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Effect of salinity stress in *Setaria viridis* (L.) P. Beauv. accession A10.1 during seed germination and plant development

Effeito do estresse salino em *Setaria viridis* (L.) P. Beauv. acesso A10.1 durante a germinação das sementes e desenvolvimento das plantas

Thalita Massaro Malheiros Ferreira[1], Mariana de Lima Santos[1], Cecilia Lima Lopes[3], Carlos Antonio Ferreira de Sousa[2], Manoel Teixeira Souza Junior[1*]

[1]Universidade Federal de Lavras/UFLA, Lavras, MG, Brasil
[2]Embrapa Meio-Norte, Teresina, PI, Brasil
[3]Embrapa Agroenergia, Brasília, DF, Brasil
*Corresponding author: manoel.souza@embrapa.br
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ABSTRACT

Setaria viridis (L.) P. Beauv. is a species proposed to be used as model plant in reverse genetics studies for the validation of gene function. Soil salinity is a recurring problem present in more than a 100 countries worldwide, and approximately 20% of the agricultural land in the world has saline and/or sodium soils. Saline stress affects all the main processes of the plant, such as germination, growth, and, consequently, the yield. The present study aimed at determining the tolerance levels of *S. viridis* A10.1 to saline stress and identify its potential as a model plant to validate salt-tolerance candidate genes/alleles as well as promoter sequences from salt-responsive genes. In an initial experiment, the seeds of the plant were sown on a germination medium containing an increasing concentration of NaCl (0, 30, 60, 90, 120, or 150 mM), and maintained there during the initial growth stage; and, in another experiment, the plants at the vegetative growth stage were submitted to increasing doses of NaCl (0.0, 0.2, 0.4, 0.6, 0.8, and 1.0g per 100g of the substrate). The germination rate was found to be affected a little by the salinity, while the seedlings development was impaired right after germination. Plant in the vegetative growth stage experienced a reduction in the evapotranspiration rates and pigment levels, along with an impairment in the system of capture and use of light, and a decrease in the leaf gas exchange rates, resulting in less accumulation of dry and fresh plant biomass proportional to the salt dose used. Plants started to die within a week at doses ≥19.4 dS/m. In conclusion, A10.1 is a glycophyte plant with some level of salt-tolerance and might be used as a model plant to validate salt-tolerance candidate genes/alleles, as well as promoters salt-responsive genes, depending on the right combination of plant age and level of stress. As seed germination is affected only little by salt stress at NaCl doses of about 15 dS/m or less, A10.1 might not be used to validate genes/alleles with a putative role regarding this trait.

Index terms: Abiotic stress; phenomics; biosaline agriculture; plant biotechnology; salt responsive genes.

RESUMO

Setaria viridis (L.) P. Beauv. é uma espécie indicada para ser usada como planta modelo em estudos de genética reversa visando a validação da função de genes. A salinidade do solo é um problema recorrente, presente em mais de 100 países em todo o mundo, e aproximadamente 20% das terras agrícolas do mundo possuem solos salinos e / ou sódicos. O estresse salino afeta todos os principais processos da planta, como germinação, crescimento e, conseqüentemente, a produtividade. O presente estudo teve como objetivo determinar os níveis de tolerância de *S. viridis* A10.1 ao estresse salino e identificar seu potencial como planta modelo para validar genes / alelos candidatos à tolerância ao sal, bem como sequências promotoras de genes responsivos ao sal. Em um experimento inicial, as sementes foram semeadas em meio de germinação contendo concentração crescente de NaCl (0, 30, 60, 90, 120 ou 150 mM), e mantidas neste durante a fase inicial de crescimento; e, em outro experimento, as plantas em crescimento vegetativo foram cultivadas com doses crescentes de NaCl (0,0, 0,2, 0,4, 0,6, 0,8 e 1,0g por 100g do substrato). A taxa de germinação foi um pouco afetada pela salinidade, enquanto o desenvolvimento das mudas foi prejudicado logo após a germinação. A planta em fase de crescimento vegetativo experimentou uma redução nas taxas de evapotranspiração e níveis de pigmentos, juntamente com um prejuízo no sistema de captura e uso da luz, e uma diminuição nas taxas de trocas gasosas foliares, resultando em menor acúmulo de biomassa fresca e seca, proporcional à dose de sal utilizada. As plantas começaram a morrer em uma semana com doses ≥19,4 dS / m. Em conclusão, A10.1 é uma planta glicófita com algum nível de tolerância ao sal e pode ser usada como planta modelo para validar genes / alelos candidatos à tolerância ao sal, bem como genes promotores responsivos ao sal, dependendo da combinação certa de idade da planta e nível de estresse. Como a germinação da semente é pouco afetada pelo estresse salino em doses de NaCl de cerca de 15 dS / m ou menos, A10.1 não pode ser usado para validar genes / alelos com um papel potencial em relação a essa característica.

Termos para Indexação: Estresse abiótico; fenômica; agricultura biosalina; biotecnologia vegetal; genes responsivos a salinidade.

INTRODUCTION

Soil salinity is a recurring problem in several parts of the world, with a predominance in arid and semi-arid regions (Food and Agricultural Organization of The United Nations, FAO, 2019). More than a hundred countries are affected by the presence of salt in their soils, and the global trend indicates even more areas to be affected with the current climatic changes (Zaman; Shahid; Heng, 2018). Approximately 20% of the agricultural land in the world has saline and/or sodium soils, among which 25% to 30% of the irrigated land is commercially unproductive due to the salinity conditions of the soil (Shahid; Zaman; Heng, 2018). In Brazil, the saline and sodium soils occur in the State of Rio Grande do Sul, in the Pantanal region of the State of Mato Grosso and, with predominance in the semi-arid part of the Northeast Region.

Saline soils, from an agricultural point of view, can be defined as those containing sufficient neutral soluble salts that would negatively affect the growth of most cultivated plants. A priori, soils that have electrical conductivity (CE) of the saturation extract >4 dS/m at 25 °C are considered as saline; however, since many fruits, vegetables, and ornamental species suffer from the adverse effects of salinity in a range of 2 to 4 dS/m, soils with CE> 2 dS/m at 25 °C are also considered to be saline (Bresler; Mc Neal; Carter, 1982).

According to plant response to salt stress, there are two groups of terrestrial plants, namely, glycophytes or halophytes. Approximately 99% are glycophytes, or salt-sensitive includes the majority of the crops. Whereas, halophytes account for only less than 1% of terrestrial plant species and can complete their life cycle in an environment with a salt concentration equal to or higher than 200 mM NaCl - approximately 20 dS/m (Flowers; Colmer, 2008).

Salts have a direct or indirect, slow or abrupt, and total or partial effects on the development and production of plants. These effects are related to the responses of plants, both in physiology and metabolism, being observed at all levels of development, causing reduction or loss of productivity (Ribeiro; Barros; Freire, 2009). Saline stress affects all the main processes of the plant, such as germination, growth, levels of photosynthetic pigments, photosynthesis rate, water absorption, nutrient homeostasis, and consequently the yield (Parihar et al., 2015).

The inhibition of growth and plant production is due to the reduction in the osmotic potential of the soil solution, caused by an excess of salts and their toxic effects (Silva et al., 2000). In the initial phase of salt stress, known as the osmotic phase, the water absorption by the root decreases (Munns, 2002), and physiological changes such as nutrient imbalance, decreased ability to detoxify reactive oxygen species (ROS), decreased photosynthetic activity, and stomatal opening also takes place (Munns; Tester, 2008). In the later phase, saline stress triggers ionic stress, one of the most harmful effects, as it generates an accumulation of Na^+ and Cl^- ions in the tissues of plants grown in soils with high concentrations of NaCl (James et al., 2011).

Due to its importance, salinity has been the focus of the studies that aim to prospect, validate and use the genes/alleles that confer tolerance to that abiotic stress in the long term (Udawat et al., 2016). The validation of salt-tolerance candidate genes/alleles, as well as promoters from genes responsive to salt stress, usually occurs through the use of a reverse genetic strategy in the model plants.

