

TOLERANCE OF ALFALFA VARIETIES SUBJECTED TO SALINITY

RODRIGO MOURA E SILVA¹; WELSON LIMA SIMÕES²; MIGUEL JULIO MACHADO GUIMARÃES³; JULIANE RAFAELE ALVES BARROS⁴; KAIO VINICIUS FERNANDES BARBOSA⁵ E FRANCISLENE ANGELOTTI⁶

¹ Universidade de Pernambuco, Rodovia BR 203, Km 2 s/n - Vila Eduardo, 56328-900, Petrolina, Pernambuco, Brasil, e-mail: moura.pnz@gmail.com.

² Embrapa Semiárido, Rodovia BR-428, Km 152, s/n - Zona Rural, 56302-970, Petrolina, Pernambuco, Brasil, e-mail: welson.simoese@embrapa.br.

³ Instituto Federal de Educação, Ciência e Tecnologia, BR-230, 65840-000, São Raimundo das Mangabeiras, Maranhão, Brasil, e-mail: mjnguimaraes@hotmail.com.

⁴ Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco, Rua Benfica, 150, Madalena, 50720-001, Recife, Pernambuco, Brasil, e-mail: juliane-ab@hotmail.com.

⁵ Universidade de Pernambuco, Rodovia BR 203, Km 2 s/n - Vila Eduardo, 56328-900, Petrolina, Pernambuco, Brasil, e-mail: kaioviniciusfernandesbarbosa@gmail.com.

⁶ Embrapa Semiárido, Rodovia BR-428, Km 152, s/n - Zona Rural, 56302-970, Petrolina, Pernambuco, Brasil, e-mail: francislene.angelotti@embrapa.br

1 ABSTRACT

Saline water can be an alternative for the production of salt-tolerant forages. Thus, the objective was to evaluate the tolerance of alfalfa varieties to salinity, aiming at their inclusion in production systems that use irrigation water with high levels of salt or salinized soils. The experiment was conducted in a greenhouse, in a 3x5x3 factorial, three alfalfa varieties (LE-N4, Crioula and DK-192), five salinity levels (0; 3.5; 5.0; 7.0; 10.5 and 14.0 dS m⁻¹) and three cuts of the aerial part, with four replications. For irrigation management, lysimeters were installed. Fresh and dry plant biomass, chlorophyll, water use efficiency and enzymatic activity were analyzed. Increased salinity reduced fresh and dry biomass of shoots and roots, chlorophyll content and water use efficiency. Salinity altered the biochemical responses of alfalfa. DK-192 showed higher production of fresh and dry biomass of the shoot, while LE-N4 was more sensitive to saline stress, reducing production from the level of 7.0 dS m⁻¹. Increased catalase activity in Crioula and DK-192 under saline stress contributed to the production of fresh and dry biomass of the shoot.

Keywords: *Medicago sativa* L, saline water, biomass, enzymes, oxidative stress.

SILVA, R. M. e.; SIMÕES, W. L.; GUIMARÃES, M. J. M.; BARROS, J. R. A.;
BARBOSA, K. V. F.; ANGELOTTI, F.
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2 RESUMO

Água salina pode ser uma alternativa para produção de forrageiras tolerantes ao sal. Assim, objetivou-se avaliar a tolerância de variedades de alfafa à salinidade, visando sua inclusão em

sistemas de produção que utilizam água de irrigação com teores de sais elevados ou solos salinizados. O experimento foi conduzido em casa de vegetação, em fatorial 3x5x3, três variedades de alfafa (LE-N4, Crioula e DK-192), cinco níveis de salinidade (0; 3,5; 5,0; 7,0; 10,5 e 14,0 dS m⁻¹) e três cortes da parte aérea, com quatro repetições. Para o manejo da irrigação foram instalados lisímetros. Analisou-se a biomassa fresca e seca da planta, clorofila, eficiência do uso da água e atividade enzimática. O aumento da salinidade reduziu a biomassa fresca e seca da parte aérea e da raiz, conteúdo de clorofila e a eficiência no uso de água. A salinidade alterou as respostas bioquímicas da alfafa. A DK-192 apresentou maior produção de biomassa fresca e seca da parte aérea, a LE-N4 foi mais sensível ao estresse salino, reduzindo a produção a partir do nível de 7,0 dS m⁻¹. O aumento da atividade da catalase na Crioula e DK-192 sob estresse salino contribuiu para produção de biomassa fresca e seca da parte aérea.

Palavras-chave: *Medicago sativa* L, água salina, biomassa, enzimas, estresse oxidativo.

3 INTRODUCTION

Salinity is a global concern because it directly affects agricultural production, putting food security at risk (FAO, 2018). Currently, the expansion of salt-affected areas has reached approximately one billion hectares in more than one hundred countries (IVUSHKIN *et al.*, 2019). In the Brazilian semi-arid region, salinization affects about 30% of the soils in irrigated perimeters (CODESVASF, 2021). In view of this, research has shown that there are salinity-tolerant plants and that the proper management of this type of water can become an option for regions with scarcity of good quality water (DIAS, 2016), since the lack of knowledge about the use of salinized water in agricultural cultivation generates negative consequences, such as crop loss and damage to soils in irrigated areas (OLIVEIRA *et al.*, 2019; SILVA *et al.*, 2018; SOUZA *et al.*, 2018;).

In this context, one of the alternatives for producers in the Brazilian semi-arid region would be the production of salinity-tolerant forage crops. Considering the intrinsic conditions of the Brazilian semi-arid region, where the available waters normally have a high salinity level (CASTRO; SANTOS, 2020), the choice of tolerant forage crops may be a strategy for agriculture, contributing as a source of nutritional improvement of the herds.

Alfalfa (*Medicago sativa* L.) is a crop that stands out in this group, because it has the ability to adapt to the most varied environmental conditions and can develop under temperature up to 54 °C (HUGHES; HEATH; METCALFE, 1966) and under salinity conditions of up to 8 dS m⁻¹ (PUTNAM *et al.*, 2017). In addition, this plant is considered one of the most important forage crops in the world for animal feed, containing high crude protein content, palatability and good digestibility, and it can be supplied to herds in the forms of hay, silage or pre-dried (RUGGIERI; MARQUES; REIS, 2020).

