http://journal.unoeste.br/index.php/ca/index
DOI: 10.5747/ca.2021.v17.n4.a450
ISSN on-line 1809-8215

Colloquium Agrariae

Submetido: 30/04/2021 Revisado: 25/05/2021 Aceito: 15/06/2021

# *Urochloa brizantha* cv. Marandu presents a better response to *in vitro* salt stress than other commercial cultivars

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**Abstract**
*Urochloa brizantha* is the main forage grass to raise cattle in Brazil, but salt stress can reduce yield. Physiological and molecular mechanisms of adaptation to salt stress remain poorly understood in this species. The objective of this study was to evaluate the responses of three cultivars of *U. brizantha* to *in vitro* salt stress. Seeds of three cultivars (Piatã, Marandu, and Xaraés) germinated in filter paper and then transferred to growth on culture media *in vitro* containing 0, 50, 100, and 200 mg $L^{-1}$ of sodium chloride (NaCl). Biometric parameters and proline content were determined after 28 days. The data were subjected to analysis of variance and the separation of means was performed by the LSD test ($p<0.05$). Semi-quantitative expression of the $\Delta^1$-pyrroline-5-carboxylate synthase (*P5CS1*) gene was performed. In all cultivars, increase of NaCl concentration in the media affected roots and shoots growth. Xaraes cultivar presented the greater biomass reduction while Marandu cultivar was the least affected. Salt stress increased by approximated 0.6 folds transcription of the *P5CS1* gene in all cultivars. However, Marandu cultivar presented a higher proline content and least biomass reduction suggesting a better response to *in vitro* to salt stress.
**Key words:** abiotic stress; brachiaria grass; salinity.

## *Urochloa brizantha* cv. Marandu apresenta uma melhor resposta ao estresse salino *in vitro* comparada a outras cultivares comerciais

**Resumo**
*Urochloa brizantha* é a principal gramínea forrageira para a pecuária no Brasil, mas o estresse salino pode reduzir a produtividade. Os mecanismos fisiológicos e moleculares de adaptação ao estresse salino permanecem pouco conhecidos nesta espécie. O objetivo desse estudo foi avaliar as respostas de três cultivares de *U. brizantha* ao estresse salino *in vitro*. Sementes de três cultivares (Piatã, Marandu e Xaraés) germinaram em papel filtro e foram transferidas para cultivo em meio *in vitro* contendo 0, 50, 100 e 200 mg L-1 de cloreto de sódio (NaCl). Os parâmetros biométricos e o conteúdo de prolina foram determinados após 28 dias. Os dados foram submetidos à análise de variância e separação de médias realizada pelo teste LSD ($p <0,05$). Foi realizada a expressão semiquantitativa do gene da $\Delta^1$-pirrolina-5-carboxilato sintase (*P5CS1*). Em todas as cultivares, o aumento da concentração de NaCl no meio afetou o crescimento das raízes e da parte aérea. A cultivar Xaraes apresentou a maior redução na biomassa enquanto Marandu foi a menos afetada. O estresse salino foi aumentado pela transcrição de aproximadamente 0,7 vezes do gene *P5CS1* em todas as cultivares. No entanto, a cultivar Marandu apresentou maior teor de prolina e menor redução de biomassa, sugerindo melhor resposta ao estresse salino *in vitro*.
**Palavras-chave:** estresse abiótico; capim braquiária; salinidade.

## Introduction

*The Paniceae* tribe is originally from Africa, counts on 100 species, among them the important forage grasses from *Urochloa* P. genus (sin. Brachiaria) Beauv. spp. (SORENG *et al.,* 2017). The *Urochloa* sp composes an agamic

complex with different levels of ploidy (VALLE *et al.,* 2009). *Urochloa brizantha* produce apomictic seeds that are clones and few cultivars are commercially available (VALLE *et al.,* 2010). In Brazil, it is estimated that about 100 M ha are cropped with tropical forage grasses (PEREIRA *et al.,* 2018) and from the total, 85 % consist of *Urochloa* species (JANK *et al.,* 2014). One of the main goals for breeding of tropical forages in Brazil is to develop or to select cultivars tolerant to the climate changes (PEREIRA *et al.,* 2018).