Parihar et al. (2015), Munns and Tester (2008), among others, highlighted the existence of three model plants that are well known and used in the scientific community, aiming mainly at the studies related to abiotic stresses: *Arabidopsis thaliana* (L.) Heynh., *Oryza sativa* L. and *Solanum lycopersicum* L. Specifically, in most experiments rice, tobacco, and Arabidopsis have been used to study salt stress using the genomic approach (Flowers, 2004). All of these plants share the same C3 photosynthetic mechanism among them.

Setaria viridis (L.) P. Beauv. (green foxtail millet) belongs to the order Cyperales, the genus *Setaria,* and the family Poaceae (Gramineae). It is a diploid plant ($2n = 2x = 18$), with a relatively smaller genome, reaching approximately 510 Mb. The species has many characteristics of genetic interest such as short stature, short life cycle, abundant seed production, the existence of protocols for tissue culture, and genetic transformation (Brutnell et al., 2010). *S. viridis* is a rapidly emerging model plant for the study of gene validation for C4 plants (Brutnell et al., 2010; Li; Brutnell, 2011; Brutnell; Bennetzen; Vogel, 2015; Martins et al., 2019).

Among the glycophytes plants, most species are sensitive to salt, but there are also some highly tolerant species (Borsai et al., 2018). Almost all the crops of agricultural importance are glycophytes and highly sensitive to salt, whereas there are just a few halophyte species that are already domesticated and used on a large scale in agriculture. Sugarcane and maize, two of the most economically important energy crops with approximately 25 million hectares harvested in 2019 in Brazil are C4 glycophytes and are sensitive to salt. These species are also potential candidates for a future horizontal transfer of salt tolerance genes/alleles validated previously in a C4 model plant such as Setaria.

However, studies evaluating the responses of *S. viridis* accessions to abiotic stresses are still scarce (Huang et al., 2016). For instance, the reports on which of them are salt-tolerant and which are not are still unknown. This work was carried out to determine the tolerance levels of *S. viridis* A10.1 to saline stress and identify its potential as a model plant to validate salt-tolerance candidate genes/alleles as well as promoter sequences from salt-responsive genes.

MATERIAL AND METHODS

Seed germination and initial seedling growth under salt stress

All experiments were carried out at the Genetics and Biotechnology Laboratory - Embrapa Agroenergia, in Brasília, DF, Brazil (S-15,732º, W-47,900º).

The seeds of *S. viridis* A10.1 were initially submitted for chemical scarification in sulfuric acid PA (1mM) for 15 minutes, with manual shaking for 5 min, to break the dormancy. After the scarification, the seeds were removed from the acid and washed three times in deionized water and were further processed in a fume hood, where they were disinfected in a solution of 2% sodium hypochlorite plus two drops of Tween20® for 5 min, under continuous agitation. At the end of the process, the seeds were removed from the disinfection solution and washed thoroughly with autoclaved deionized water until the resulting foam and the characteristic hypochlorite odor dissipated; then were further dried on a sterile filter paper.

Increasing amounts of NaCl were added to a standardized volume of the germination medium [MS 1/2 strength, pH 5.8, supplemented with 1 mL/L of vitamins, 20 g/L sucrose, 2.0 g/L Phytagel®, and 100 mg/L inositol (Murashige; Skoog, 1962)] to obtain the salt concentrations of 0, 30, 60, 90, 120, and 150mM NaCl. After autoclaving for 20 min at 121 ºC, 1 atm, and cooled to 25 ºC, the electric conductivity (EC) was measured at each of the saline concentrations.

For the germination and initial growth, 20 seeds per plate and four plates per treatment were placed. After they were seeded on the sterile germination medium, they were kept for nine days in a Conviron® growth chamber model Adaptis 1000TC (Controlled Environments Inc., Winnipeg, Canada) under a 16/8 hour photoperiod (light/dark), temperature 25 ±2 ºC with an intensity of 150 µmol/m²/s. Seven days after the sowing, the number of seeds that were germinated per plate was counted. In case the shoot and root systems were visible, the seed was considered germinated. At nine days after sowing, the leaf area and the variables related to the seedling root system morphology were also measured. This experiment was repeated four times, by two different persons.

The leaf area was determined by the chlorophyll fluorescence imaging technique, using a fluorometer model, IMAGING-PAM version Maxi from Walz (Heinz Walz GmbH, Effeltrich, Germany). For this, the seedlings were initially kept in the dark for 30 min and then subjected to a routine that firstly measures the initial fluorescence (Fo), with the lowest possible light intensity, and the maximum fluorescence (Fm), from a pulse of saturating light (2800 µmol/m²/s). The Imaging Win software, which controls the equipment, allows the determination of the area that generates a fluorescence signal from the image of any parameter. For *S. viridis* seedlings, the leaf area was estimated from the area of maximum fluorescence signal emission (Fm). This calculation of the area is independent of the chlorophyll concentration and the intensity of the fluorescence signal.

The variables related to the morphology of the root system were determined using the Regent WinRHIZO v.4.0 (Regent Systems, Quebec, Canada), coupled with a professional Epson XL10000 scanner, and equipped with an additional light unit (TPU). The seedlings were placed in an acrylic vat having a 20 x 30cm length, containing a water film. The images thus generated were evaluated in two plants of each plate, and the following variables were eventually determined: total length of roots (cm), total projected area (cm²), total surface area (cm²), the average diameter (mm), length by volume (cm/m³), and the root volume (cm³).

Submission of plants at the vegetative stage to salt stress

To obtain plants at the vegetative stage, seeds of *S. viridis* A10.1 were submitted for breakage of dormancy, disinfection, and germination using the same protocol as listed previously; however, no NaCl was added to the germination medium. Seven days after sowing, the seedlings were transplanted individually to the pots (0.2 L) containing 100g of the substrate and transferred to a Conviron® growth chamber model PGW40 (Controlled Environments, Winnipeg, Canada) under a 16/8 hr photoperiod (light/dark), temperature 25 ±2 ºC, 60% relative humidity and a light intensity of 500 µmol/m²/s.

When plants reached the second stage of development (vegetative phase), 14 days after sowing, and 7 days after transplanting (Martins et al., 2015), they were subjected to salinity stress. We used the doses of 0, 2, 4, 6, 8, and 10 g NaCl/100g of the substrate, in five replicates per treatment.

The used substrate consisted of soil, a commercial substrate (Bioplant®), and vermiculite, in a 2:1:1 ratio (by volume). Before mixing, all the three substrate constituents were sterilized at 121 °C and 1 atm for 30 min. Diluted saline solutions were used to apply NaCl evenly to the surface of the substrate. The amount of water to dissolve the NaCl was standardized to achieve the field capacity of the substrate, which was previously determined.

We applied such a procedure to ensure that there was no leakage of the solution and, therefore, loss of Na^+ or Cl^-. The daily maintenance of the water content was performed by replacing evapotranspirated water using deionized water up to the limit of the field capacity. The plants remained under the salt stress for 12 days. Eight days after applying the salt to the substrate, two plants from each treatment were collected to determine their weight, which was added to the final weight of the pots, to correct the calculation of the daily amount of water needed to make the substrate return at 100% of field capacity.

Twelve days after applying the saline solution, the substrate contained in each plastic vessel was sucked, with the aid of a Büchi® model V-700 vacuum pump (Sigma-Aldrich, Missouri, United States), to collect the samples of aqueous extract. We determined the EC and the water potential (Ψw) of these samples using a Hanna conductivity meter model, HI98311 (Hanna Instruments, Rhode Island, USA), and a Decagon water potential meter model WP4C (Decagon Devices, Pullman, USA), respectively.

The evaluation of plants under the salt stress was performed by measuring gas exchange, using the ADC infrared gas analyzer model LCpro-SD (ADC Bioscientific, Hoddesdon, United Kingdom). Photosynthetically active radiation was set to 1,500 µmol/m^2/s, the CO_2 inside the chamber was maintained at around 400 µmol CO_2 mol^{-1}, and the temperature was set at 25 ºC. The following variables were obtained: *A* - Net CO_2 assimilation rate (µmol CO_2 m^{-2}/s); *gs* - stomatal conductance rate to water vapor (mol of H_2O m^{-2}/s); *E* - transpiration (mmol of H_2O m^{-2}/s); and *Ci* - intracellular CO_2 concentration (µmol CO_2 mol^{-1}). The evaluations were carried out on the youngest leaf, completely expanded. The measurements took place 12 days after applying the saline solution.