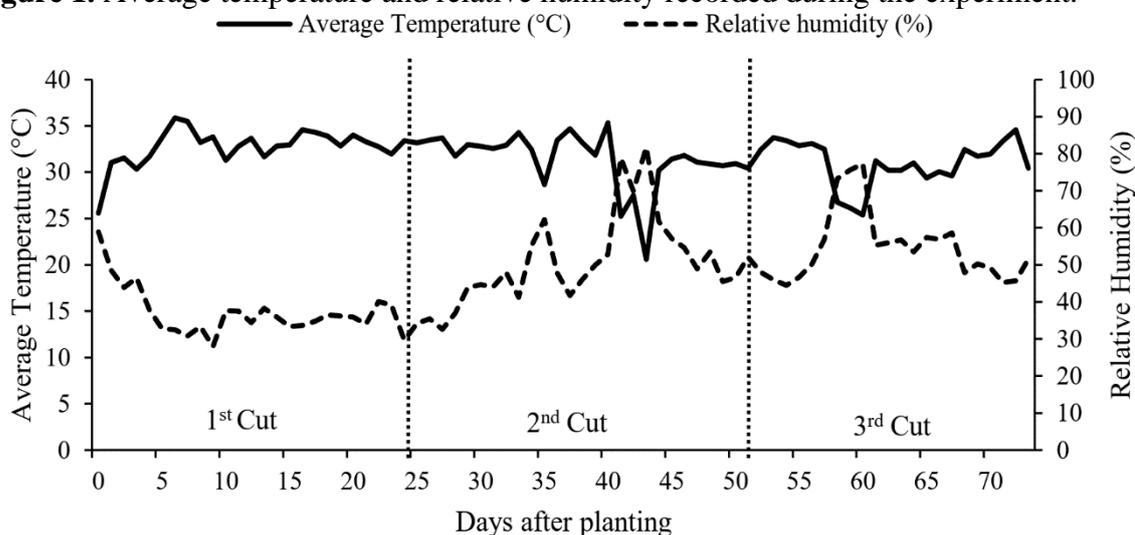
The different genetic inheritances, even within the same species, respond differently to the stress caused by excess of salts in the environment. In this context, plants have biochemical mechanisms as a defense against this condition, through the use of enzymatic systems, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), that play a key role in protecting plant cells against the indirect effects of salinity (GUIMARÃES *et al.*, 2020). Selecting saline stress-tolerant alfalfa varieties will be of great importance, especially for animal feed, as it reduces damage to forage quality (EL-SHARKAWY *et al.*, 2017). In the specific case of alfalfa, there are still few studies that have evaluated its production in saline conditions in the Brazilian semi-arid

region. Therefore, it is necessary to investigate the salinity tolerance of alfalfa varieties to identify those that are more suitable for cultivation in areas with high levels of salts in the soil or irrigation water. This knowledge can contribute to the development of more sustainable and resilient production systems in arid and semi-arid regions. Thus, the objective was to evaluate the tolerance of alfalfa varieties to salinity, aiming at their inclusion in production systems that use irrigation water with high levels of salt or salinized soils.

4 MATERIALS AND METHODS

The experiment was conducted in a greenhouse in Petrolina – PE, Brazil (9°8'.9" S/40°18'33.6" W, 373 m), in the period from June 2020 to January 2021. The values of average temperature and relative humidity were obtained by means of an automatic weather station installed on site, throughout the experimental period (Figure 1).

Figure 1. Average temperature and relative humidity recorded during the experiment.



Fonte: Authors (2021)

The experimental design used was randomized blocks, with treatments distributed in a 3 x 5 x 3 factorial arrangement, corresponding to three varieties of alfalfa (LE-N4, Crioula and DK-192), five salinity levels of irrigation water (0; 3.5; 5.0; 7.0; 10.5 and 14.0 dS m⁻¹) and three cuts of the shoots, with four replicates.

Alfalfa seeds were sown at 2 cm depth in planting trays filled with organic substrate. Two weeks after sowing, two seedlings of each variety were transplanted into plastic pots with capacity of 20 liters filled with 3 cm of crushed stone at the base and 15 kg of collected soil.

The fertility of the soil, an Argissolo Amarelo Distrófico latossólico (Oxisol) with

sandy loam texture, was corrected with applications of 500 mL of nutrient solution prepared according to the recommendation of Novais, Neves and Barros (1991) for greenhouse experiments. The recommended amount was divided into three applications with an interval of twenty days between them, initiated after each of the cuts. Each pot received 5.4, 4.4 and 4.5 mL of NaHPO₄, KH₂PO₄ and (NH₄)₂SO₄, as well as 28.0, 31.5, 1.6, 67.5, 10.6, 3.0 and 4.0 mg per 125 mL pot⁻¹ of HBO, CuSO₄.5HO, (NH₄)₂MoO₄.4HO, MnSO₄.HO, ZnSO₄.7HO, FeSO₄.7HO and Na EDTA, respectively.

For irrigation management, weighing lysimeters were installed in all treatments of one block, in which pots were fixed onto

load cells (TSD AEPH) with capacity of up to 50 kg. The load cells were placed under metal bases with devices to collect excess water drained from each pot. The cells were connected to two AM16/32B multiplexers, linked to a CR1000 datalogger, with readings every 15 seconds, recording them every 15 minutes. The lysimeters were calibrated by obtaining a curve of signal readings (mV) from the permanent wilting point to the maximum soil water holding capacity in each pot.

Twenty days after transplanting to the pots, irrigations with saline solutions were initiated. The solutions were prepared in polyethylene drums with capacity of 100 L, from the salts NaCl, CaCl₂.2H₂O and MgSO₄.7H₂O, in order to obtain an equivalent ratio of 7:2:1 relative to Na:Ca:Mg, which is the salinity ratio of most wells in the semi-arid region (AQUINO *et al.*, 2007).

Irrigations were performed every two days, replacing the volume of water consumed by the plants, in order to maintain the moisture content in the soil at its maximum water holding capacity. An additional leaching depth of 15% was added in the volume of the maximum capacity retained in each pot, in order to avoid the accumulation of salts in the soil profile.

Harvest was carried out respecting the limit of at least 80% flowering of the varieties (1st cut: 48 days, 2nd cut: 76 days, 3rd cut: 96 days), and the plants were cut at 10 cm height from the soil. Only the shoots (S) were removed in the first and second cuts, whereas in the third cut, shoots (S) and roots (R) were removed and weighed to obtain fresh mass. Then, the material was placed in an oven at 60°C and kept until reaching constant weight to determine the dry mass. Water use efficiency (WUE) was calculated through the ratio between total dry mass production (shoot + root) and plant water consumption.

For the enzymatic analyses, samples of leaves without injuries, with green color

and fully expanded, located between the apex and the collar of the plant, were collected (1st cut: 47 days, 2nd cut: 75 days). The samples were immediately stored in aluminum foil envelopes and immersed in liquid nitrogen (N₂). The plant extracts were prepared using 1 g of plant material macerated in liquid nitrogen with the addition of 0.01 g of polyvinylpyrrolidone and 3 mL of extraction buffer (pH 7.5) at the concentration of 100 mM of potassium phosphate. Then, the extract was centrifuged at 15,000 g for 15 minutes at 4 °C, and the obtained supernatant was used as crude enzymatic extract.

Catalase (CAT) activity was determined following the decomposition of H₂O₂ for 60 seconds, through spectrophotometric readings at 240 nm, at 25 °C, according to the method described by Havir and Mchale (1987). Ascorbate peroxidase (APX) activity was determined as described by Nakano and Asada (1981), by monitoring the oxidation rate of ascorbate using a spectrophotometer at wavelength of 290 nm, at 25 °C, for 60 seconds. Superoxide dismutase (SOD) activity was determined according to the methodology of Giannopolitis and Ries (1977), with readings in spectrophotometer at wavelength of 560 nm, and defining the SOD unit as the amount of enzyme necessary to inhibit NBT photoreduction by 50%.

