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Interspecific Variations in the Growth, Water Relations and Photosynthetic Responses of Switchgrass Genotypes to Salinity Targets Salt Exclusion for Maximising Bioenergy Production

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Abstract: The expansion in the cultivation of bioenergy crops to saline lands is of importance for ensuring food security as long as high productivity is maintained. The potential of switchgrass to grow under saline conditions was examined in three genotypes from a early seedling growth to full maturity at 50, 100, 200 and 300 mM of sodium chloride (NaCl). The carbon assimilation rates were generally lower and correlated to stomatal closure in plants exposed to salinity in all the tested genotypes. Based on the results of ion concentrations in different parts of the plant, switchgrass genotypes differed in their responses to NaCl. The Alamo genotype excluded salt from the roots, whereas Trailblazer and Kanlow accumulated it in the root, stem and leaf tissues. The increased leaf salt concentration was accompanied by a higher proline concentration in the 200 and 300 mM NaCl treatments toward the end of the experiment. Overall, Alamo showed the highest yields at all salinity levels, indicating that excluding salt from the roots may result in a better performance in terms of biomass production. The accumulation of salt observed in Kanlow and Trailblazer resulted in lower yields, even when other mechanisms, such as the production of salt glands, were observed, especially in Kanlow. These results suggest that the Alamo genotype has the ability to maintain high yields under saline conditions and that this characteristic could be further exploited for maximizing bioenergy production under saline conditions.

Keywords: bioenergy crop; cation balance; CO_2 assimilation; salt stress; *Panicum virgatum*

1. Introduction

Climate change will have a major impact on agricultural systems within the next decades [1] and expose many ecosystems to atmospheric and soil water deficits [2]. In many arid or semi-arid areas, due to a decreased water availability and increases in salinity related to inadequate irrigation practices [3,4], the use of crops with an enhanced tolerance to salt stress will be required [5]. However, many conventional crops are largely intolerant to even small increases in soil salinity [6]. It is anticipated that a reduction in the availability of land suitable for conventional agriculture will cause a direct competition between the land used for the production of energy crops and that used for food production, forestry and/or conservation measures [7]. Given this scenario, any expansion in the cultivation of bioenergy crops is likely to place an increasing emphasis on the use of land that is unsuitable for conventional agriculture, including areas subjected to a high salinity. Consequently, the wider exploitation of bioenergy crops may require an enhanced salt tolerance.

Salinization is a product of natural interactions among geological, hydrological and vegetation processes, as well as being due to anthropogenic influences, including irrigation and grazing practices [8]. Saline soils can be characterized as those soils that have an electrical conductivity above 4 dS m^{-1} [4], and they are often linked to increased concentrations of sodium chloride (NaCl). An increase in salinity is usually associated with decreases in the productivity of sensitive plants/crops (glycophytes), even at low concentrations, while in more resistant species (halophytes), the productivity may even be stimulated at low concentrations. However, considerable variations in salt tolerance exist both among glycophytes and halophytes, and even halophytes can show growth reductions at a higher salinity [9]. Saline conditions can affect growth through reductions in leaf initiation, leaf expansion, reproduction and root growth [10–12]. Part of the reason for a reduction in growth is associated with lower substrate water potentials [13] that can limit water and nutrient uptake (physiological drought), with the toxic effects of high concentrations of sodium (Na^+) and chlorine (Cl^-) ions on plant metabolism, as well as with competition with nutrient ions, particularly potassium (K) [12]. Saline conditions also have an indirect effect on photosynthetic activity due to reductions in stomatal conductance, as a consequence of lower soil water potentials, restricting the availability of CO_2 for photosynthesis [14]. However, there is also evidence of salt-related non-stomatal inhibition of photosynthesis, through reductions in the chlorophyll content of leaves [15], an increased resistance to CO_2 diffusion to the site of reduction in the chloroplast, a reduced RUBISCO (ribulose 1,5–bisphosphate) activity [16], decreases in the stability of photosystem II (PSII) and inhibition of photosynthetic electron transport [17].