We applied the chlorophyll fluorescence technique to evaluate the plants - Saturation Pulse Method, using an image fluorimeter model IMAGING-PAM Maxi from Walz (Heinz Walz GmbH, Effeltrich, Germany), powered by the ImaginWin software version 2.40b. An induction curve approach was used. The IMAG-MAX/L LED lighting head and an IMAGMAX/K4 CCD camera were mounted on a 15 mm diameter metal bar on the optional support so that the measurement sensor was at a standard distance of 18.5 cm for all the plants. The following settings were used: measurement light = 1; saturation pulse = 10 (2800µmol/m^2/s); gain = 1; damping = 2; red gain = 25; red intensity = 4; NIR intensity = 7; factor Fm = 1.055; factor F = 0.999; actinic light = 9. The induction curve was 315 s long and started with the measurement of Fo and Fm, with the actinic light switched on after 40 sec, applying a saturation pulse after every 20 sec. The measurements were performed on plants kept in the dark for 30 min, on the same leaf used for gas exchange evaluations. The evaluated parameters were: Fm; Fo; Y(II); Fv/Fm; Y(NPQ); Y(NO). After measuring the initial parameters, all the derived ones were calculated by the software. For this, an area of interest (AOI) that did not include the central rib was marked. The measurements were taken daily for 12 consecutive days.

A Resonnon Pika XC hyperspectral camera (Resonon, Bozeman, MT, USA), which was activated by the Spectronon software version 2.1 to scan the leaves, was applied to measure the pigments. The system was composed of the hyperspectral camera, with a linear translation phase and a fixed lighting system in the assembly tower. Hyperspectral images were generated, maintaining the same distance for all the plants, and the settings used were made according to the recommendations in the manual. Five regions of interest for the average reflectance spectrum were marked on each plant. The calculated parameters were: chlorophyll index [CI = $(R_{660}-R_{930})$ x R_{930}] (Gitelson *et al.,* 2005), photochemical reflectance index [PRI = $(R_{531}-R_{570})/(R_{531}+R_{570})$] (Gamon *et al.,* 1992), carotenoid index [CRI = $(R_{510})-(R_{550})xR_{800}$] (Gitelson *et al.,* 2002). The measurements were taken daily for 12 consecutive days.

The plant aerial parts were harvested 12 days after beginning the stress and were weighed to determine the fresh weight. Then, they were placed in an oven at 65 ºC, with forced ventilation, until a constant weight was obtained, and then the dry mass was determined.

Experimental design and data analysis

A completely randomized design was used in all the experiments. Before proceeding with the statistical analysis, we verified that whether or not the data presented the homogeneity of variance, and error independence. Once these requirements were satisfied, we subjected the data to an analysis of variance according to Snedecor and Cochran (1967). After the analysis of variance, the regression equations were adjusted to the data whose

means were significantly different, using the SISVAR software (Ferreira, 2011).

RESULTS AND DISCUSSION

Effects of the saline stress on seed germination and the early growth of the Setaria viridis seedlings

As the saline concentration in the germination medium was increased, the EC value was also observed to rise, thereby, reaching 14.7326 dS/m at a concentration of 150 mM NaCl (Figure 1A). The increase in EC led to a linear decrease in the seed germination percentage, which dropped from 100% in the control treatment to around 70% in the highest NaCl concentration (Figure 1B). Guo et al. (2011) reported that the saline stress began to prevent the seed emergence to a significant extent already at 8.3 dS/m, dropping the germination rate to less than 40%. Although there are some similarities between this present study and their study (such as the temperature, EC range, and time applied for germination), Guo and colleagues did not report the specific accession of green foxtail millet that was employed in their study; making it difficult to explain this difference in the results.

These results showed that the germination of seeds from the A10.1 accession would not be significantly affected in the soils with the higher levels of salinity than the minimum threshold level that is considered to be saline (CE > 2 dS/m at 25 °C) (Bresler; Mc Neal; Carter, 1982). It was not possible to use the salt doses equivalents to the ones latter used to input stress in the plants at their vegetative stage (14 days after sowing) because at a concentration higher than 150 mM NaCl, the medium did not polymerize after being sterilized in the autoclave (data not shown).

The most drastic effect occurred at the early stages of the seedlings development, where from 90 mM NaCl up, the reduction in the leaf area was approximately 90% in comparison to that of the control plants (Figure 1C). Visually, it was possible to observe the differences in the leaf area either by digital (Figure 2A) or by chlorophyll fluorescence (Figure 2B) images.

As the salt concentration in the germination medium increased, it resulted in a drastic reduction in the size of the root system (Figure 3). The variables most affected by salinity stress were volume, length by volume, total length, and total projected area with a drop of 99%, 96%, 91%, and 88%, respectively; while the least affected variables were the average diameter and the total surface area, that dropped by 27% and 78%, respectively (Figure 4).

When applied to the sensitive plant species, the salinity stress generally slows down and reduces the seed germination, in addition to restricting the seedling growth (Chartzoulakis; Loupassaki, 1997; Guo et al., 2012). At first, the effect of salts on the soil resulted in the reduction of its water potential, which interfered with the absorption of the water by the seed (Pereira et al., 2012). Subsequently, the entry of solutes can lead to ionic toxicity, changes in the composition of lipids, and interference with the functioning of the plasma membrane, affecting, for example, permeability and transport (Bliss; Platt-Aloia; Thomson, 1984; Cordeiro et al., 2014).

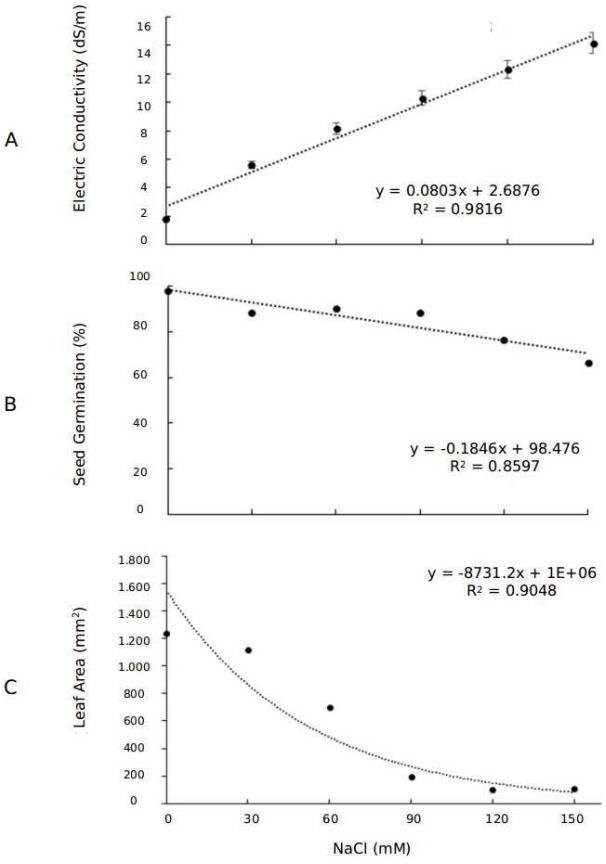

Figure 1: The electric conductivity in the germination medium, (A) the seed germination percentage, (B) and the leaf area of *Setaria viridis* A10.1 seedlings, (C) as a function of the NaCl concentration in the germination medium at the ninth day after sowing. The values represent an average of three, (A) sixteen (four per experiment), (B), and four (C) measurements. The bars in A indicate the standard error of the mean.