The data obtained were subjected to analysis of variance (ANOVA) using the Sisvar 5.0 program. To compare salinity levels, first- and second-degree regression models were evaluated when significant at 1 or 5% probability level. Varieties and cuts were compared by Tukey Test at 0.05 probability level.

5 RESULTS AND DISCUSSION

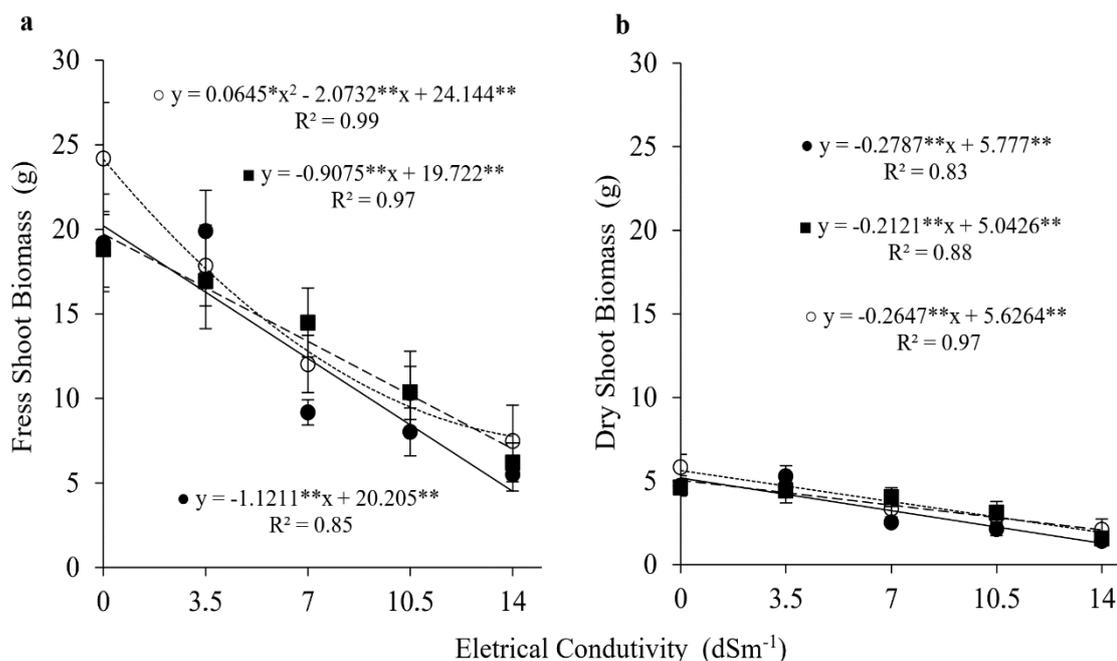
The interactions variety x salinity levels and cut x salinity levels were significant for shoot fresh biomass (SFB), shoot dry biomass (SDB), total chlorophyll and water use efficiency (WUE). There was an interaction between Cut and Variety for water use efficiency (WUE). For root fresh and dry biomass, only the isolated effect of the salinity level was significant.

The fresh and dry biomass of the varieties LE-N4 and Crioula showed a decreasing linear behavior with increasing salinity levels of irrigation water. On the other hand, the production of fresh and dry

biomass of the variety DK-192 showed a quadratic behavior (Figure 2a, b). The values of shoot fresh biomass of the variety LE-N4 varied between 19.20 and 5.51 g.plant⁻¹ at the salinity levels of 0 and 14 dS m⁻¹, respectively, with a reduction of 71.31% (Figure 2a). For the Crioula variety, the values of shoot fresh biomass ranged from 18.82 to 6.22 g.plant⁻¹, for salinity levels of 0 and 14 dS m⁻¹, respectively, with a reduction of 66.95% (Figure 2a). For the variety DK-192, the values of fresh biomass production varied from 24.18 g.plant⁻¹ to 7.48 g.plant⁻¹, for salinity levels of 0 and 14 dS m⁻¹, respectively, with a reduction of 69.06% (Figure 2a).

Figure 2. Fresh (a) and dry (b) aerial part (P.A) biomass of alfalfa varieties submitted to different levels of salinity of irrigation water.

● LE-N4 ■ CRIOULA ○ DK-192



Fonte: Authors (2021)

Significant regression coefficients with $p < 0,01$ (**) and $p < 0,05$ (*).

The increase in salinity level also caused reductions in the shoot dry biomass of all varieties, with the lowest averages at the salinity level of 14 dS m⁻¹, with 1.43, 1.58 and 2.10 g.plant⁻¹ for LE-N4, Crioula and DK-192, respectively (Figure 2b). The reduction of biomass in alfalfa plants may be

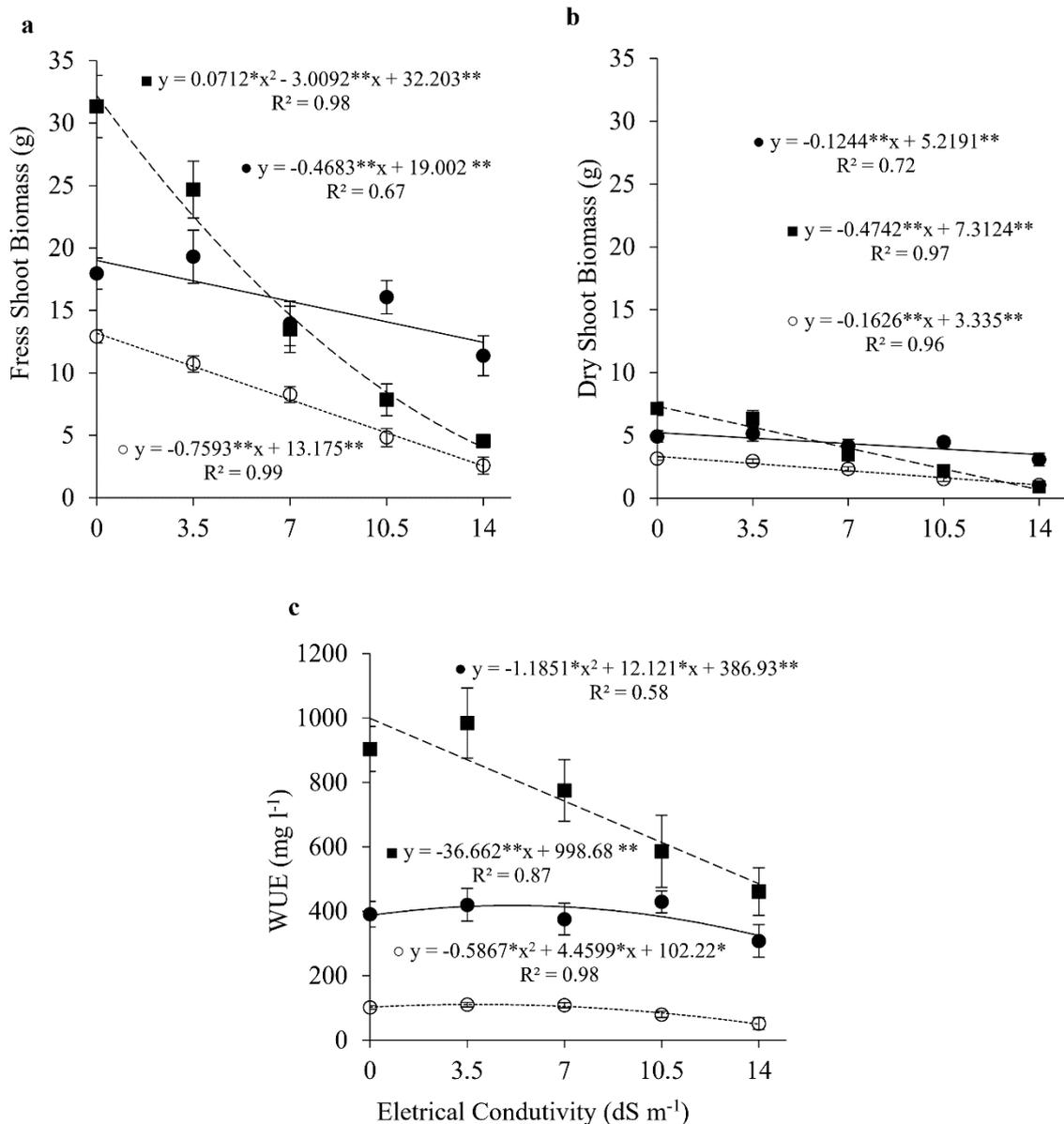
associated with their energy expenditure to adapt to the salt stress imposed (VILLA; SANTOS; SECCO, 2019). Stomatal closure, for example, causes a blockage in the concentrations of carbon dioxide that is assimilated by the leaves, leading to a decrease in the accumulation of