Salt stress is one of the most threatening abiotic stresses that limits plant growth and development. Several efforts have been made to understand the responses to salt stress and exploring genotype tolerance-associated will be particularly essential in the future (ZHAO *et al.,* 2020). During salt stress, the reduction in water availability causes an increase in the respiration rate and poor distribution of minerals causing instability of the membranes and failure to maintain the cell turgor pressure (BABU *et al.,* 2012). Tolerance to salinity consists of numerous processes at physiological, cellular, biochemical, and molecular levels, allowing plants survival (ACOSTA-MOTOS *et al.,* 2017). Such changes include increases in the root/shoot ratio and chlorophyll content, as well as changes in leaf anatomy, preventing toxicity by the accumulation of ions in the leaves, preventing water loss, and protect the photosynthesis process (ARIF *et al.,* 2020). At the molecular level, the expression of several genes related in response to salt stress. One of these genes is $\Delta^1$-pyrroline-5-carboxylate synthase (*P5CS1*) whose transcription in plants is induced by osmotic stresses including dehydration, salinity, low and high temperatures (SIDDIQUE *et al.,* 2018; TROVATO *et al.,* 2019). The proline biosynthesis is mainly via the glutamate pathway, which includes two enzymes and non-enzymatic reactions that occur in the cytoplasm or chloroplast and are catalyzed by $\Delta^1$-pyrroline-5-carboxylate synthase (*P5CS*) and $\Delta^1$-pyrroline-5-carboxylate reductase (*P5CR*), respectively (AMINI *et al.,* 2015). The increase in the transcription of the *P5CS1* gene can result in a greater accumulation of proline and consequently, plants are more resistant to salt stress (BENITEZ *et al.,* 2016). Therefore, the genes involved in the accumulation of proline are the key to progress in understanding this process and the impact on stress tolerance.

The imposition of salt stress *in vitro* is an effective strategy for the selection and characterization of plant responses to salinity in several species including *Oryza sativa* (TEH *et al.*, 2015), *Camelina sativa* (KHALID *et al.,* 2015), *Carthamus tinctorius* (HAMEDI *et al.,* 2016), *Lathyrus sativus* (PIWOWARCZYK *et al.,* 2016), *Amsonia orientalis* (ACEMI *et al.,* 2017). Therefore, the *in vitro* cultivation of *Urochloa* sp cultivars under salt stress can be an alternative for the initial evaluation concerning salinity tolerance. The objective of this study was to evaluate the biometric parameters and the semi quantitative expression of *P5CS* gene and the proline content of three *U. brizantha* cultivars subjected to different levels of salt stress under *in vitro* culture.

## Material and Methods

### Plant material

Before starting the experiment with salinity, the percentage of germination of the seeds of the commercial cultivars of *U. brizantha*, (Marandu, Xaraes, and Piatã) was tested. This step was carried out to ensure that all seeds tested in the saline media were able to germinate normally. The seeds were scarified by immersion in concentrated sulfuric acid ($H_2SO_4$) for 15 minutes, shaking with a glass stick, washed in running water to remove excess acid, and then the seeds were dried on paper towels at room temperature. After drying, the scarified seeds were manually peeled, and immersed in 70% ethanol for 15 s. After the seeds were transferred for a 5% sodium hypochlorite (NaOCl) solution containing three drops of Tween 80, for 15 minutes, and then rinsed 5 times in sterile distilled water. All the disinfection process was conducted in a superficial laminar flow chamber.

### Growth conditions and stress treatment

One hundred seeds of each cultivar were pre-germinated in Petri dishes containing filter paper and water in a proportion of 2 times the weight of the paper. The plates were kept in the dark for seven days. After that period, the germinated seeds were transferred to the basic culture medium composed of macro, microelements, and vitamins from the MS culture medium (MURASHIGE; SKOOG, 1962) with 30 g $L^{-1}$ of sucrose agar. To impose salt stress, sodium chloride (NaCl) was added in concentrations of 0, 50, 100 and 200 mg $L^{-1}$ to the basic MS medium. The pH was adjusted to 5.8 and 8 g $L^{-1}$ of agar was added to solidify the media. The culture media were distributed in test tubes, 10 ml in each.