In general, conventional crops show limited growth under saline conditions [18]. In contrast, some bioenergy crops, such as *Arundo donax* [19,20] and *Miscanthus* x *giganteus* [21], have been reported to show an increased tolerance to moderately high levels of salinity at least in the short term. For switchgrass (*Panicum virgatum*), reductions in aboveground and belowground biomass have been reported in response to salinity, but almost all the available information is based on germination and seedling studies [22]. There are, however, some long-term exposure studies with switchgrass that have highlighted high emergence rates and biomass production in both lowland and upland switchgrass types exposed to moderate to high salinity [23,24]. This suggests that switchgrass may be a good candidate for bioenergy production in marginal lands affected by salinity.

Salinity tolerance in plants is related to different mechanisms, primarily associated with either salt exclusion and/or compartmentation [9,25]. Previous studies have indicated that switchgrass populations may show different responses to salt, such as a selective exclusion from the roots, as well as the accumulation of salts in aerial plant parts [23]. Increases in organic solutes (i.e., proline and soluble sugars), often related to salt tolerance, have also been found in response to increasing salt concentrations in switchgrass [26,27]. Other mechanisms, for example the elimination of salt through salt glands [22,28], have also been suggested, but in general information on the mechanisms and/or variability of salt tolerance among switchgrass genotypes is limited.

To examine the response of switchgrass to salinity, three different genotypes were compared in order to investigate the effects on (1) biomass production, (2) leaf-level photosynthesis, (3) plant water balance, (4) proline and sugar concentrations in leaves, (5) cation concentrations in roots, culms and leaves and (5) stomatal density andsize and salt gland production. The results provide insights into the different mechanisms of salt tolerance found in switchgrass and how these contribute to final biomass yields.

2. Material and Methods

2.1. Plant Material, Growth Conditions Experimental Design

Switchgrass (*Panicum virgatum* L.) is an important, largely warm-season, perennial grass of the prairies of the United States, and is usually used as a forage crop and for preventing soil erosion. Three switchgrass genotypes, i.e., Alamo, Kanlow and Trailblazer, were used for this experiment. Alamo and Kanlow are lowland ecotypes, whereas Trailblazer is an upland ecotype. The seeds were obtained from Ceres College

Station, College Station, TX, USA and CERES Inc., Thousand Oaks, CA, USA. The seeds were sterilized with a 2% solution of sodium hypochlorite for two minutes, rinsed with sterilized distilled water and germinated in Petri dishes with filter paper, and water was applied. After seven days, the seedlings were planted in 0.5 L pots with a mixture of John Innes No. 2, perlite and vermiculite (2:1:1; *v*:*v*:*v*) and grown in a greenhouse under natural light conditions (~500 µmol photon m^{-2} s^{-1}) with a day/night temperature of ~27/20 °C. Manual watering was applied to the plants as needed (3–4 times per week). When the second/third leaf was fully developed, the plants were transplanted into 4.5 L pots with the same substrate (one plant per pot, 84 plants per genotype).

Two months after planting, the seedlings were divided into five groups and salt stress was induced adding NaCl in the irrigation water. Five different concentrations of NaCl, i.e., control, 50, 100, 200 and 300 mM, were used. In order to avoid an osmotic shock, the salt concentrations were gradually increased. For the highest concentrations (200 and 300 mM NaCl), the whole process took a total of three weeks, and the plants were subsequently exposed to these treatments for 6 months. A total of five harvests were performed over the duration of the experiment; at 60, 90, 135, 180 and 240 days after planting (DAP). The mean values of the electric conductivity of the growing media throughout the experiment, measured with a WET sensor (WET2, Delta-T Devices, Ltd., Cambridge, UK), are shown in Table 1.