photoassimilates, which results in the reduction of plant growth and yield. In a study conducted with 36 alfalfa genotypes subjected to salinity level of 150 mM using NaCl, it was possible to observe reductions of 39.79% for fresh biomass (SFB) and 35.17% for dry biomass (SDB) (BENABDERRAHIM; GUIZA; HADDAD, 2020).

In the interaction cut x salinity levels, the shoot fresh biomass of the first and third cuts showed a decreasing linear behavior, with reductions of 36.62 and 80.03%, when comparing the salinity levels of 0 and 14 dS m⁻¹, respectively (Figure 3a). In the second cut, fresh biomass showed a decreasing quadratic behavior, with reduction of 85.57% for the use of saline water with 14 dS m⁻¹ (Figure 3a).

Figure 3. Fresh (a), dry (b) shoot biomass and water use efficiency (c) of alfalfa varieties subjected to different levels of irrigation water salinity and cutting time.

● LE-N4 ■ CRIOULA ○ DK-192



Fonte: Authors (2021)

Significant regression coefficients with $p < 0.01$ (**) and $p < 0.05$ (*).

Shoot dry biomass accumulation showed a decreasing linear behavior between the cuts at the different salinity levels, with reductions of 37.19, 87.5 and 67.08% for the first, second and third cuts, respectively. However, the values found at 3.5 dS m^{-1} were the closest ones to those of the treatment without salinity, both in the second and in the third cuts. The highest dry biomass production was verified in the first cut (Figure 3b). The increase in salinity reduces the availability of water and nutrients in plant tissues (SCHOSSLER *et al.*, 2012). This reduction occurs due to the destructuring of soil properties caused by sodicity, reducing its capacity for water storage and absorption, also interfering in the root-ion mechanism, promoting a reduction in the absorption of nutrients by the plant (PRADO, 2008). Excess salts cause an increase in osmotic pressure and, as a result, reduces the absorption of water and nutrients. Depending on the degree of salt stress, the plant may also lose the water that is stored inside its roots, due to changes in the osmotic potential of the soil, which is one of the major causes of reduction in plant growth (DIAS; BLANCO, 2010).

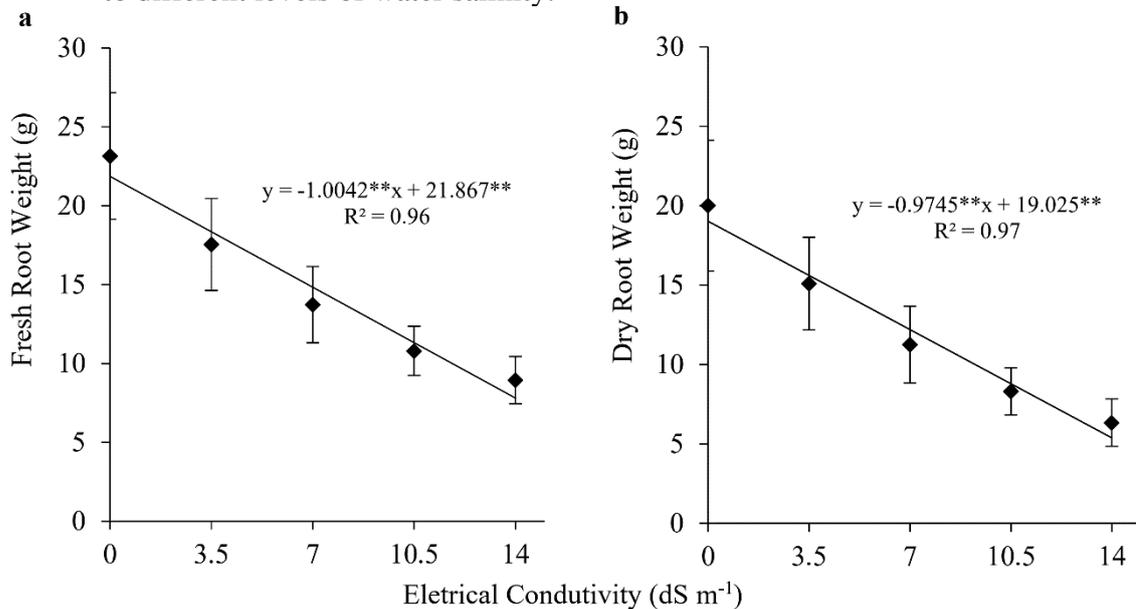
For water use efficiency, the maximum values were 388.91 mg L^{-1} under salinity level of 0.32 and 110.70 at 3.80 dS m^{-1} , in the first and third cuts, respectively. There were reductions of 1.18 and 0.58 mg L^{-1} in the first and third cuts, respectively, at each unit of salinity levels added in the treatments, totaling reductions of 21% for the first cut and 54% for the third cut (Figure

3c). Studies conducted by Pereira Filho (2020) corroborate what was observed in this study, as these authors verified that the increase of salts in irrigation water reduced the water absorption capacity by the plant, due to the osmotic and toxic effects of the ions, with reduction in plant growth. In the results obtained for shoot dry biomass in studies conducted by Díaz, Grattan and Reyes (2018) and Ashrafi, Zahedi and Razmjoo (2015), the reduction was also related to the lower absorption of water and nutrients by the plant. However, these responses will vary depending on the variety and salinity level to which the plant was subjected.