Seeds pre-germinated on filter paper for 7 days were distributed in the test tubes containing the different concentrations of NaCl. The experiment was carried out with six replicates for each cultivar and treatment. All plant materials were grown in a culture room at 25 ± 1 °C under a photoperiod of 16 / 8h of light and luminous intensity of 30 µmol $m^{-2}$ $s^{-1}$. After 28 days in the culture media with salt treatments, the plants were removed from the test tubes. The shoots were separated from the roots, and were weighed on an electronic scale to determine the fresh mass. The length of the aerial part and roots were measured with the aid of a graduated ruler.

Semi Quantitative PCR

Total RNA was extracted from 50 mg of leaf tissue from treatments using the Pure Link Plant™ RNA Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The samples were treated with the Turbo DNAse enzyme (Ambion) to eliminate contamination with genomic DNA, following the manufacturer's protocol. The concentration of the total extracted RNA was measured by spectrophotometry at 260 nm, its quality estimated at the 260/280 nm ratio, and the integrity by 1.2% (w/v) agarose gel electrophoresis.

The synthesis of cDNA was performed following the protocol of the Ready Script Kit cDNA Synthesis Mix™ (Sigma Aldrich) from the total RNA extracted from the leaves. This cDNA was subjected to a PCR reaction with the specific primers of the normalizing gene *eIF4A*, to verify whether they amplified the expected fragment. This gene was chosen among a panel of seven reference genes previously tested in our laboratory in *U. brizantha* submitted to salt stress.

For the PCR reaction, the protocol of the SuperMix PCR Kit™ (Invitrogen, Carlsbad, CA, USA) was followed and the samples were subjected to electrophoresis in 1.5% (w/v) agarose gel. The design of the primer to amplify a fragment of the *P5CS1* gene was based on orthology in species of the *Panicoideae* family (same as that of *U. brizantha*) since this species does not have its genome sequenced and available in the databases. Firstly, we use the keyword *P5CS1* in the database: The Arabidopsis Information Resource (https://www.arabidopsis.org/) and found the sequence corresponding to *P5CS1* (AT2G39800). This sequence was used as a query to search for sequences from *Panicoideae* species, in the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html) using the blast tool. We found six sequences *Panicum halli* (C02762.1), *Panicum virgatum* (J06546.1), *Setaria italica* (3G220500.1), *Setaria viridis* (3G225500.1), *Sorghum bicolor* (009G160100) and *Zea mays* (GRMZM2G375504). These sequences were aligned and a pair of primer to amplify a fragment of (91 bp) was designed: 5'-CCAGTGGGTGTTGAAGGTCT-3' and 5'GGGTGTAGGCAACATCCTTG-3' at conserved region. The gene *eIF4A* (eukaryotic initiation factor) was used to normalize the data (TAKAMORI *et al.,* 2017). The primers were used to evaluate the relative expression of the *P5CS1* gene in the three *U. brizantha* cultivars grown *in vitro* in different concentrations of NaCl.

RT-PCR reactions (reverse transcriptase polymerase chain reaction) were performed in a Multigene Biosystems™ thermal cycler. The 35 times cycle was chosen for both *P5CS1* genes and the normalizer *eIF4A* (eukaryotic initiation factor) with the following conditions: 95 °C for 2 min and 35 times 95 °C for 30 s, 60 °C for 30, 1 min at 72 °C and 2 min at 72 °C of final extension. The amplicons of both genes were submitted to electrophoresis in 1.5% agarose gel. The gels were ethidium bromide-stained photographed under UV light. The quantification of amplicons was calculated by densitometry using the ImageJ software. The relative expression was calculated by quantifying the *P5CS1* gene in each treatment divided by the normalizing (*eIF4A* gene) quantification. The data were calibrated by the zero treatment (without the addition of NaCl) in each cultivar.