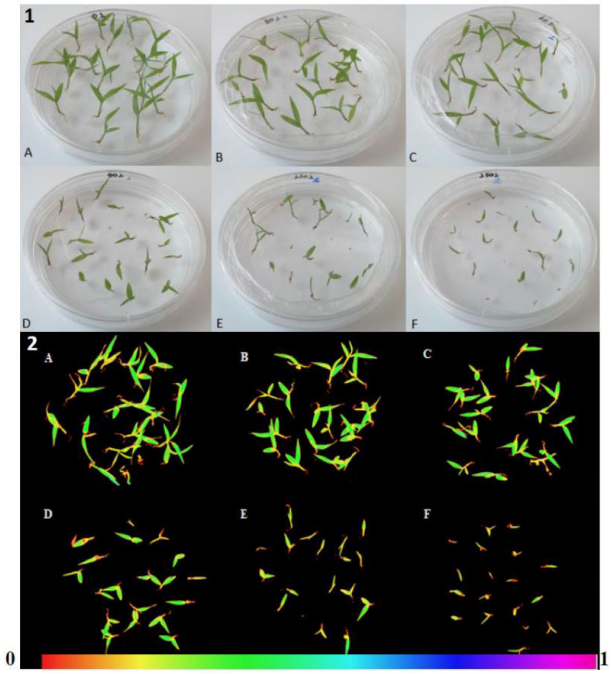

Figure 2: General view (RGB Image) of *Setaria viridis* A10.1 seedlings nine days after sowing, as a function of the NaCl in the germination médium, (1) and derived from the chlorophyll fluorescence technique (saturation pulse method) (2). The data values for each plant in the image correspond to the maximum fluorescence parameter (Fm) and mapped with the help of the false-color bar located below. NaCl concentration: (A) 0 mM, (B) 30 mM, (C) 60 mM, (D) 90 mM, (E) 120 mM, and (F) 150 mM.

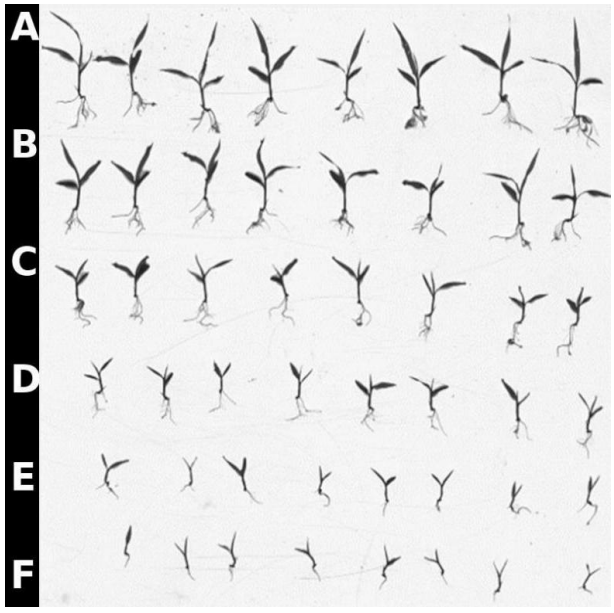

Figure 3: General view of *Setaria viridis* A10.1 seedlings nine days after sowing, as a function of the NaCl in the germination medium. Image obtained with the Regent WinRHIZO v.4.0 (Regent Systems, Quebec, Canada), coupled with a professional Epson XL10000 scanner, equipped with an additional light unit (TPU). NaCl concentration: (A) 0 mM, (B) 30 mM, (C) 60 mM, (D) 90 mM, (E) 120 mM, and (F) 150 mM.

Setaria viridis accession A10.1 showed a considerable high germination rate under saline conditions (Figure 1B). Salinity does not affect the seed germination to a greater extent in some salt-sensitive species (Flowers, 2004); for instance, some salt-sensitive accessions of Arabidopsis can be germinated on the germination substrate with a saline concentration up to 125 mM (Galpaz; Reymond, 2010). In experiments carried out with the plant species of the Poaceae family, of which *S. viridis* is a part of, the results were not different. Rice grass seeds, for example, when submitted to the increasing salt concentrations, showed a germination rate above 80%, even at 150 mM NaCl concentration. In this species, germination was completely inhibited in only 400 mM NaCl concentration (Sadeghloo; Asghari; Ghaderi-Far, 2013). In corn, the experiments were carried out with the concentrations ranging up to 200 mM of NaCl and KCl, and the results showed that the salts did not directly affect the seed germination (Conus et al., 2009).

Muscolo, Panuccio and Eshel (2013) stated that most of the crops have a greater intolerance to salinity in the initial post-germination growth as compared to the others. Some studies show that even species that present high germination in a saline environment, in general, does suffer a reduction in the leaf area, number of leaves, height of the seedlings, and the biomass of the aerial parts (Chartzoulakis; Loupas-saki, 1997; Cavalcanti et al., 2005a, 2005b; Conus et al., 2009). Therefore, the percentage of seed germination cannot be considered as a good indicator of tolerance to salt stress in the plants (Cavalcanti et al., 2005a, 2005b; Conus et al., 2009).

For instance, in this study, although *Setaria* seeds have a reasonable germination rate under salt stress, the initial seedling development in the case of the roots and the shoots, was found to be highly impaired. Galpaz and Reymond (2010) state that the response to the saline stress can be better quantified using the percentage of reduction in the length of the roots, concerning the control. In this study, both roots and shoots of *Setaria* were drastically affected by salt stress. In conclusion, the findings of this study suggest that this *Setaria* accession does not indicate to be a viable

model plant for the validation of candidate genes/alleles playing a role in the improvement of germination rates under salinity stress. Instead, it would be of great use for the validation of candidate genes/alleles having a role in conferring tolerance at early developmental stages.

Morphophysiological responses of S. viridis accession A10.1 plants to saline stress

The increase in the levels of NaCl concentration in the substrate led to a proportional rise in the EC and a reduction in the water potential of the saturation extract (Figure 5). Doses of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 g NaCl per 100g of substrate resulted in an EC of 3, 9, 14, 20, 25, and 31 dS/m, respectively as measured on the day 12 of the stress-induced conditions. The treatment group that did not receive any additional NaCl (represented by the saline level of 0.0 g NaCl) showed an EC greater than zero. This could be explained due to the ionic effect of the salts present in the fertilizers used.