The second cut showed a decreasing linear behavior (Figure 3c), with considerable gradual reduction from the salinity level of 7.0 dS m^{-1} . However, there was a 53% reduction at the salinity level of 14 dS m^{-1} . The alteration of the photoassimilates obtained from photosynthesis in the different parts of the plant (roots, growing tissues and storage organs) also promotes the decrease in the production of total biomass (SILVA; KLAR; SILVA, 2013), since the distribution of these photosynthetic assimilates is important to determine the type of plant growth, which may oscillate between the biomass of the shoot and/or the root (TAIZ *et al.*, 2017).

Root fresh and dry biomass showed a linear and decreasing behavior, with reductions of 61.3 and 68.3%, respectively, as a function of the increase in salinity level (Figure 4a and b).

Figure 4. Fresh (a) and dry (b) root biomass of LE-N4, Crioula and DK-192 varieties subjected to different levels of water salinity.



Fonte: Authors (2021)

Significant regression coefficients with $p < 0.01$ (**) and $p < 0.05$ (*).

In a study conducted with 15 alfalfa populations under salt stress, it was observed that the root biomass per plant under salinity levels of 18.3 and 24.5 dS m⁻¹ were reduced, respectively, by 18 and 49% (CORNACCHIONE; SUAREZ, 2015). These reductions are related to several mechanisms such as the reduction of cell turgor (COSTA; PINHO; PARRY, 2008), etiolation of plants (AGUIAR *et al.*, 2008), restriction in the water absorption process (COELHO, 2013), and reduction in the number of leaves (OLIVEIRA *et al.*, 2006).

In addition, the excess of sodium and chlorine ions in the soil caused by high salinity affects the translocation of indispensable hormones such as abscisic acid and cytokinins from the roots to the shoots of the plant, restricting its growth (KUNZ *et al.*, 2007), since abscisic acid is one of the responsible for the alteration of genes induced by salt stress, being considered the hormone related to stress, participating in several physiological processes in plants, such as stimulation of stomatal movements, increased root growth,

accumulation of proline and inhibition of shoot growth (CREELMAN *et al.*, 1990; BRUXELLES *et al.*, 1996). This reduction occurs due to osmotic stress, which reduces the capacity to absorb water and essential minerals such as potassium and calcium (MUNNS *et al.*, 2006; TUTEJA, 2007). In addition, when exposed to severe conditions of salt stress, there may be permanent wilting or senescence of the plant, because the solution in the soil becomes a hyperosmotic solution.

Farissi *et al.* (2018), in a study with three levels of saline water and two varieties of alfalfa, observed that there was a decrease in root growth, with reductions of 24.5% for the *Tata* variety and 41.3% for the *Taf1* variety, consequently reducing dry biomass as the salinity levels increased.

In the first and third cuts, the varieties showed no significant difference for water use efficiency (Table 1). However, the second cut showed higher water use efficiency for the varieties LE-N4, Crioula and DK-192, with values of 590.67, 853.81 and 781.66 mg L⁻¹, respectively.

Table 1. Water Use Efficiency (mg l^{-1}) of alfalfa varieties subjected to different cutting times.

Cuts	WUE		
	LE-N4	CRIOULA	DK-192
1 st cut	331.08 bA	381.82 bA	441.09 bA
2 nd cut	590.67 aB	853.81 aA	781.66 aA
3 rd cut	89.27 cA	85.00 cA	96.68 cA

Fonte: Authors (2021)

*Averages followed by the same lowercase letter in the column and uppercase in the row do not differ at 5% probability.

For forage sorghum (*Sorghum bicolor* (L.) Moench), a reduction of up to 50% was also observed in water use efficiency with salinity level from 6.77 dS m^{-1} (GUIMARÃES *et al.*, 2020). Under salt stress, plant tissues absorb less water due to the reduction of their water potential (HASEGAWA *et al.*, 2000). Therefore, this process indicates the need for greater water consumption for cell growth and development to occur, as the salinity levels increase (SANTOS JÚNIOR *et al.*, 2013).

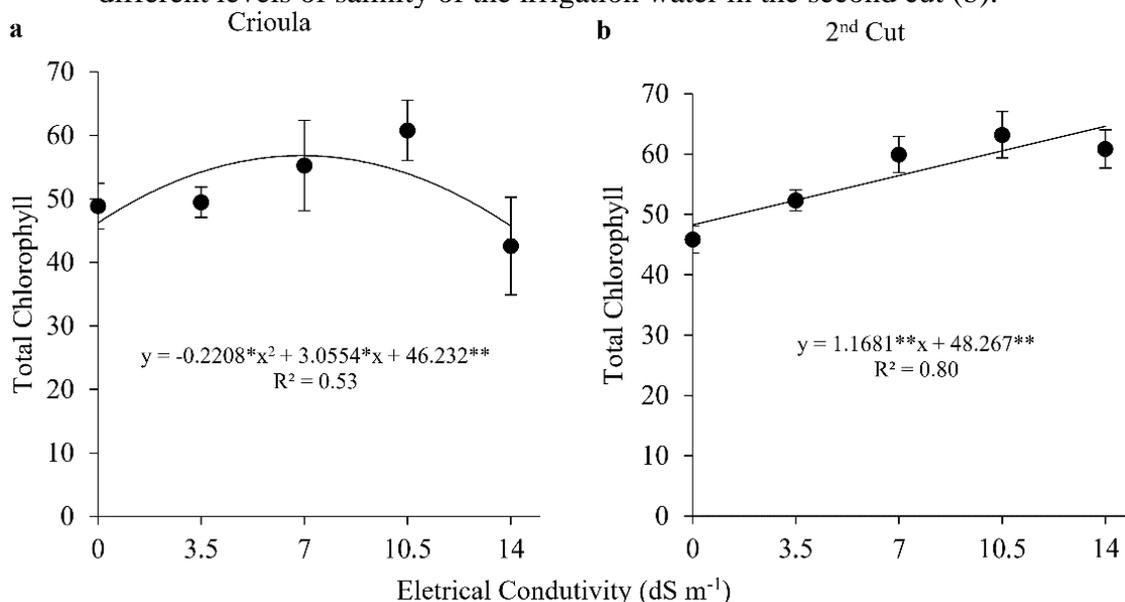
In the initial stage, the plant demands greater energy expenditure for root and shoot production, as opposed to an already developed plant. Depending on the stage of development that the plant is in, there may be acclimatization due to the time of exposure to stress, being a mechanism of tolerance (COMINELLI, 2013). Therefore, in the present study it was possible to observe that the energy expenditure was higher in the first cut due to the production of roots and shoots, which justifies the lower

values of water use efficiency found when compared to the second cut, when the plant already had an established development of the root system.

Alfalfa plants are classified as moderately tolerant to salt stress with a salinity threshold of 2.0 dS m^{-1} , and these levels of salinity tolerance may vary between varieties. Therefore, some authors already demonstrate the existence of this variation in the alfalfa crop, and it is relevant to conduct new studies aiming at this new classification regarding salinity tolerance (DIAS *et al.*, 2016).

In the analysis of total chlorophyll, it was found that the varieties showed different behaviors. For LE-N4 and DK-192, the mathematical models were not significant, with mean chlorophyll contents of 48.16 and 58.96, respectively (Figure 5a). However, the chlorophyll content showed quadratic behavior for the Crioula variety, reaching a maximum point of 56.83, at the salinity level of 6.94 dS m^{-1} (Figure 5a).