Table 1. The electric conductivity of the growing media (mS cm^{-1}) of the control, 50, 100, 200 and 300 mM NaCl treatments at different harvest dates.

Days After Planting (DAP)	Control	50 mM	100 mM	200 mM	300 mM
DAP 60	1.02	-	-	-	-
DAP 90	1.06	2.23	3.02	3.00	2.87
DAP 135	1.24	2.87	4.99	6.12	9.01
DAP 180	1.67	3.65	5.23	6.89	9.54

Values are means (n = 6).

2.2. Plant Growth Parameters

The shoot and root biomass (oven dried to a constant weight for a minimum 2 days at 80 °C), total leaf area (LA) and mean leaf area (MLA) were determined for each harvest interval. Plants exposed to 200 and 300 mM NaCl were only harvested at 135 and 180 DAP. The leaf areas were calculated on three representative leaves from the middle part of the canopy using a portable leaf area meter (ADC Bioscientific, Hoddesdon, UK) and then multiplied by the leaf number to estimate the total leaf area. The values of LA are absent for the last harvest (240 DAP) due to plant senescence.

2.3. Gas Exchange and Chlorophyll Fluorescence Measurements

Leaf gas exchange measurements were conducted with a portable infrared gas analyser (LI-COR 6400, Lincoln, NE, USA) at 25 °C (leaf temperature) on the last fully expanded leaf from the bottom to the top. Light response (A/PPFD) curves and the response of CO_2 assimilation to intercellular CO_2 concentration (A/Ci curves) were conducted for each harvest following the method described in Cordero & Osborne [29]. The light-saturated assimilation rate (A_{sat}), stomatal conductance (g_s), the ratio of the intercellular to ambient CO_2 concentration (Ci/Ca) and instantaneous water use efficiency (WUE_{inst}) were calculated. The average vapour pressure deficit (VPD) during the measurements varied from 1.05 to 1.27 kPa.

Chlorophyll fluorescence measurements on the adaxial surface of the leaves were made using a fluorometer (FMS 2, Hansatech, King's Lynn, UK) and coincided with the gas exchange measurements. The maximum quantum efficiency of photosystem II (Fv/Fm) was calculated from the fluorescence parameters after dark adaptation of the leaves for 20 min. The values for leaf gas exchange and chlorophyll fluorescence parameters were calculated for all genotypes and treatments for the first

four harvests. Because plants started senescing, some values for the photosynthetic and chlorophyll fluorescence parameters are absent at 180 DAP and at the end of the experiment (240 DAP).

2.4. Leaf Water Status and Epidermal Impressions

The pre-dawn leaf water potential (Ψ_{pd}) was determined using a Scholander-type pressure chamber (SKPM 1400 Series, SKYE Instrument, Dole, Powys, UK), and the relative water content (RWC) was estimated via a modification of Weatherley's method [30]. Both parameters were first measured on fully developed leaves from the top of the main culm.

Epidermal impressions were obtained by applying transparent nail polish over an approximately 0.5×1 cm area on both the adaxial and abaxial surfaces of one leaf per plant, avoiding the midrib. These impressions were placed on a microscope slide and observed under 40× using a Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany) with Syncroscopy Automontage (Syncroscopy, Cambridge, UK) digital imaging software. The number of stomata was counted, and the lengths of three different stomata were measured within a visual field of 0.09 mm^2 in each sample. The stomatal density, stomatal size and the presence of salt glands were examined with ImageJ (Wayne Rasband, Washington, DC, USA).

2.5. Proline and Total Soluble Sugars in Leaves

Total soluble sugars (TSS) and proline were quantified in potassium phosphate buffer (KPB) (50 mM, pH = 7.5) extracts of fresh tissue (0.2 g) after being manually ground with liquid nitrogen. The extracts were filtered through four layers of cheesecloth and centrifuged at 28,710× *g* for 15 min at 4 °C. The supernatant was collected and stored at 4 °C for further TSS and proline determinations. The total soluble sugars were analyzed spectrophotometrically with the anthrone reagent [31]. Free proline was estimated via a spectrophotometric analysis at 515 nm with the ninhydrin reaction [32]. An analysis of the data was performed on the youngest full-mature leaves harvested at midday, frozen in liquid nitrogen and stored at −20 °C for each harvest for later quantification.