Proline content

The determination of proline content was carried out as previously described (BATES *et al.,* 1973) with modifications. In brief, 25 mg of plant tissue (shoots) grown *in vitro* were macerated in liquid nitrogen, 2.5 ml of sulfosalicylic acid (3%) added, vortexed followed by 15 min. The samples were centrifuged at 5000 g for 3 min. 1 ml of the supernatant was removed and 1 ml of acid ninhydrin and 1 ml of glacial acetic acid were added. The samples remained in a water bath at 100 °C for 1 h and then cooled on ice. Two ml of toluene was added to each sample and vortexed for 20 s. The samples remained for 5 min at room temperature to separate the

phases. 1 ml of the aqueous phase (supernatant) was removed to quantify proline content by spectrophotometry at 520 nm. The analyzes were performed in triplicate and the concentration of proline was determined using a standard curve (0 - 100 µg $mL^{-1}$) and expressed in micromol of proline per gram of fresh mass (µmol g $MF^{-1}$).

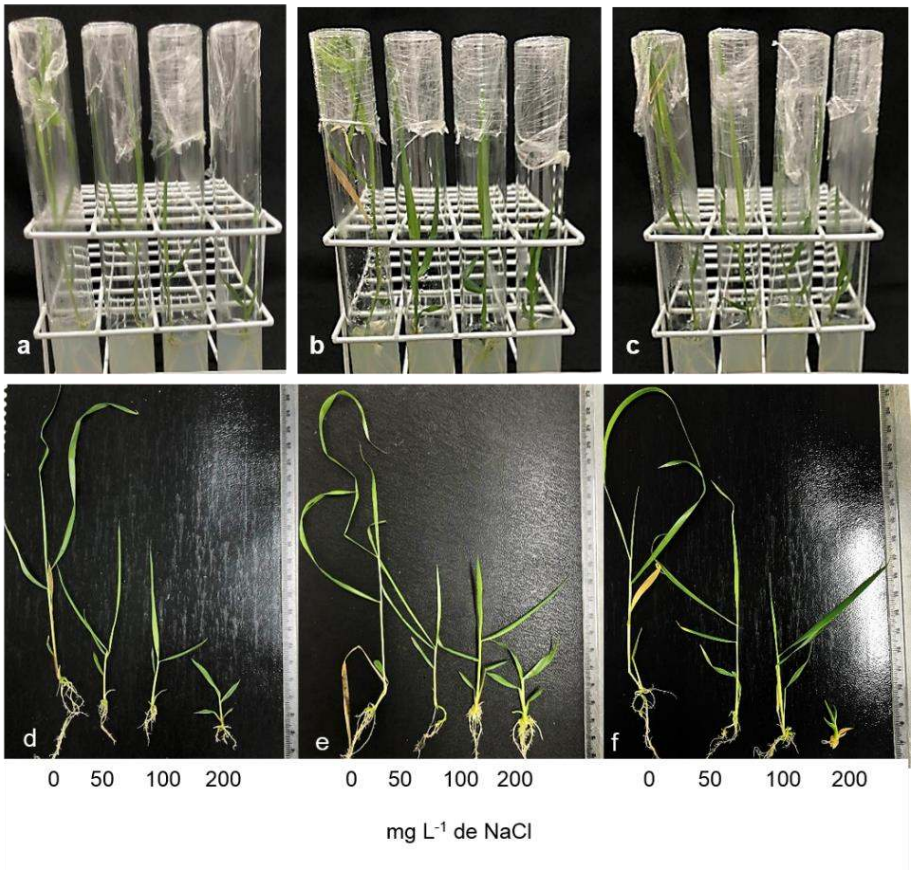
Statistics Analysis

All results were subjected to analysis of variance (ANOVA). Significant differences between means were determined using the Least Significant Difference (LSD test) at a level of $p < 0.05$.

