X.2006.00186.x

Cha-Um, S., Wangmoon, S., Mongkolsiriwatana, C., Ashraf, M., & Kirdmanee, C. (2012). Evaluating sugarcane (*Saccharum* sp.) cultivars for water deficit tolerance using some key physiological markers. *Plant Biotechnology, 29*, 431-439. https://doi.org/10.5511/plantbiotechnology.12.0726a

Cia, M. C., Guimarães, A. C. R., Medici, L. O., Chabregas, S. M., & Azevedo, R. A. (2012). Antioxidant responses to water déficit by drought-tolerant and sensitive sugarcane varieties. *Annals of Applied Biology, 161*, 313-324. https://doi.org/10.1111/j.1744-7348.2012.00575.x

Errabii, T., Gandonou, C. B., Essalmani, H., Abrini, J., Idaomar, M., & Senhaji, N. S. (2007). Effects of NaCl and mannitol induced stress on sugarcane (*Saccharum* sp.) callus cultures. *Acta Physiologiae Plantarum, 29*, 95-102. https://doi.org/10.1007/s11738-006-0006-1

Filippou, P., Bouchagier, P., Skotti, E., & Fotopoulos, V. (2014). Proline and reactive oxygen/nitrogen species metabolism is involved in the tolerant response of the invasive plant species *Ailanthus altissima* to drought and salinity. *Environmental and Experimental Botany, 97*, 1-10, 2014. https://doi.org/10.1016/j.envexpbot.2013.09.010

Gupta, B., & Huang, B. (2014). Mechanism of salinity tolerance in plants: Physiological, biochemical, and molecular characterization. *International Journal of Genomics, 1*, 1-18. https://doi.org/10.1155/2014/701596

Inman-Bamber, N. G. (2004). Sugarcane water stress criteria for irrigation and drying off. *Field Crops Research, 89*, 107-122. https://doi.org/10.1016/j.fcr.2004.01.018

Inman-Bamber, N. G., & Smith, D. M. (2005). Water relations in sugarcane and response to water deficits. *Field Crops Research, 92*, 185-202. https://doi.org/10.1016/j.fcr.2005.01.023

Jaleel, C. A., Manivannan P., Sankar, B., Kishorekumar, A., & Panneersevam, R. (2007). Calcium chloride effectS on salinity-induced oxidative stress, proline metabolism and indole alkaloid accumulation in *Catharanthus roseus*. *Comptes Rendes Biologies, 330*(9), 674-683. https://doi.org/10.1016/j.crvi.2007.07.002

Joshi, R., Wani, S. H., Singh, B., Bohra, A., Dar, Z. A., Lone, A. A., ... Pareek-Singla, S. L. (2016). Transcription factors and plants response to drought stress: Current understanding and future directions. *Frontiers in Plant Science, 7*, 1029. https://doi.org/10.3389/fpls.2016.01029

Kacem, N. S., Delporte, F., Muhovski, Y., Djekoun, A., & Watillon, B. (2017). *In vitro* screening of durum wheat against water-stress mediated trought polyethylene glycol. *Journal of Genetic Engineering and Biotechnology, 15*, 239-247. https://doi.org/10.1016/j.jgeb.2017.04.004

Karuppanapandian, T., Moon, J. H., Kim, C., Manoharan, K., & Kim, W. (2011). Reactive oxygen species in plants: Their generation, signal transduction, and scavenging mechanisms. *Australian Journal Crop Science, 5*(6), 709-725.

Kramer, P. J. (1980). Drought stress, and the origins of adaptations. In N. C. Turner, & P. J. Kramer (Eds.), *Adaptation of plants to water and high temperature stress* (pp. 7-22). New York, Wiley.

Liu, J. H., Peng, T., & Dai, W. (2014). Critical cis-acting elements and interacting transcription factors: Key players associated with abiotic stress responses in plants. *Plant Molecular Biology Reporter, 32*, 303-317. https://doi.org/10.1007/s11105-013-0667-z

Lokhande, V. H., Nikam, T. D., & Suprasanna, P. (2010a). Differential osmotic adjustment to iso-osmotic NaCl and PEG stress in the *in vitro* cultures of *Sesuvium portulacastrum* (L.) L. *Journal of Crop Science Biotechnology, 13*, 251-256. https://doi.org/10.1007/s12892-010-0008-9

Lokhande, V. H., Nikam, T. D., & Suprasanna, P. (2010b). Biochemical, physiological and growth changes in response to salinity in callus cultures of *Sesuvium portulacastrum* L. *Plant Cell Tissue and Organ Culture, 102*, 17-25. https://doi.org/10.1007/s11240-010-9699-3

Mccree, K. J., Kallsen, C. E., & Richardson, S. G. (1984). Carbon balance of sorghum plants during osmotic adjustment to water stress. *Plant Physiology, 76*, 898-902. https://doi.org/10.1104/pp.76.4.898

Medeiros, D. B., Silva, E. C., Nogueira, R. J. M. C., Teixeira, M. M., & Buckeridge, M. S. (2013). Physiological limitations in two sugarcane varieties under water suppression and after recovering. *Theoretical and Experimental Plant Physiology, 25*(3), 213-222. https://doi.org/10.1590/S2197-00252013000300006.