2.6. Mineral Analysis

The samples from the youngest fully mature leaves (0.5 g dry weight) were dry-ashed and dissolved in hydrogen chloride (HCl), as described in Duque [33]. The potassium, magnesium, calcium and sodium concentrations were determined using a Perkin Elmer Optima 4300 inductively coupled plasma optical emission spectroscopy (ICP-OES) (Perkin Elmer, Billerica, MA, USA). The operating parameters of the ICP-OES were: radio frequency power 1300 W, nebulizer flow 0.85 L min^{-1}, nebulizer pressure 30 psi, auxiliary gas flow 0.2 L min^{-1}, sample introduction 1 mL min^{-1}. The cation balance was calculated as the ratio of the potassium, calcium and magnesium concentrations divided by the sodium concentration in tissues with the following formula: ((K + Ca + Mg)/Na) [23].

2.7. Statistical Analysis

One and two-factor analyses of variance (ANOVA) were performed in SPSS 20.0 (IBM Corp, Armonk, NY, USA). The variance was related to the main treatments (salinity and genotype) and the interaction between them. The Levene's test was used to check for homoscedasticity. The means ± SE were calculated, and, when the F-ratio was significant, the least significant differences were evaluated via the Tukey-b posthoc test. The linear regression and Pearson product-moment correlation coefficients were calculated to study the relationships between the variables. The significance levels were always set at 5%.

3. Results

3.1. Biomass Production

Reductions in the shoot biomass at higher salinity levels were generally found at all harvest dates, and these were significant for all the genotypes at 135 DAP, although there was a smaller impact

with Trailblazer (Figure 1a–c). At the final harvest (240 DAP), there was a 50–88% reduction in yield. Overall, Alamo was the highest yielding genotype across all the treatments, although the biomass production for Trailblazer was less impacted by salinity. The leaf area was reduced as the salinity increased in a similar way to that of the shoot yield (Figure 1d–f), and a larger reduction was found in the lowland Alamo and Kanlow types. The mean leaf area (MLA) (Figure 1g–i) was, in most cases, significantly higher in the lowland types and largely unaffected by the salinity. The root biomass was significantly lower in the high salinity treatments at the end of the experiment (180 and 240 DAP), especially in Kanlow and Traiblazer (Table 2). However, Kanlow showed a higher root to shoot ratio (Table 2), related to the strong impact of the salinity on the shoot biomass (Figure 1a–c).