**Results and Discussion**

Plant growth under salt stress activates several responses to survive including physiological, biochemical, and molecular changes that affect plant growth and development (SZABADOS; SAVOURE, 2009). In this work, we evaluated three cultivars of *U. brizantha* (Marandu, Piatã and Xaraés) to *in vitro* salt stress. After seven days seeds germinated in filter paper were transferred to test tubes with or without NaCl and remained for 28 days old (Figure 1A). After this time, the plantlets were removed from the test tubes (Figure 1B). The shoots and roots growth were inversely affected by the increase in the concentration of NaCl in the media culture. This result was observed in all cultivars tested. Seedlings showed a vitrified aspect at 200 mg $L^{-1}$ NaCl in all the three cultivars (Figure 1B).

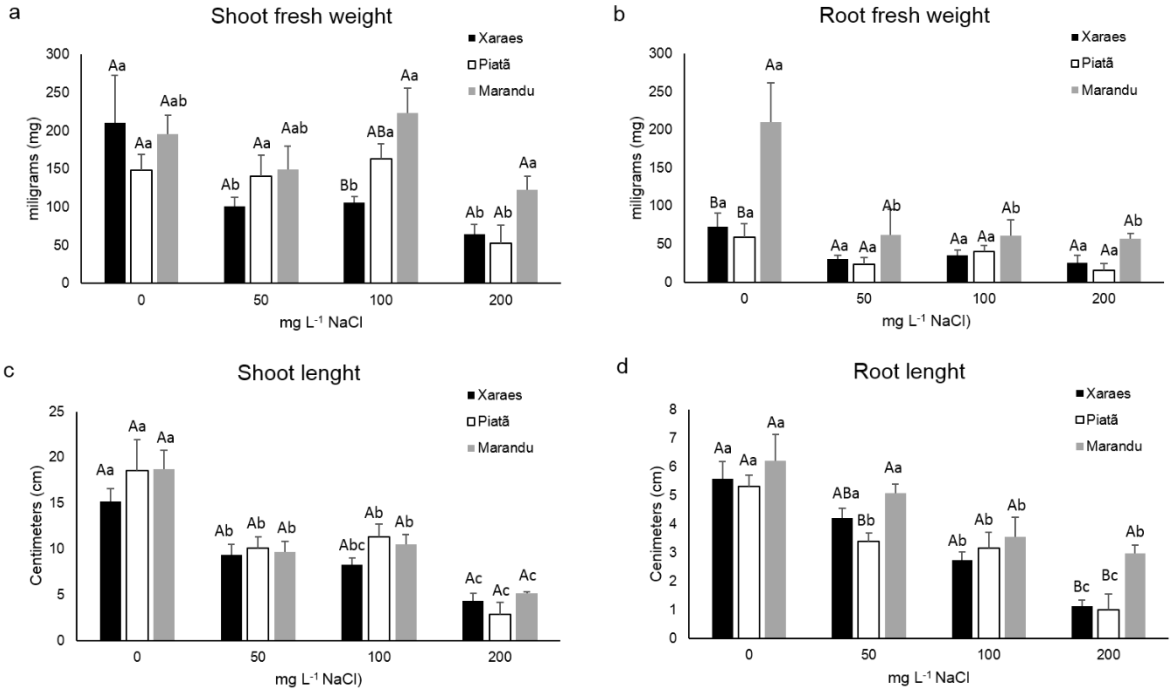
**Figure 1**. Seedling growth characteristics of the three cultivars of *U. brizantha* in MS medium with different NaCl concentrations after 28 days. Xaraes (A, D); Marandu (B, E); Piatã (C, F).




The Xaraés cultivar showed significant reductions in fresh weight of shoots from 50 mg $L^{-1}$ while Piatã and Marandu only at 200 mg $L^{-1}$ of NaCl (Figure 2A). The root fresh weight was only significant for the Marandu cultivar (Figure 2B). The shoots length were significantly reduced by the addition of 50 mg $L^{-1}$ NaCl to the media in all cultivars. The reduction in growth was 73, 88, and 72% for the cultivars Xaraés, Piatã, and Marandu, respectively (Figure 2C). The Piatã cultivar showed significant reductions in root length from 50 mg $L^{-1}$, while for Xaraés and Marandu from

100 mg $L^{-1}$ (Figure 2D). The most drastic reduction was observed at 200 mg $L^{-1}$ NaCl in all cultivars in the order of 80% for cultivars Xaraés and Piatã and 51% for Marandu (Figure 2D).