Molinari, H. B. C., Marur, C. J., Daros, E., Campos, M. K. F., Carvalho, J. F. R. P., Bespalhok-Filho, J. C., … Vieira, L. G. E. (2007). Evaluation of the stress-inducible production of proline in transgenic sugarcane (*Saccharum* spp.) osmotic adjustment, chlorophyll fluorescence and oxidative stress. *Physiologia Plantarum, 130*, 218-229. https://doi.org/10.1111/j.1399-3054.2007.00909.x

Munawarti, A., Taryono, Semiarti, E., & Sismindari, S. (2014). Morphological and Biochemical Responses of Glagah (*Saccharum spontaneum* L.) Accessions to Drought Stress. *Journal of Tropical Life Science, 4*(1), 61-66. https://doi.org/10.11594/jtls.04.01.10

Murashige, T., & Skoog, F. A. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum, 15*, 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nakano, Y., & Asada, K. (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology, 22*, 867-880. https://doi.org/10.1093/oxfordjournals.pcp.a076232

Noctor, G., & Foyer, C. H. (1998). Ascorbate and glutathione: Keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology, 49*, 249-79. https://doi.org/10.1146/annurev.arplant.49.1.249

Oliveira, E. C. A., Oliveira, R. I., Andrade, B. M. T., Freire, F. J., Júnior, M. A. L., & Machado, P. R. (2010). Crescimento e acúmulo de matéria seca em variedades de cana-de-açúcar cultivadas sob irrigação plena. *Revista Brasileira de Engenharia Agrícola e Ambiental, 14*(9), 951-960. https://doi.org/10.1590/S1415-43662010000900007

Passioura, J. B. (1997). Drought and drought tolerance. In E. Belhassen (Ed.), *Drought tolerance in higher plants. Genetical, physiological and molecular biological analysis* (pp. 1-5). Dordrecht, Kluwer.

Patade, V. Y., Bhargava, S., & Suprasanna, P. (2012). Effects of NaCl and iso-osmotic PEG stress on growth, osmolytes accumulation and antioxidant defense in cultured sugarcane cells. *Plant Cell Tissue and Organ Culture, 108*, 279-286. https://doi.org/10.1007/s11240-011-0041-5

Patade, V. Y., Bhargava, S., & Suprasanna, P. (2014). Better osmotic adjustment mediates salt and PEG stress tolerance in primed plants of contrasting cultivars of sugarcane. *Sugar Tech., 17*(4), 348-355. https://doi.org/10.1007/s12355-014-0350-1

Patade, V. Y., Suprasanna, P., & Bapat, V. A. (2008). Effects of salt stress in relation to osmotic adjustment on sugarcane (*Sacharum officinarum* L.) callus cultures. *Plant Growth Regulation, 55*, 169-173. https://doi.org/10.1007/s10725-008-9270-y

Peixoto, P. H. P., Cambraia, J., Sant'Anna, R., Musquim, R., & Moreira, M. A. (1999). Aluminum effect on lipid peroxidation and on the activities of enzymes of oxydative metabolism in sorghum. *Revista Brasileira de Fisiologia Vegetal, 11*(3), 137-143.

Pérez-Clemente, R. M., Vives, V., Zandalinas, S. I., López-Climent, M. F., Muñoz, V., & Gómez-Cadenas, A. (2013). Biotechnological approaches to study plant responses to stress. *Biomed Research International, 2013*, 654120. https://doi.org/10.1155/2013/654120

Pimentel, C. (2004). *A relação da planta com a água* (p. 191). Seropédica: EDUR.

Puente-Garza, C. A., Meza-Miranda, C., Ochoa-Martínez, D., & García-Lara, S. (2017). Effect of *in vitro* drought stress on phenolic acids, flavonols, saponins, and antioxidant activity in Agave salmiana. *Plant Physiology Biochemistry, 115*, 400-407. https://doi.org/10.1016/j.plaphy.2017.04.012

Ramesh, P. (2000). Effect of different levels of drought during the formative phase on growth parameters and its relationship with dry matter accumulation in sugarcane. *Journal of Agronony & Crop Science, 185*, 83-89. https://doi.org/10.1046/j.1439-037x.2000.00404.x

Rampino, P., Pataleo, S., Gerardi, C., Mita, G., & Perrotta, C. (2006). Drought stress response in wheat: Physiological and molecular analysis of resistant and sensitive genotypes. *Plant, Cell & Environment, 29*, 2143-2152. https://doi.org/10.1111/j.1365-3040.2006.01588.x

Rao, C. M., Kumar, M. V., & Reddy, L. K. (2005). Management of sugarcane clones under limited moisture situations (drought) at formative stage. *Indian Sugar*, 949-953.