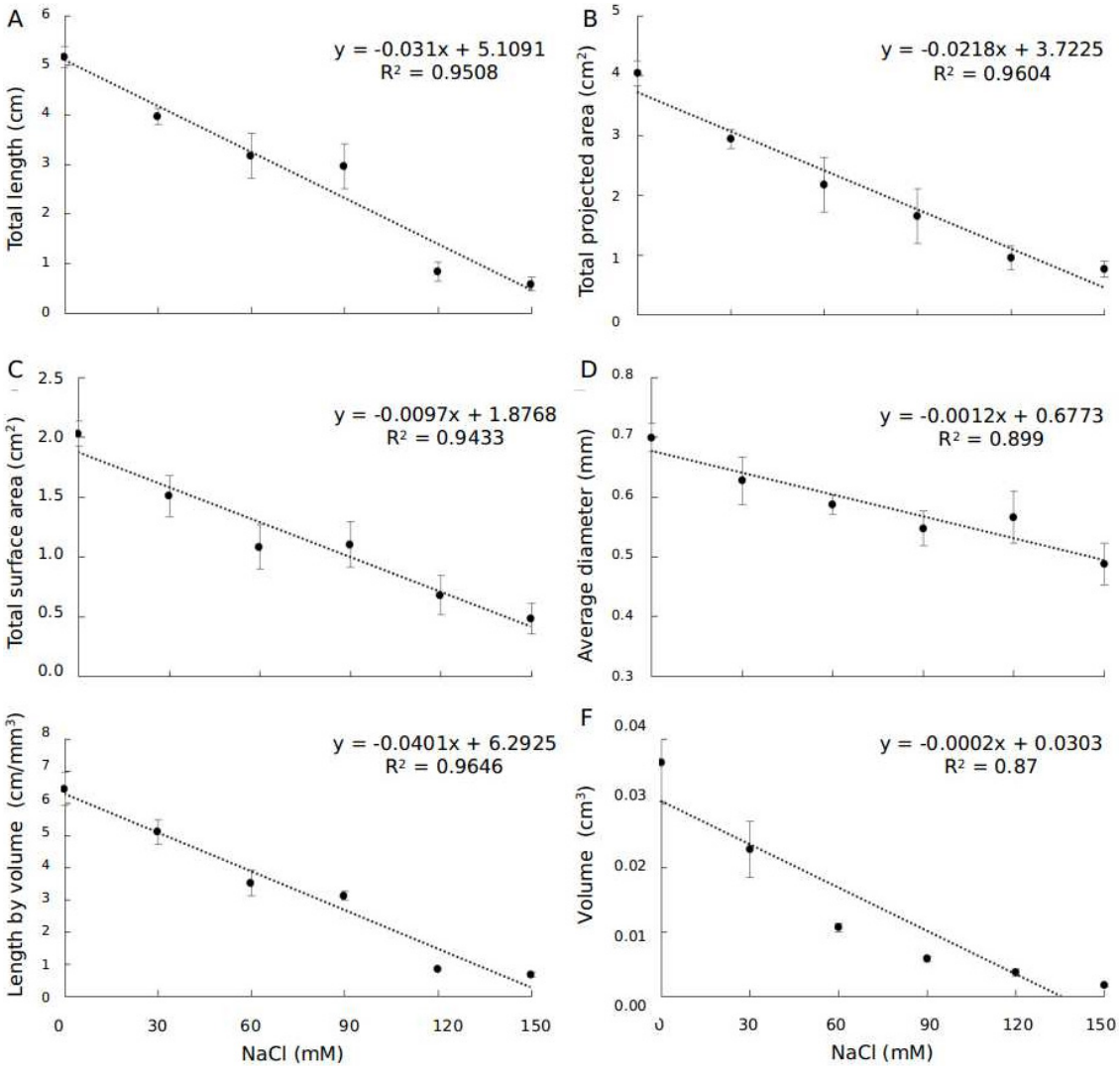

Figure 4: The morphological variables of *Setaria viridis* A10.1 seedlings root system, as a function of the NaCl concentration in the germination medium at the ninth after sowing. Variables: (A) total length of roots (cm), (B) total projected area (cm^2), (C) total surface area (cm^2), (D) the average diameter (mm), (E) length by volume (cm/m^3), and (F) root volume (cm^3). The values represent an average of eight seedlings. The bars indicate the standard error of the mean. Where the error bars do not appear, it is because they are smaller than the symbols.

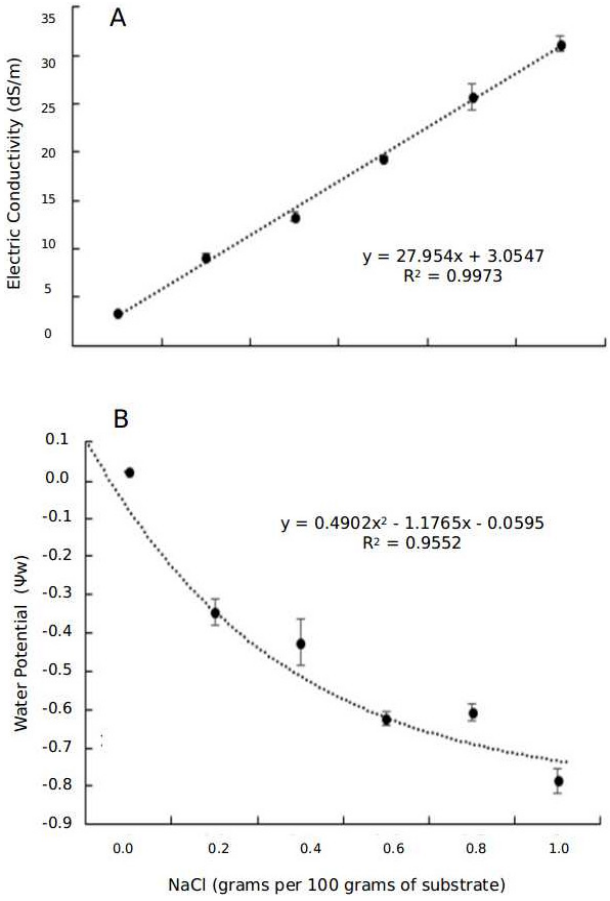

Figure 5: The electric conductivity (A) and the water potential (B) of the cultivation substrate where *Setaria viridis* A10.1 plants - on the vegetative stage - were cultivated. Measurements made on the 12th day after the onset of the salinity stress, and the values represent an average of five plants. The bars indicate the standard error of the mean. Where the error bars do not appear, it is because they are smaller than the symbols.

Due to the robustness of the salinization protocol used where the total control of the amount of salt and water was available in each treatment, the evaporation and evapotranspiration rates were able to efficiently discriminate the salt levels in the substrate used (Figure 6). Thus, the highest rates of evaporation and evapotranspiration occurred in the control treatment, while, on increasing the doses of NaCl in the substrate, both the rates decreased proportionally. With the plant growth over time, the differences in the evapotranspiration rates among the different treatment groups became more pronounced (Figure 6B).

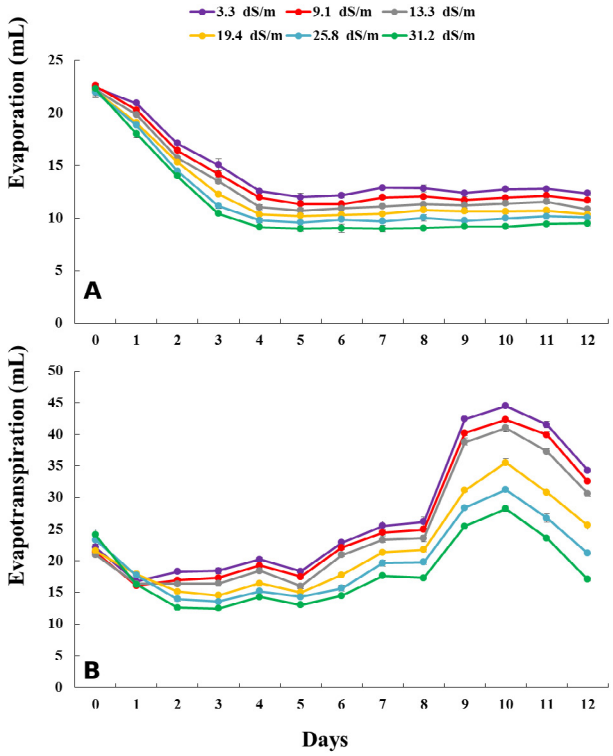

Figure 6: The daily rate of water loss as a function of the electric conductivity, EC (dS/m) in the cultivation substrate. Water evaporation rate of the substrate; the values represent an average of three replicates. Plant evapotranspiration rate; the values represent an average of five seedlings. The bars indicate the standard error of the mean. Where the error bars do not appear, it is because they are smaller than the symbols.

In the control plants, the chlorophyll fluorescence variables relatively remained stable throughout the evaluation period (Figure 7). However, a general increase was observed in the values of initial fluorescence in the dark (Fo), and in the quantum yield of regulated [Y(NPQ)] and unregulated [Y(NO)] dissipation of light energy; as more pronounced the treatment was, the higher it increased. In general, it was observed that only at a value equal or higher than 19.4 dS/m, the leaves began to show problems in the apparatus for light capture and its use. Also, there was a reduction in the values of maximum fluorescence in the dark (Fm), the effective quantum yield of photosystem II [Y(II)], and the maximum quantum yield of photosystem II (Fv/Fm). Such reduced values accentuated throughout the stress period, specifically, up to day 5 of the stress-induced conditions, mainly at EC value equal or higher than 19.4 dS/m. At the dose value of 25.8 dS/m or higher, 100% of the plants were observed to be dead by the end of the experiment (Figure 8).