**Figure 2**. Biometric measurements of shoots and roots at three cultivars of *U. brizantha* after 28 days in vitro with different concentrations of NaCl. (AB) Fresh weight (mg), CD Length (cm). Capital letters compare the three cultivars at each NaCl concentration and lower-case letters compare each cultivar on the four NaCl concentrations. Different letters indicate the statistical difference between the means of treatments by the LSD test ($p < 0.05$). The error bar indicates the mean (n = 6) standard error. Bars=SE.

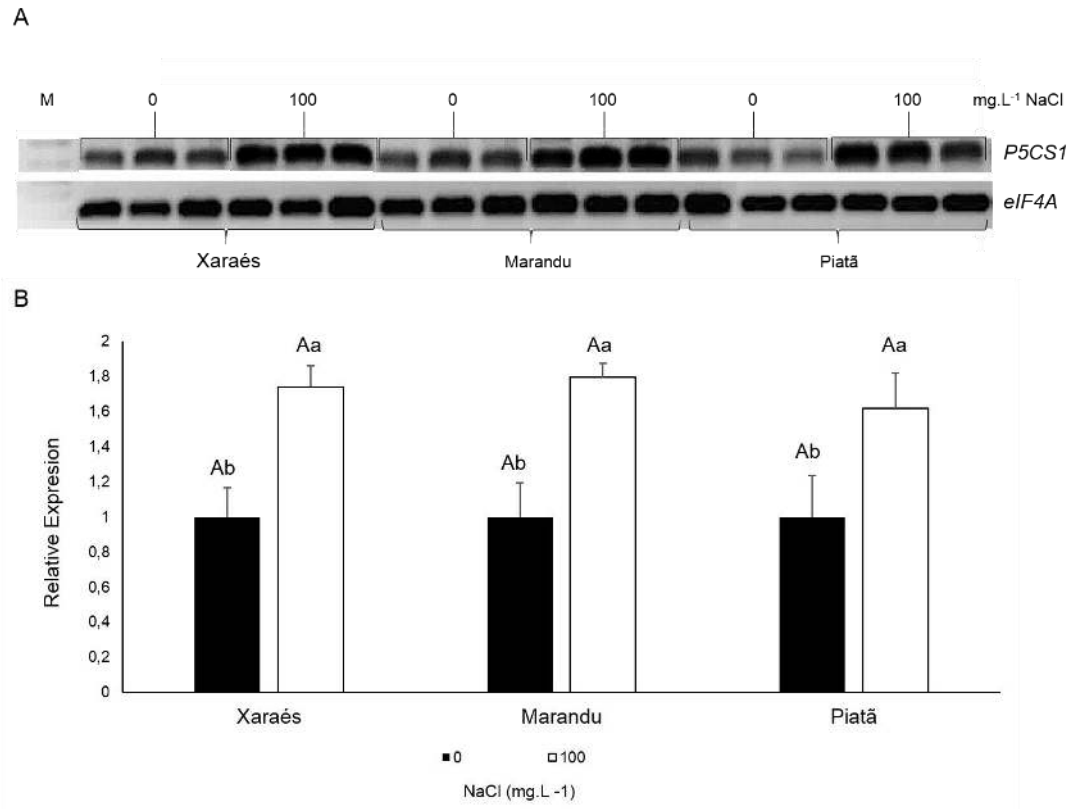



Plants under salt stress immediately start a rapid osmotic phase resulting in an increase of sodium concentration ($Na^+$) in plant cells that inhibit potassium ($K^+$) uptake (CABOT *et al.,* 2014). Potassium is an indispensable element, consequently, plants reduce growth and development when it is deficient (GUPTA; HUANG, 2014). Tolerance to salinity varies with plant species and even within the same species. Although, Marandu cultivar also decreased growing when the NaCl increased; it was the least affected by salinity because their leaves became wider at concentrations of 100 and 200 mg $L^{-1}$ NaCl, resulting in a less reduction in fresh mass weight (Figure 1B and E). In *Lathyrus sativus* (chícharo), exposure to salt *in vitro* significantly reduced the length of the roots and the shoot, but did not influence the dry mass of the shoot and even increased in the roots (PIWOWARCZYK *et al.,* 2016). It is believed that tolerance to salinity may be associated with increased or not reduced mass in stressed plants compared to the control (TALUKDAR, 2011). Plants can tolerate low levels of salt stress by extrusion of $Na^+$ ions out of the cell and or trapping it at the vacuoles (MUNNS; TESTER, 2008). Comparing two cultivars of basil (*Ocimum basilicum*) under salt stress it was found that the cultivar more tolerant improved water balance preserving photosynthesis, accumulated lower abscise acid and higher $Na^+$ in leaves that can function as an osmoticum resulting in less reduction on the biomass accumulation than the sensitive cultivar (MANCARELLA *et al.,* 2016). Salt stress reduced the growth in the three *U. brizantha* cultivars however we observed that Marandu cultivar leaves were wider resulting in the least reduction of shoots biomass. We hypothesize that it could be a result of compartmentalization of $Na^+$ íons on the vacuoles increasing leaves area.