Figure 5. Total chlorophyll content of the Crioula alfalfa variety (a) and total chlorophyll at different levels of salinity of the irrigation water in the second cut (b).



Fonte: Authors (2021)

Significant regression coefficients with $p < 0.01$ (**) and $p < 0.05$ (*).

In response to salinity, plants may exhibit photosynthetic changes due to the increase in chlorophyll-degrading enzymes (chlorophyllases), which destroy chloroplasts, altering the chlorophyll concentration (MATEOS-NARANJO *et al.*, 2010). The reduction in photosynthetic pigments due to increased salinity has been observed by other authors such as Aquino *et al.* (2007) and Silva *et al.* (2017) for sorghum (*Sorghum bicolor* (L.) Moench).

It is emphasized that chlorophylls are essential pigments for the photosynthetic process and are directly linked to the efficiency of photosynthesis, and because they have an unstable chemical structure, they are easily degraded (STREIT *et al.*, 2005). Thus, when the plant has a high chlorophyll rate, it indicates a higher photosynthetic rate, as well as its growth and adaptation to different environments (CASTRO *et al.*, 2014; JESUS; MARENCO, 2008). According to Blankenship (2014), there are internal and external factors that can influence the processes of synthesis and degradation of chlorophylls.

External factors include CO₂ concentration, excess and limitation of soil water, temperature, light intensity, nitrogen content and salinity. In addition, the reduction of chlorophyll content may be associated with factors such as the loss of chloroplast membranes (CECCARELLI *et al.*, 2010) and damage to chloroplasts caused by reactive oxygen species (ROS) (GILL; TUTEJA, 2010).

For the first cut, no statistically significant difference was observed in the total chlorophyll content of the leaves as a function of salinity levels. However, in the second cut, the total chlorophyll content showed increasing linear behavior with increasing salinity (Figure 5b). It is possible to observe the decrease in chlorophyll concentration in the Crioula variety, with maximum chlorophyll content at 6.94 dS m⁻¹, also having the highest value of fresh biomass at the same salinity level, when compared with the varieties LE-N4 and DK-192 (Figure 5A).

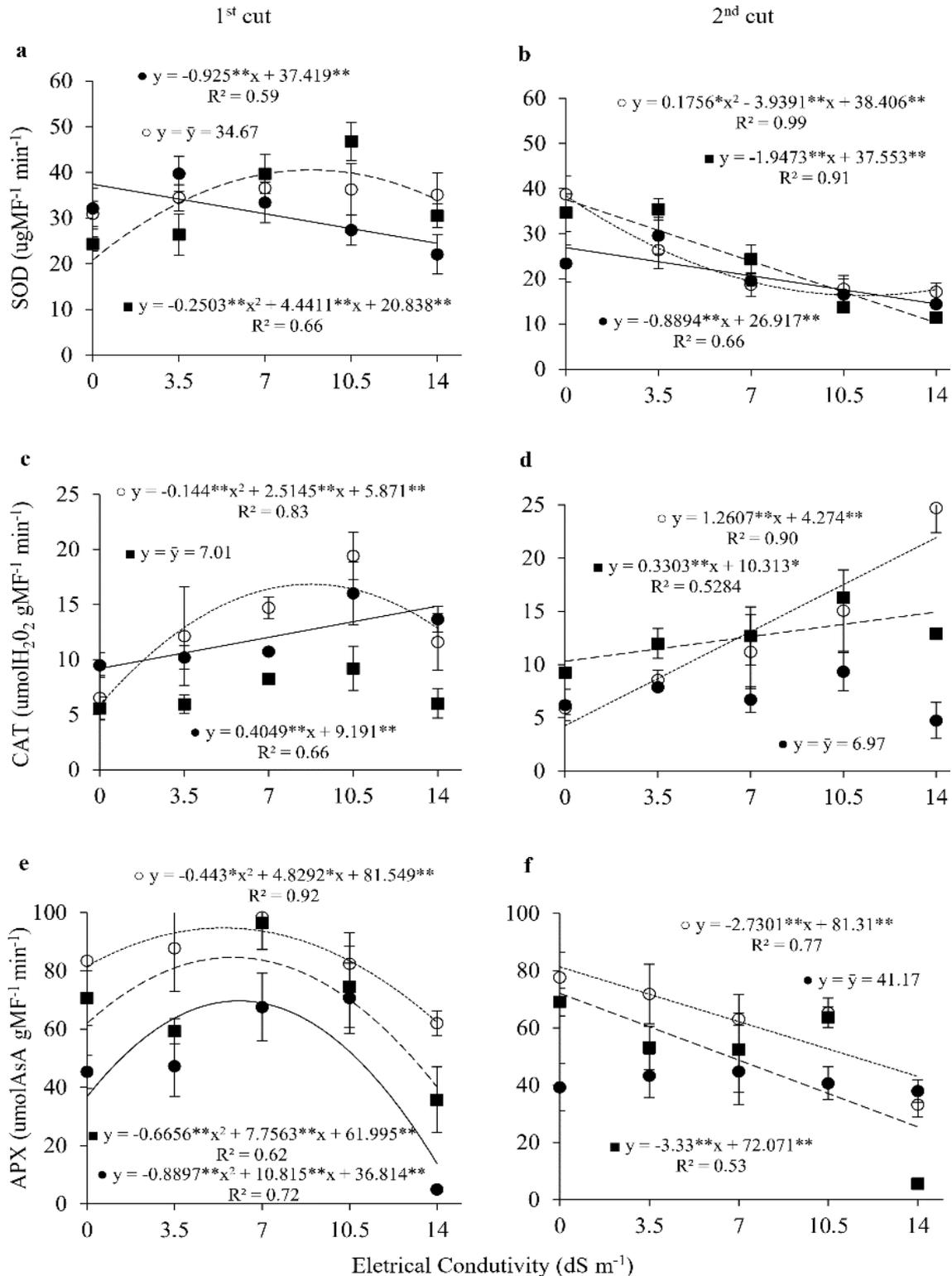
The reduction of dry biomass is related to the reduction of chlorophyll contents, as can be seen for the Crioula

variety. The lowest production, with a 65.78% reduction for the dry biomass (Figure 2a), was observed in plants subjected to the salinity level of 14 dS m⁻¹. The lowest chlorophyll content (Figure 5a) was also observed at this salinity level. As an example, in the study with the forage crop Asian pigeonwing (*Clitoria ternatea* L.), reductions in chlorophyll of up to 51.73% were observed when plants were subjected to a salinity level of 6 dS m⁻¹, demonstrating that salinity negatively affected chlorophyll content (MARTINS *et al.*, 2012).

In the evaluation of enzymatic activity, there was a triple interaction of Cut x Variety x Salinity Levels for superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). The LE-N4 variety showed a decreasing linear behavior of SOD activity with the increase in the salinity level applied in the two cuts evaluated, with reductions of 31.25% and 38.58% in the first and second cuts, respectively.

Figure 6. Enzymatic activity of superoxide dismutase (SOD) (a, b), catalase (CAT) (c, d) and ascorbate peroxidase (APX) (e, f) of alfalfa varieties subjected to different levels of salinity of irrigation water and cutting time.