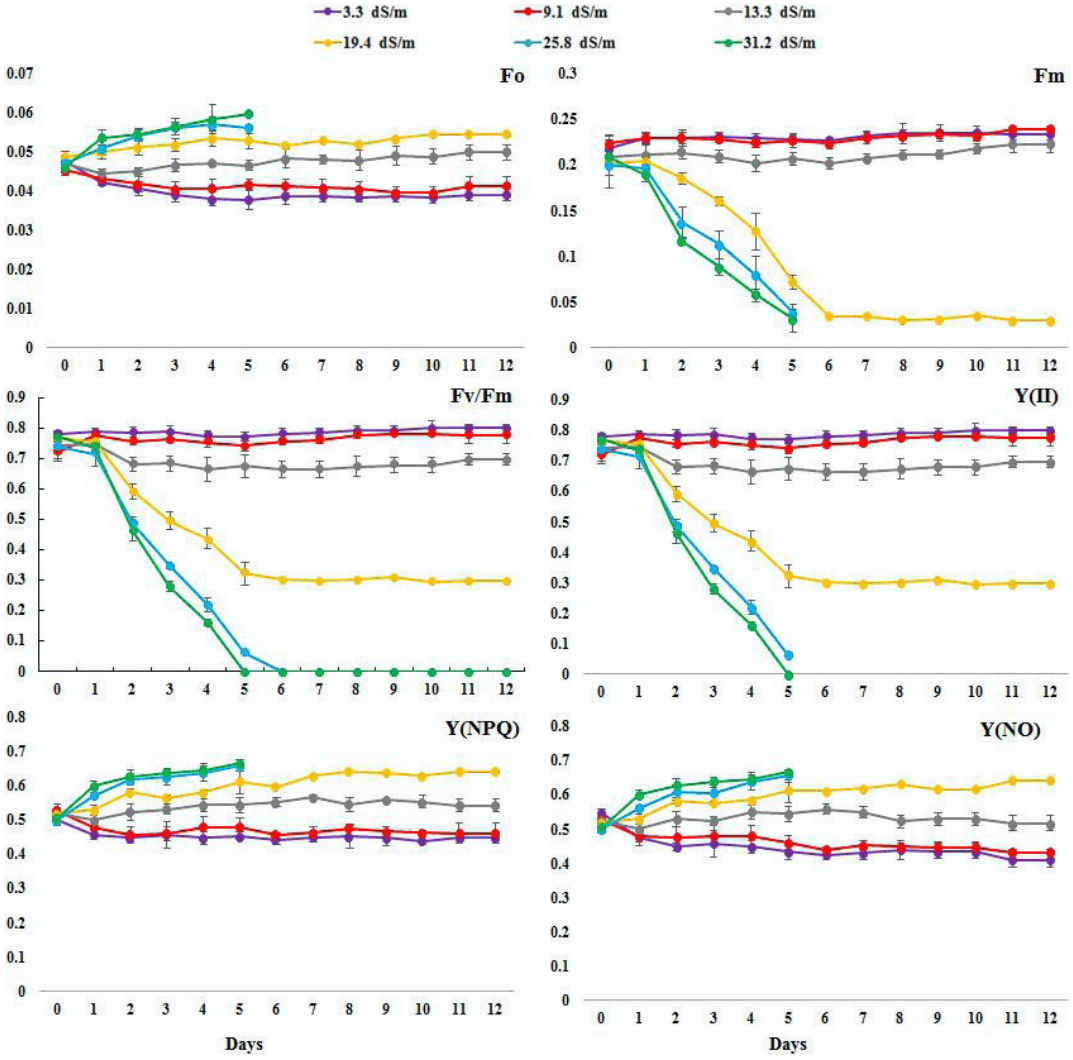

Figure 7: Chlorophyll fluorescence technique (saturation pulse method) variables collected daily from *Setaria viridis* A10.1 plants, as a function of the electric conductivity (dS/m) in the cultivation substrate. Measurements were made daily for 12 days. Variables: Fo - minimum fluorescence yield on dark-adapted leaves; Fm - maximum fluorescence yield on the dark-adapted leaf; Fv/Fm - maximum quantum yield of photosystem II; Y(II) - effective quantum yield of photosystem II; Y(NPQ) - quantum yield of regulated energy dissipation; and Y(NO) - quantum yield of nonregulated energy dissipation. The values represent the average of five plants. Plants cultivated in a substrate with electric conductivity (EC) of 25.8 and 31.2 dS/m died between the 5th and 6th days after the onset of the salinity stress; therefore, data from these plants were not collected from the 6th day.

The chlorophyll index (CI), the carotenoid reflectance index (CRI), and the photochemical reflectance index (PRI) were found to be efficient in discriminating the plants based on the different salt doses applied to their cultivation substrate (Figure 9). No differences were observed practically among the variables before the application of saline stress (day 0). However, a drop was observed in the CI, CRI, and PRI values as a function of the salt doses added to the substrate. A downward trend was observed in the CI, CRI, and PRI values as the three highest doses levels of NaCl were used. These indices reached their lowest around the 5th and the 7th day, staying on this stable plateau thereafter. The other NaCl doses used did not show any change in the CI throughout the entire duration of the experiment, while an upward trend was observed in their CRI and PRI values before reaching a stable plateau.

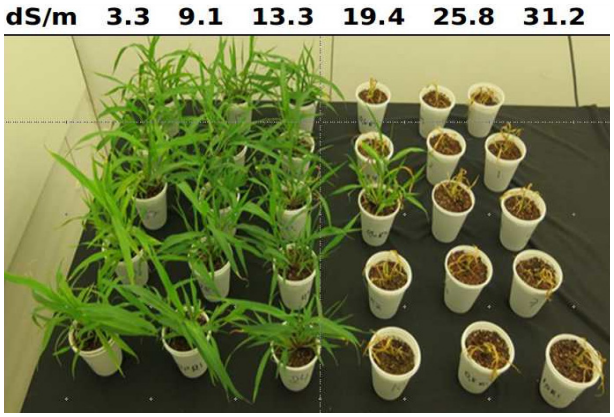

Figure 8: General view of *Setaria viridis* A10.1 plants - on the vegetative stage - as a function of the electric conductivity, EC (dS/m) in the cultivation substrate. The RGB image was taken on the 12th day after the onset of the salinity stress.

Also, a linear drop was observed in the accumulation of fresh and dry biomass in the aerial part of the *Setaria* plants as the NaCl doses increased proportionally (Figure 10). The fresh and dry biomass values from plants subjected to NaCl doses equal to or higher than 19.4 dS/m were mostly from the dead plants. Whereas, the fresh and dry biomass values from plants subjected at doses of 9.1 and 13.3 dS/m doses were from alive plants. There were no visual differences observed between the control and the stressed plants (at 9.1 and 13.3 dS/m doses) in terms of leaf color. However, the amount of biomass was reduced by 22% and 38% in these doses, respectively.

The drop in all the four gas exchange variables measured was increased as the salt level got higher (Figure 11). The drop in the leaf gas exchange rates is a usual response to salt, and its intensity depends on the plant tolerance levels to the salinity stress (Parihar et al., 2015). The drop in gas exchange rates for *Setaria* plants seems to be due to stomatal closure, as CO_2 assimilation, stomatal conductance, and transpiration rates decreased in the same proportion (Figure 11A, B, and D).

The water available to the plants is directly related to the salts dissolved in it, which increases their EC and decreases their osmotic potential and, consequently, hinders and reduces their absorption (Parihar et al., 2015). In this respect, the osmotic effect of the salt was striking in both the substrate and the substrate grown with the plants, because the higher the salt concentration, the lower is the evaporation (Figure 6A) and evapotranspiration (Figure 6B) rates, respectively. The reduction in the evapotranspiration rates was basically due to the drop in the gas exchange rates as a whole (Figure 11), especially in the stomatal conductance and transpiration rates.