Several abiotic stresses including salinity lead to biochemical changes that increase the biosynthesis of compatible solutes such as proline, which play an important role in mitigating the effects of stress, helping to maintain the integrity of membranes, and

eliminating oxygen-reactive species (ALI *et al.,* 2017). To estimate the semi-quantitative expression of the *P5CS1* gene and proline content we use the samples from the three cultivars (Marandu, Piatã and Xaraés) growing *in vitro* at 0 and 100 mg $L^{-1}$ NaCl. This concentration was chosen because in most cases, there was no significant difference between 50 and 100 mg.$L^{-1}$ for the biometric parameters and the decrease in growth at 200 mg $L^{-1}$ NaCl was so drastic that there was not enough material for RNA extraction. Since there is no genome available for *U. brizanha*, we designed a primer to amplify a fragment of the *P5CS1* gene on a conserved sequence from six species from the *Panicoideae* family. The sequence alignment showed that the *P5CS1* gene is highly conserved among them. A conventional PCR was conducted using the designed pair of primers and polled samples from all cDNAs. Agarose gel electrophoresis revealed the presence of a single band of the expected size 81 bp for *P5CS1* (Figure 3A). In this work, the addition of 100 mg.$L^{-1}$ NaCl to the culture media led to an increase in the transcription of the *P5CS1* gene (0.6 fold) in all cultivars (Figure 3B). The *P5CS* gene encodes is a bifunctional enzyme that catalyzes proline biosynthesis, an osmoprotectant that protects the cell against abiotic stresses (RAI; PENNA, 2013).

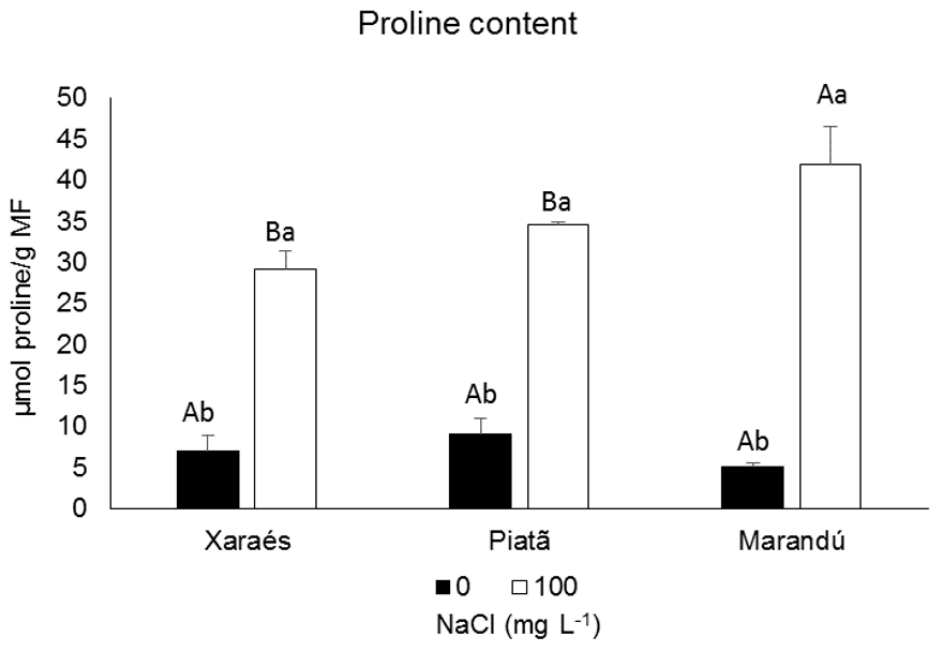
**Figure 3.** Semi-quantitative PCR on 1.5% agarose gel stained with ethidium bromide (A). Relative expression of the *P5CS1* gene normalized with the *eIF4A* gene and calibrated with the zero treatment in each cultivar (B). Capital letters compare the three cultivars at the same NaCl concentration and lower case letters compare each cultivar at 0 and 100 mg.L-1 NaCl. Different letters indicate the statistical difference between the means of treatments by the LSD test ($p < 0.05$). Error bar indicates the standard error of the mean (n = 3). Bars=SE.