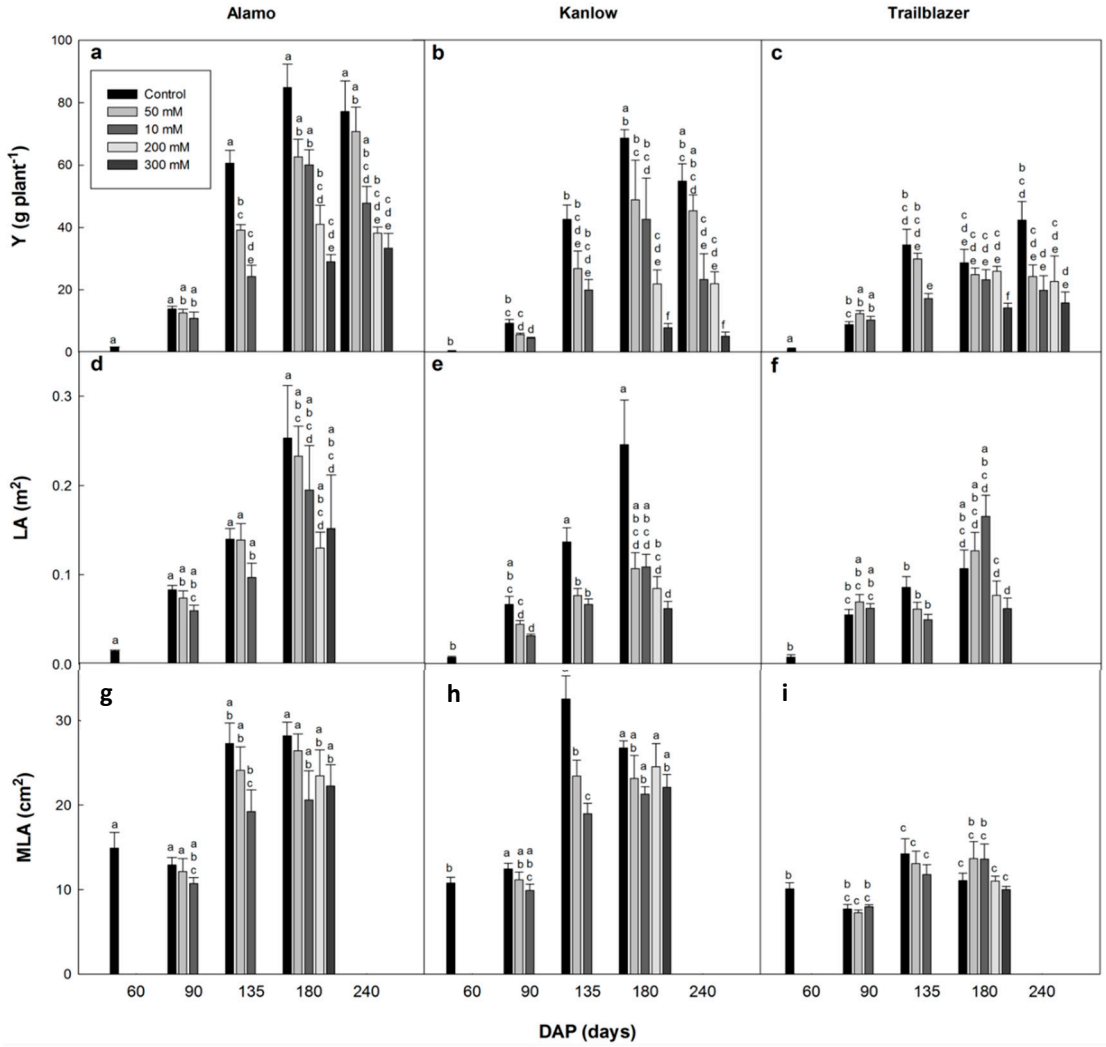

Figure 1. (**a**–**c**) The shoot yield (Y), (**d**–**f**) total leaf area (LA) and (**g**–**i**) mean leaf area (MLA) in *Panicum virgatum* (Alamo, Kanlow and Trailblazer genotypes) for the control, 50, 100, 200 and 300 mM NaCl treatments. The values are the means ± SE (n = 3–6). Within each days after planting (DAP), the values that are significantly different are identified with different letters ($p < 0.05$).

Table 2. The root dry matter (DM) and root to shoot ratio in *Panicum virgatum* (Alamo, Kanlow and Trailblazer genotypes) for the control, 50, 100, 200 and 300 mM NaCl treatments.