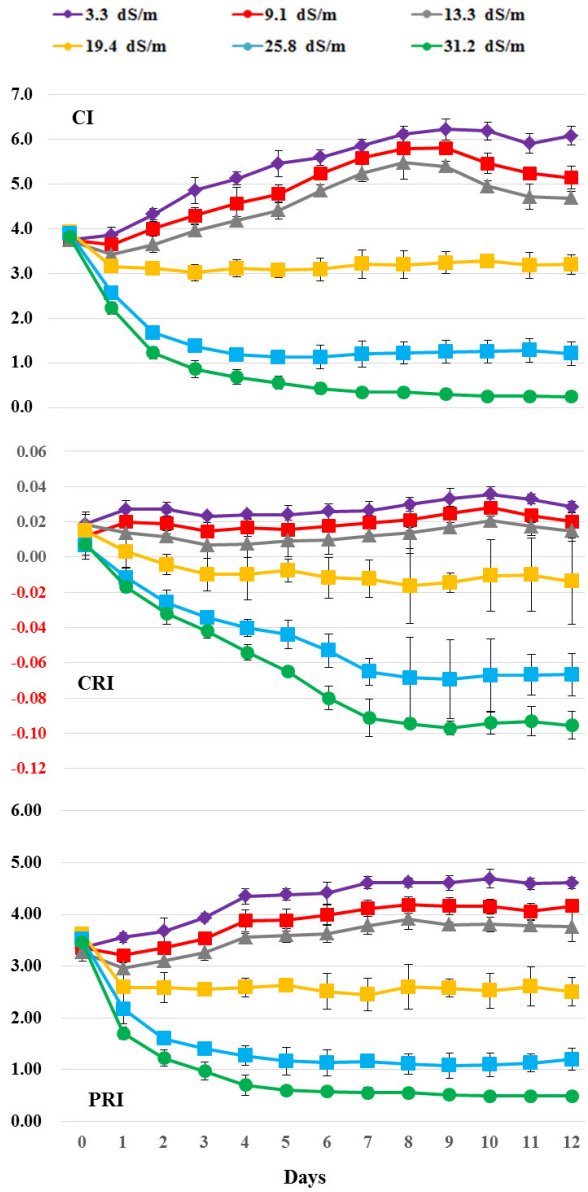

Figure 9: The chlorophyll index (CI), the carotenoid reflectance index (CRI), and photochemical reflectance index (PRI) values from *Setaria viridis* A10.1 plants - on the vegetative stage - as a function of the electric conductivity (dS/m) in the cultivation substrate. Measurements were made daily for 12 days, and the values represent an average of five plants. The bars represent the standard error of the mean. Where the bars do not appear, it is because they are smaller than the symbols.

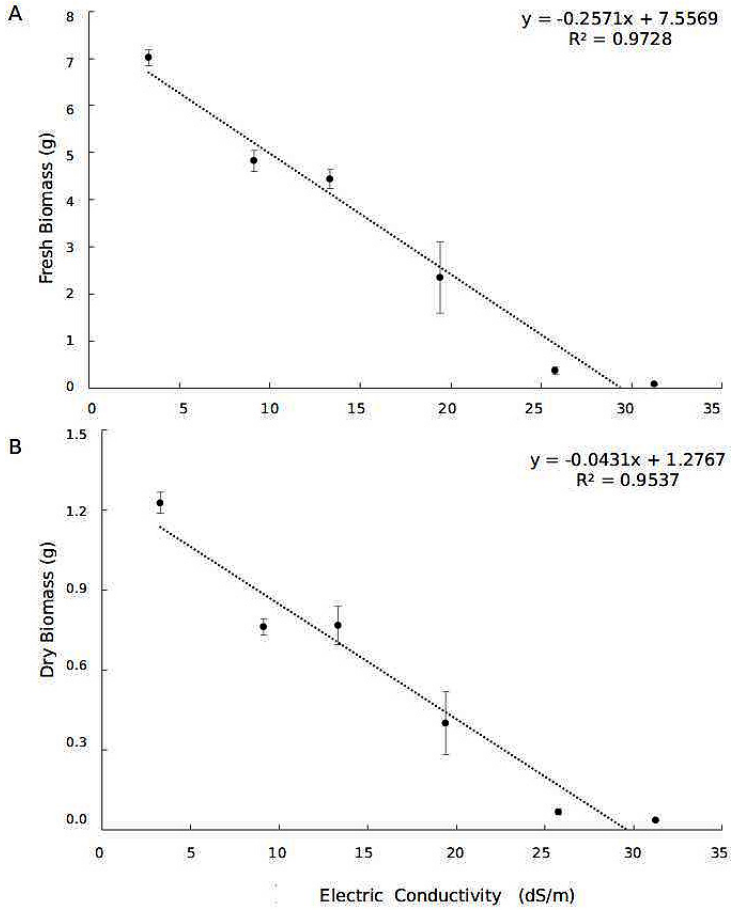

Figure 10: Fresh biomass (A) and Dry biomass (B) as a function of the electric conductivity (dS/m) in the cultivation substrate. Measurements made at the end of the experiment, after cultivating the *Setaria viridis* A10.1 plants - on the vegetative stage - for 12 days on salinity stress. The values represent an average of five plants. The bars represent the standard error of the mean. Where the bars do not appear, it is because they are smaller than the symbols.

Studies with the electron microscopy have shown that, under salt stress, there is disorganization in the thylakoid structure (Parida; Das, 2005). In this study, the presence of NaCl in the substrate, starting at approximately 13.3 dS/m, caused damage to the chloroplast membrane system and, consequently, to the reaction centers and the capture antenna complexes of light from photosystem II (Figure 7). Such inference was observed due to both the increase in the Fo and the reduction in the Fm and Fv/Fm values. Consequently, there was a drop in the electron flow in the Z scheme of photosynthesis, which was proportional to the salt concentration in the substrate. The light energy that is not used to flow the electrons in the Z scheme goes directly for the generation of heat, and the remaining part is re-emitted as fluorescence. The increase of this last variable under salt stress means that the photochemical apparatus was not able to dissipate light energy in a regulated manner and, therefore, may have collapsed, contributing to the death of the plants.

Salinity affects the chlorophyll content of the plants (Atlassi Pak; Nabipour; Meskarbashee, 2009). In the present study, the drop in the levels of leaf pigments in *Setaria* plants (represented by CI and CRI in Figure 8) is a recurrent result in the literature for several plant species that are susceptible to salinity (Saleh, 2012; Shah; Houborg; Mccabe, 2017). A similar drop appears in *Oryza sativa* when it is subjected to salt stress. In this species, it was observed that there was a decrease in the content of chlorophyll a (33%) and b (41%), with 200 mM NaCl concentration (Amirjani, 2012). In *Vigna radiata*, 150 mM NaCl concentration caused a 31% decline in the chlorophyll levels (Saha; Chatterjee; Biswas, 2010).