In most plant species, increased transcription of the *P5CS* gene and concentration of proline are positively related to stresses (ZHANG; BECKER, 2015). This increase in the transcription of the *P5CS1* gene depends on the species, the growth stage, and the salt concentration used (ANNUNZIATA *et al.,* 2016). We observed that all *U. brizantha* cultivar increased transcription of *P5CS1* under salt stress at the same level. Estimation of proline content revealed that it significantly increased in the three cultivars of *Urochloa* sp grown at 100 mg $L^{-1}$ NaCl on culture media. The increase was 4.2, 3.8, and 8.2 times for cultivars Xaraés, Piatã, and Marandu, respectively, when compared to the treatment without salt addition (Figure 4). Plants

under abiotic stress increase the concentration of endogenous proline. Cultivar Marandu accumulated significantly more proline than the other two cultivars (Figure 4).

**Figure 4.** Determination of proline content in 3 cultivars of *U. brizantha* cultivated *in vitro* without and with the presence of 100 mg.L-1 NaCl. Capital letters compare the three cultivars at the same NaCl concentration and lower case letters compare each cultivar at 0 and 100 mg.L-1 NaCl. Different letters indicate the statistical difference between the means of treatments by the LSD test ($p < 0.05$). Error bar indicates the standard error of the mean (n = 3). Bars=SE.




In general, gene transcription increases a few hours after the salt stress imposition (PER, 2017). Although the transcriptional level of the *P5CS1* genes was similar at the concentration of 100 mg $L^{-1}$ NaCl in the three cultivars, proline content was higher in cv. Marandu. This may have occurred because the relative expression of the *P5CS1* gene and the determination of proline content were performed after 28 days of *in vitro* growth in the presence or absence of NaCl. In *Arabidopsis*, for example, seedlings grown at 170 mM NaCl, the accumulation of *P5CS* transcripts started 4 h after treatment, reached its maximum level after 8 hours, and then decreased for the next 24 hours (AMINI *et al.,* 2015). In this way, it is possible that the transcriptional level of *P5CS1* in *U. brizantha* was already stabilized at that time and the possible differences between cultivars could not be observed.

**Conclusion**

In this work, the addition of any concentration of NaCl to the *in vitro* culture media reduced the growth of shoots and roots and increased the transcription of *P5CS* gene in all three cultivars of brachiaria assayed. However, Marandu cultivar showed a lower reduction in the fresh weight of shoots and roots compared to Piatã and Xaraés. This could be the result of the greater accumulation of proline in Marandu cultivar, since this amino acid act as an osmoprotectant against salt stress. The results presented here suggest that Marandu could present a better response of adaptation to *in vitro* salt stress.

**Acknowledgment**

Paula Beatriz Ramos Guimarães is grateful for a grant from the National Council for Scientific and Technological Development (PIBIC-CNPq) – Brazil. We are grateful to Cristiane Aparecida de Souza for technical assistance in the tissue culture laboratory- UNOESTE.

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