● LE-N4 ■ CRIOULA ○ DK-192



Fonte: Authors (2021)

Significant regression coefficients with $p < 0.01$ (**) and $p < 0.05$ (*).

It can be observed that, for the catalase enzyme, the LE-N4 variety in the first cut showed an increasing linear behavior, with an increment of $0.4049 \text{ umol H}_2\text{O}_2 \text{ gFM}^{-1} \text{ min}^{-1}$ as the salinity levels increased (Figure 6a). However, at the salinity level of 10.5 dS m^{-1} , an increase of up to four times was observed in comparison to the salinity level of 0 dS m^{-1} . For ascorbate peroxidase, the activity showed a quadratic behavior, reaching a maximum point of $69.68 \text{ umolAsA gFM}^{-1} \text{ min}^{-1}$ at 6.07 dS m^{-1} (Figure 6e). In the second cut, the enzymes catalase and ascorbate peroxidase showed no statistically significant response (Figure 6b and f).

The amount of salt in the soil can reduce or aggravate the intensity of salt stress, causing a greater generation of reactive oxygen species (ROS) and, as a consequence, there may be an increase in the activation of enzymatic defense systems (SHAHZAD *et al.*, 2019). To mitigate oxidative stress caused by excess salt, plants produce different types of antioxidant enzymes, especially superoxide dismutase, ascorbate peroxidase and catalase.

The Crioula variety showed quadratic behavior for the enzyme superoxide dismutase, with maximum activity of $40.58 \text{ ugFM}^{-1} \text{ min}^{-1}$ under salinity level of approximately 8.87 dS m^{-1} , and for the enzyme ascorbate peroxidase, reaching a maximum point of $84.59 \text{ umolAsA gFM}^{-1} \text{ min}^{-1}$ under salinity level of 5.82 dS m^{-1} (Figure 6c and e). The catalase enzyme did not show statistically significant results in the first cut (Figure 6a). However, in the second cut there was an increasing linear behavior with increment of $0.3303 \text{ umolH}_2\text{O}_2 \text{ gFM}^{-1} \text{ min}^{-1}$ for each unit of salinity level added, totaling an increase of 28.16% in its enzymatic activity when comparing the salinity levels of 0 and 14 dS m^{-1} (Figure 6b, d and f).

Different behaviors can be observed among the varieties in the different cuts performed. The variety LE-N4 showed a

decrease in the activity of superoxide dismutase as the salinity level increased in the two evaluated cuts. Lower reductions in the activity of this enzyme in the second cut may be related to the acclimatization of this variety to the applied stress. However, it is important to point out that the activity values observed in the second cut were, in absolute terms, lower than those of the first cut, thus demonstrating that a longer time of exposure to stress can lead to decreased activity of this enzyme (WILLADINO; CAMARA, 2010).

The enzymes superoxide dismutase and ascorbate peroxidase showed a decreasing linear behavior, with reductions of -1.94 and $-3.33 \text{ umolAsA gFM}^{-1} \text{ min}^{-1}$, respectively, for each salinity level added, leading to reductions of 66.94 and 92.03%, respectively, when comparing the treatment of 14 dS m^{-1} with the control treatment (Figure 6d and f). Superoxide dismutase is an important enzyme in the process of combating the toxic effects of ROS, representing the plant's first line of oxidative defense against stresses, besides catalyzing the dismutation of two $\text{O}_2^{\cdot-}$ radicals, generating H_2O_2 and O_2 (BHATTACHARJEE, 2012; MITTLER, 2002; MOHARRAMNJAD *et al.*, 2016).

In addition, Wang *et al.* (2009) reported in their study the effect of salt stress on the activities of superoxide dismutase in alfalfa leaves and observed a significantly greater increase in SOD activity under treatment with salt. Keeping the activity of superoxide dismutase high under salt stress conditions can help the plant protect itself from oxidative damage and possibly from the accumulation of $\text{O}_2^{\cdot-}$. Alfalfa varieties more tolerant to salinity showed higher levels of superoxide dismutase when compared to more sensitive varieties (ASHRAF, 2009; GILL; TUTEJA, 2010; VALIZADEH *et al.*, 2013; WANG *et al.*, 2009).

For the enzyme superoxide dismutase, it was observed that the variety LE-N4 in the first cut showed a decreasing

linear behavior, with a reduction of $-0.925 \text{ ugFM}^{-1} \text{ min}^{-1}$ at each increase in the salinity level. It must be highlighted that there was a reduction of 31.25% when comparing the treatment without salinity with the treatment of 14 dS m^{-1} (Figure 6c).

In addition, in the second cut the superoxide dismutase showed a decreasing linear behavior with reduction of $-0.889 \text{ ugFM}^{-1} \text{ min}^{-1}$ in its activity at each level of salinity added, with a reduction of up to 38.58%, comparing the levels of 0 and 14 dS m^{-1} (Figure 6d).

For the variety DK-192 in the first cut, the enzyme superoxide dismutase did not have statistically significant results (Figure 6c). The enzymes catalase and ascorbate peroxidase, however, showed decreasing quadratic behaviors, with maximum points of 16.848 and $94.710 \text{ umolAsA gFM}^{-1} \text{ min}^{-1}$ under salinity levels of 8.7 and 5.45 dS m^{-1} , respectively (Figure 6a and e). However, in the second cut, superoxide dismutase showed a decreasing quadratic behavior with minimum point equal to $16.315 \text{ ugFM}^{-1} \text{ min}^{-1}$ under salinity level of 11.216 dS m^{-1} (Figure 6d).

As for the enzyme catalase, the behavior obtained was increasing linear, with an increment of $1.2607 \text{ umolH}_2\text{O}_2 \text{ gFM}^{-1} \text{ min}^{-1}$ at each level of salinity added, resulting in an increase of 76.06% in its activity when the control treatment is compared to the one with the highest salinity (Figure 6b).

Unlike catalase, the enzyme ascorbate peroxidase showed a decreasing linear behavior with a reduction of $-2.730 \text{ umolAsA gFM}^{-1} \text{ min}^{-1}$ as the salinity levels increased, resulting in a reduction of 57.24% in its enzymatic activity, when comparing the salinity levels of 0 and 14 dS m^{-1} (Figure 6F). It was observed that ascorbate peroxidase had a decreasing quadratic behavior, with maximum activity reaching $98.33 \text{ umolAsA gFM}^{-1} \text{ min}^{-1}$ under salinity level of 7.0 dS m^{-1} (Figure 6e).

For the LE-N4 variety, a 30.55% increase in the catalase enzyme in the first cut and a 23.38% reduction in its activity in the second cut under salinity level of 14 dS m^{-1} , when compared with the level of 0 dS m^{-1} , were observed in the present study. Ascorbate peroxidase had reductions of 89.18 and 3.26% in its activity for the first and second cuts, respectively, with increased salinity, when comparing the salinity levels of 0 and 14 dS m^{-1} .