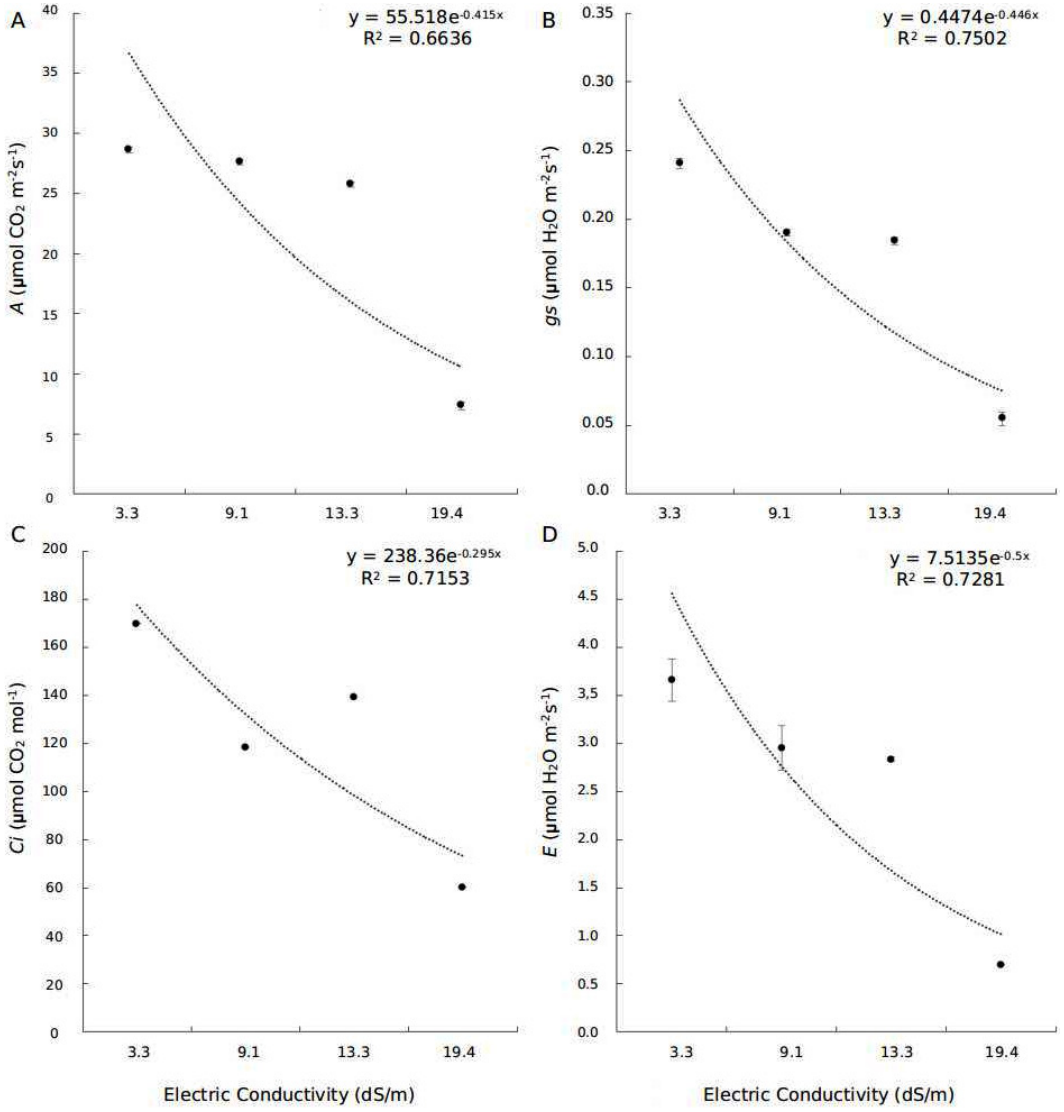

Figure 11: Gas exchange rates in leaves of *Setaria viridis* A10.1 plants - on the vegetative stage - as a function of the electric conductivity (dS/m) in the cultivation substrate. (A) A - Net assimilation rate of CO_2; (B) gs - Stomatal conductance; (C) Ci - Intercellular CO_2 concentration; and (D) E - Transpiration rate. Measurements made at the end of the experiment, after cultivating the plants for 12 days on salinity stress, and the values represent an average of five plants. The bars represent the standard error of the mean. Where the bars do not appear, it is because they are smaller than the symbols.

It is interesting to note that at the end of the stress period, the drop in the PRI was proportional to the salt stress (Figure 9), which indicated that probably the xanthophyll cycle was functioning in the plants at all the studied salt doses. The activation of the xanthophyll cycle is a response observed in some halophyte species under salt stress (Qiu; Lu; Lu, 2003; Rabhi et al., 2012) and is related to the protection of the photosynthetic apparatus against the photoinhibition damage (Qiu; Lu; Lu, 2003). In the case of *S. viridis* A10.1, the functioning of the xanthophyll cycle was not sufficient to allow the flow of electrons that is not used for photosynthesis to flow. Otherwise, there would not have been an increase in the fluorescence emission at the highest doses of NaCl, as can be seen with the increase in Y(NO) (Figure 7).

The effect of the salt stress on the accumulation of biomass in the plants was most likely related to the osmotic effect, which resulted in the stomatal closure and restriction of CO_2 entry into the mesophyll of the leaves. The reduction of dry matter due to high salinity is a response that has been commonly cited in the literature previously for several intolerant species (Khan et al., 2010; Khan et al., 2015; Sousa et al., 2015; Li et al., 2014).

Our results indicated that *S. viridis* A10.1 could tolerate doses of at least 13.3 dS/m without any substantial morphological changes (Figure 10). However, from a dose of at least 19.4 dS/m and higher, the morphophysiological variables of the *S. viridis* A10.1 plants were strongly affected, leading to their death. According to Flowers (2004), although the tolerance to the saline stress in the plants is variable, the crop species are generally intolerant to one-third–around 16.5 dS/m - of the salt concentration found in seawater, which is about 50 dS/m. This study showed that *S. viridis* A10.1 is not a halophyte species, as it died at NaCl levels ≥20 dS/m; however, due to its response to the salinity stress at the vegetative phase of development, one can say that it is a glycophyte having some level of tolerance. Kim et al. (2014) evaluated the responses of *S. viridis* seedlings at the third-leaf stage to the salinity stress and showed that this species had some level of salt-tolerance when cultivated for a week in 100 mM of NaCl; however, the study did not report about which specific accession they had used.

The range of NaCl doses–in EC terms–used to evaluate the effects of the saline stress on the seed germination, the early growth of the *S. viridis* seedlings, and the one to be used on the plants at the vegetative phase of development differ in a way that not allows a complete analysis of equivalence between all the doses used in this study. However, considering that the EC obtained in the medium with 90 and 150 mM NaCl were very close to the values with the ones obtained in the soil with 0.2 and 0.4 g of this salt per 100 g of the substrate (Figure 1 and Figure 5), we compared the responses of A10.1 seedlings at a very early stage of growth and plants at the vegetative phase of development. The general morphological effects of an EC of about 9.5±0.5 and 14±0.7 dS/m seemed to have affected more seedlings than the plants. Most likely due to an age effect, the older the plant was, the more tolerant it was observed to be. In both cases, no death of the seedlings or the plants were observed.

In *S. viridis* A10.1, the speed of the responses seen in this present study allowed us to relate the reduction in the growth and, in the higher doses, the death of plants due to the osmotic shock; since the salt was added when the plants had already reached the stage of full development. The variables measured in this work allowed to assess the tolerance levels of the plants to saline stress, as in the case of studies done previously by Sousa et al. (2016), Atlassi Pak, Nabipour and Meskarbashee (2009), Flowers, Munns and Colmer (2015).

The objective of this study was to determine whether the A10.1 accession is salt-intolerant or not and to appreciate its potential use as a model plant to validate the salt-tolerance candidate genes/alleles and promoters from salt-responsive genes. The responses obtained here allowed us to classify this accession of *S. viridis* as a useful model plant for both the promoter sequence as well as the candidate genes/alleles validation, depending on the level of stress and the stage of plant/seedling development one will be using. Although, it will not be useful as a model plant for the validation of candidates genes/alleles with a putative role in the improvement of germination rates under salinity stress and neither for the levels of salinity found in most saline soils. However, if the goal is to validate the candidate genes/alleles with a putative role in conferring tolerance at very early developmental stages, it might prove helpful.

CONCLUSIONS

Taking into account that the objective of this study was to determine whether the A10.1 accession is salt-intolerant or not and to appreciate its potential use as a model plant to validate the salt-tolerance candidate genes/alleles as well as promoter sequences from salt-responsive genes, the following can be concluded. *Setaria viridis* accession A10.1 is a glycophyte species with some level of tolerance to salinity stress. The tolerance expresses when plants are in NaCl doses of about 15 dS/m or less, and apparently, the level of tolerance gets higher as the plants get old (comparing a 2-day-old seedling to a 20-day-old plant). Seed germination of accession A10.1 is little affected by salt stress when seeded on germination medium with NaCl doses of about 15 dS/m or less. Consequently, it cannot be considered as a model plant for the validation of candidates genes/alleles with a putative role in the improvement of germination rates under salinity stress. If the goal is to validate candidates' genes/alleles with a putative role in conferring tolerance at very early developmental stages (about two days after germination), this accession might prove to be a model plant at NaCl doses of about 15 dS/m or less. For validation of the promoter sequences from salt-responsive genes, one might use NaCl doses of about 15 dS/m or less, independent of the age of the plant. At last,

as *S. viridis* accession showed a very high plant death rate when subjected to doses of NaCl of ≥19.4 dS/m at the vegetative growth phase, it might be used as a model plant to validate candidate genes/alleles for salt tolerance, mainly those putatively responsible for increasing the level of tolerance.

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