		Root DM (g plant^{-1})					Root/Shoot				
	Treatment	**DAP 60**	**DAP 90**	**DAP 135**	**DAP 180**	**DAP 240**	**DAP 60**	**DAP 90**	**DAP 135**	**DAP 180**	**DAP 240**
Alamo	Control	0.58a	5.13a	22.08a	41.80a	34.13a	0.40a	0.38a	0.37a	0.49a	0.46b
	50 mM	-	4.91ab	18.07ab	26.99bc	24.72abcd	-	0.40a	0.46a	0.43a	0.35b
	100 mM	-	4.63ab	9.86b	23.67bcd	18.80bcde	-	0.44a	0.40a	0.41ab	0.40b
	200 mM	-	-	-	15.30cde	19.88abcde	-	-	-	0.38b	0.53a
	300 mM	-	-	-	11.13de	13.13cde	-	-	-	0.39b	0.42b
Kanlow	Control	0.19b	3.04c	17.30ab	35.05ab	30.32ab	0.31a	0.35a	0.42a	0.51a	0.55a
	50 mM	-	1.71d	9.36b	24.00bcd	26.47abc	-	0.30a	0.35a	0.60a	0.50b
	100 mM	-	1.71d	7.76b	13.99cde	16.11bcde	-	0.38a	0.37a	0.40b	0.84a
	200 mM	-	-	-	13.88cde	13.19cde	-	-	-	0.66a	0.60a
	300 mM	-	-	-	4.25e	3.04f	-	-	-	0.52a	0.75a
Trailblazer	Control	0.49a	3.61bc	18.18ab	16.50cde	34.30a	0.37a	0.38a	0.47a	0.60a	0.62a
	50 mM	-	4.56ab	16.94ab	17.38cde	13.23cde	-	0.37a	0.56a	0.69a	0.58a
	100 mM	-	4.41ab	6.41b	11.75de	11.29cde	-	0.42a	0.37a	0.50a	0.59a
	200 mM	-	-	-	10.72de	10.62cde	-	-	-	0.41ab	0.65a
	300 mM	-	-	-	5.66e	5.94f	-	-	-	0.39b	0.46b
Salinity		-	ns	***	***	***	-	ns	ns	ns	ns
Genotype		-	***	*	***	**	-	*	ns	ns	*
Interaction		-	**	ns	*	ns	-	ns	ns	ns	ns

The values are the mean ± SE (n = 3–6). Within each DAP, the statistical differences are indicated by different letters ($p < 0.05$). ns, *, **, and *** indicate, respectively, non-significant or significant at 5%, 1% and 0.1% levels for the results of the two-way ANOVA with salt stress and genotype as the main effects.

3.2. Gas Exchange and Fluorescence Measurements

Throughout the experiment, the CO_2 assimilation rates were generally lower in plants exposed to salinity (Figure 2a–c). At 135 DAP, which was the last measurement in which no apparent senescence was observed for any treatment, the reductions in assimilation in the 300 mM treatment were lower for the lowland Alamo (44%) and Kanlow (36%) types than for the upland Trailblazer (63%) type. At this DAP, there were significant differences due both to salinity ($F = 9.707$, $p < 0.001$) and genotypic variation ($F = 23.100$, $p < 0.001$). Differences found at the last harvest (180 DAP) were related to salinity ($F = 9.707$, $p < 0.001$) and the interaction of salinity and variety ($F = 2.59$, $p < 0.05$). The reductions in A_{sat} correlated with decreases in stomatal conductance (g_s) at every stage; $r = 0.729$, $r = 0.718$ and $r = 0.570$ at 90, 135 and 180 DAP, respectively (Figure 2d–f).