When the activities of catalase and ascorbate peroxidase of the LE-N4 variety were evaluated, a significant behavior could be observed only for the first cut. Catalase showed a progressive linear increase with the increment in salinity levels, while ascorbate peroxidase showed a quadratic behavior, with maximum value of activity under salinity level of 6.07 dS m^{-1} . In the second cut, no significant behaviors were observed for these enzymes in the LE-N4 variety. This result may be associated with the sensitivity of this variety to salt stress, because in the work conducted by Babakhani *et al.* (2011), the varieties that showed higher tolerance to salinity had increments in the activity of ascorbate peroxidase of 3.26 and 4.4 times when compared to the control treatments, which demonstrates that the increase in ascorbate peroxidase helped in the decomposition of hydrogen peroxide (H_2O_2), avoiding its excessive accumulation in the plant.

In addition, the enzymes catalase and ascorbate peroxidase are known as the second line of defense of plants, suppressing the H_2O_2 produced by superoxide dismutase (RUIZ-LOZANO *et al.*, 2003). The reduction of these enzymes contributed for the LE-N4 variety to have a lower biomass production (Figure 2a), demonstrating its low tolerance to increments in salinity levels.

For superoxide dismutase, there were also reductions in activity, equal to 31.25 and 38.58% in the first and second cuts, respectively (Figure 6a, b, e and f). This lack

of synchrony between enzyme activities resulted in a lower yield of shoot dry biomass (Figure 2a), symbolizing greater sensitivity to salinity. According to Sharma *et al.* (2012), the activity of superoxide dismutase results in the retention of $O_2^{\bullet-}$, preventing the oxidation of proteins, amino acids, nucleic acids and carbohydrates, which can cause cell damage, reducing crop yield. The degree of oxidative stress in cells is determined by the amount of superoxide, H_2O_2 and hydroxyl radicals. Therefore, the interaction between the activities of the enzymes superoxide dismutase, ascorbate peroxidase and catalase is extremely important in reducing the toxic levels of ROS in cells (APEL; HIRT, 2004).

The Crioula variety had a 53.14% increase in the activity of the catalase enzyme from the first to the second cut in the comparison between treatments with salinity level equal to 14 dS m^{-1} (Figure 6a, b, and f), showing the highest production of fresh biomass (Figure 3b). For superoxide dismutase, a significant reduction was observed in its activity in the second cut (Figure 6a and b).

When comparing the responses of the activities of the enzyme ascorbate peroxidase between the first and second cuts under salinity level of 14 dSm^{-1} , a reduction of 84.60% was observed. For the DK-192 variety in the first cut, the enzymes catalase and ascorbate peroxidase showed maximum points of their enzymatic activity equal to $16.84 \text{ umolH}_2\text{O}_2 \text{ gFM}^{-1} \text{ min}^{-1}$ under salinity level of 8.7 dS m^{-1} and $94.71 \text{ umolAsA gFM}^{-1} \text{ min}^{-1}$ under salinity level of 5.45 dS m^{-1} , respectively. In the second cut, the enzyme superoxide dismutase had its minimum activity under salinity level of 11.21 dS m^{-1} (Figure 6b and d), coinciding with the highest water use efficiency to compensate for the water deficit experienced due to the increase in salinity levels (Table 1).

According to Shim *et al.* (2003), the reduction of catalase occurs in several plant species when they are under oxidative stress,

which is related to the increase of salicylic acid. Results obtained by Farissi *et al.* (2018) show that salt stress caused a significant increase in H_2O_2 in the roots and leaves of alfalfa. These authors also mention that in salt stress-tolerant varieties, there is an increase in the enzymatic activity of superoxide dismutase, followed by the enzymes catalase and ascorbate peroxidase. The activities of these enzymes were higher in the roots than in the leaves of the plants, due to the sensitivity to salinity and because the root was exposed first to stress.

Chaparzadeh and Mehrnejad (2013) verified significant differences in H_2O_2 accumulation in alfalfa leaves, where there was an increase due to salt stress, consequently causing oxidation and decreased leaf expansion, a fact observed at the highest salinity levels due to the accumulation of toxic ions in older leaves, causing their death (WILLADINO; CAMARA, 2010).

In the present study, the performance of superoxide dismutase for the LE-N4 variety did not vary regardless of the cut. However, the highest biomass production (Figure 2a) coincided with the highest catalase activity (Figure 6c and d) that occurred in the first cycle. Plants that develop higher activity of this enzyme have greater capacity to reduce $O_2^{\bullet-}$, reducing its effects on plant metabolism (BARBOSA *et al.*, 2014).

A study with two varieties of alfalfa reported that the activity of ascorbate peroxidase had a significant increase as salinity increased, up to 4.4 times for one of the varieties when compared to the control (BABAKHANI *et al.*, 2011). The activity of the catalase enzyme was affected in the treatments with salinity, causing a significant increase in its production in both cultivars studied. Variations in the synchronism between enzymes are related to preservation or expansion of production and the response of varieties that are tolerant to salt stress (GUIMARÃES *et al.*, 2020).

The results presented by Benabderrahim *et al.* (2020) indicate that the antioxidant activity of the enzyme superoxide dismutase in alfalfa leaves was not efficient in the process of eliminating peroxide and superoxide radicals created during salt stress. On the other hand, the authors concluded that tolerance to the salts may be associated with the defense mechanism involving the activity of the catalase enzyme. This may justify the increase in the activity of this enzyme in the second cut of this study, coinciding with the highest biomass production (Figure 3), highest water use efficiency (Table 1) and highest chlorophyll content (Figure 5) for the Crioula and DK-192 varieties.

The divergence between the biochemical results of the studied varieties were also found by Al-Farsi *et al.* (2020) in their study with alfalfa varieties. These authors verified that the genetic diversity of alfalfa contributes to behavioral divergence, which may be an important result in breeding programs aimed at salt stress-tolerant varieties. Thus, knowing the process of plant defense against salt stress is essential for the selection of tolerant varieties, since there are few studies on the biochemical behavior of alfalfa plants.

When using enzymatic variables, it is important to observe the relationship between the behavior of enzymes and the production parameters of the studied crops, because plants that show a change in synchrony between their lines of enzymatic defense (Superoxide Dismutase: 1st line of defense and Catalase, Ascorbate Peroxidase: 2nd line of defense), combined with the reduction in their yield, can be considered stress-sensitive plants. On the other hand, varieties that have a synchronism in the behavior of the lines of enzymatic defense combined with increased production can be considered stress-tolerant plants (GUIMARÃES *et al.*, 2020). Therefore, the most sensitive variety to the increase in salinity levels was LE-N4 and the most

tolerant, with the highest production, was DK-192.

6 CONCLUSIONS

The DK-192 variety had higher production of shoot fresh and dry biomass. The LE-N4 variety was the most sensitive to salt stress, showing a significant reduction in production from the salinity level of 7.0 dS m⁻¹.

The increase in catalase enzyme activity for the Crioula and DK-192 varieties under salt stress conditions contributed to higher production of shoot fresh and dry biomass.

Thus, it can be observed that the catalase enzyme has an important function in relation to the salinity tolerance mechanism for alfalfa crop and can be used as an indicator for future studies on genetic improvement of this species, in addition to being used as a parameter for selecting alfalfa varieties that are more tolerant to salt stress.

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