Robertson, M. J., Inmam-Bamber, N. G., Muchow, R. C., & Wood, A. W. (1999). Physiology and productivity of sugarcane with early and midseason water deficit. *Field Crops Research, 64*, 211-227. https://doi.org/10.1016/S0378-4290(99)00042-8

Said, E. M., Mahmoud, R. A., Al-Akshar, R., & Safwat, G. (2015). Drought stress tolerance and enhancement of banana plantlets *in vitro*. *Austin Journal of Biotechnology & Bioengineering, 2*(2).

Sanchez-Rodriguez, R., Rubio-Wilhelmi, M. M., Cervilla, L. M., Blasco, B., Rios, J. J., Rosales, M. A., … Ruiz, J. M. (2010). Genotypic differences in some physiological parameters symptomatic for oxidative stress under moderate drought in tomato plants. *Plant Science, 178*, 30-40. https://doi.org/10.1016/j.plantsci.2009.10.001

SAS Institute Inc. (2013). *Statistical Analysis System*. SAS 9.4 Help and Documentation, Cary, NC: SAS Institute Inc. Retrieved from http://www.sas.com/en_us/software/sas9.html

Sen, A. (2012). Oxidative stress studies in plant tissue culture. In M. A. El-Missiry (Ed.), *Biochemistry, Genetics and Molecular Biology: Antioxidant Enzyme* (Chapter 3). InTech.

Shehab, G. G., Ahmed, O. K., & El-Beltagi, H. S. (2010). Effects of various chemical agents for alleviation of drought stress in rice plants (*Oryza sativa* L.). *Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 38*(1), 139-148. https://doi.org/10.15835/nbha3813627

Silva, M. A., Silva, J. A. G., Enciso, J., Sharma, V., & Jifon, J. (2008). Yield components as indicators of drought tolerance of sugarcane. *Scientia Agricola, 65*(6), 620-627. https://doi.org/10.1590/S0103-90162008000600008

Silveira, J. A. G., Viégas, R. A., Rocha, I. M. A., Moreira, A. C. O. M., Moreira, R. A., & Oliveira, J. T. A. (2003). Proline accumulation and glutamine synthetase activity are increased by salt-induced proteolysis in cashew leaves. *Journal of Plant Physiology, 160*, 115-123. https://doi.org/10.1078/0176-1617-00890

Singels, A., Donaldson, R. A., & Smit, M. A. (2005). Improving biomass production and partitioning in sugarcane: theory and practice. *Field Crops Research, 92*, 291-303. https://doi.org/10.1016/j.fcr.2005.01.022

Taiz, L., & Zeiger, E. (2009). *Fisiologia vegetal* (4th ed.). Porto Alegre: Artmed.

Torabi, S., & Niknam, V. (2011). Effects of iso-osmotic concentrations of NaCl and mannitol on some metabolic activity in calluses of two Salicornia species. *In Vitro Cellular & Developmental Biology-Plant, 47*, 734-742. https://doi.org/10.1007/s11627-011-9371-6

Tuteja, N. (2007). Mechanisms of high salinity tolerance in plants. *Methods in Enzymology, 428*, 419-438. https://doi.org/10.1016/S0076-6879(07)28024-3

Vanrensburg, L., Kruger, G. H. J., & Kruger, R. H. (1993). Proline accumulation the drought tolerance selection: its relantionship to membrane integrity and chloroplast ultra structure in Nicotiana tabacum L. *Journal of Plant Physiology, 141*,188-94. https://doi.org/10.1016/S0176-1617(11)80758-3

Verbruggen, N., & Hermans, C. (2008). Proline accumulation in plants: A review. *Amino Acids, 35*, 753-759. https://doi.org/10.1007/s00726-008-0061-6

Verma, D., Ansari, M. W., Agrawal, G. K., Rakwal, R., Shukla, A., & Tuteja, N. (2013). *In vitro* selection and field responses of somaclonal variant plants of rice cv PR113 for drought tolerance. *Plant Signaling Behavior, 8*(4). https://doi.org/ 10.4161/psb.23519

Verslues, P. E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J., & Zhu, J. K. (2006). Methods and concepts in quantifying resistance to drought,salt and freezing, abiotic stresses that affect plant water status. *The Plant Journal, 45*, 523-539. https://doi.org/10.1111/j.1365-313X.2005.02593.x

Wang, Z., & Huang, B. (2004). Physiological recovery of Kentucky bluegrass from simultaneous drought and heat stress. *Crop Science, 44*, 1729-1736. https://doi.org/10.2135/cropsci2004.1729

Wani, S. H., Sofi, P. A., Gosal, S. S., & Singh, N. B. (2010). *In vitro* screening of rice (*Oryza sativa* L) callus for drought tolerance. *Communications in Biometry and Crop Science, 5*(2), 108-115.

Yordanov, I., Velikova, V., & Tsonev, T. (2003). Plant responses to drought and stress tolerance. *Bulgarian Journal of Plant Physiology*, 187-206.