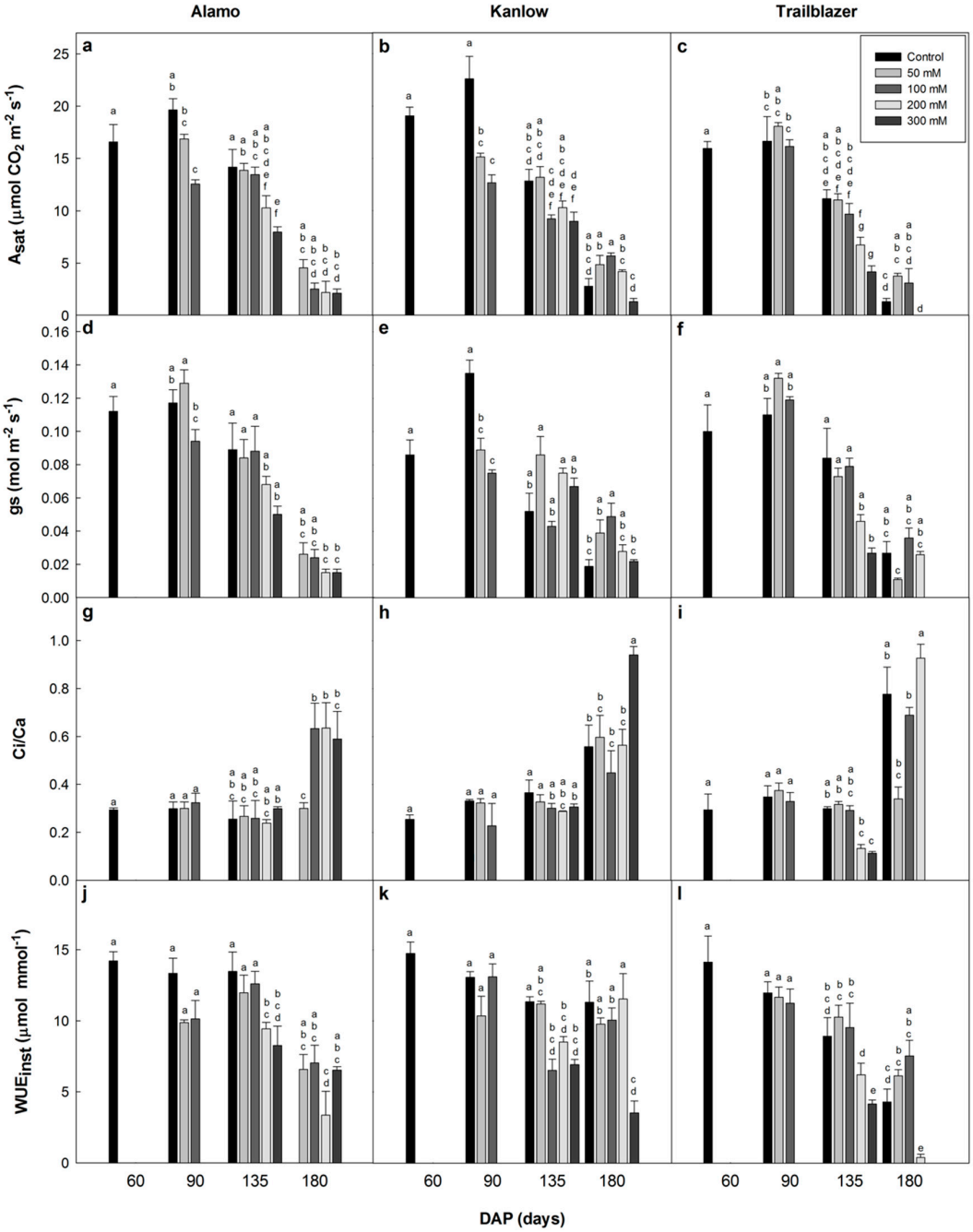

Figure 2. The (**a**–**c**) CO_2 assimilation rate (A_{sat}), (**d**–**f**) stomatal conductance to water vapour (g_s),

(**g–i**) ratio of intercellular to external CO_2 concentration (Ci/Ca) and (**j–l**) instantaneous water use efficiency (WUE_{inst}) of Alamo, Kanlow and Trailblazer genotypes of *Panicum virgatum* measured at 400 µmol mol^{-1} CO_2, 2000 µmol photon m^{-2} s^{-1} and 25 ± 2 °C for the control, 50, 100, 200 and 300 mM NaCl treatments. The values are the means ± SE (n = 3–6). Within each DAP, the values that are significantly different are identified with different letters ($p < 0.05$).

The Ci/Ca ratio did not change until 180 DAP (Figure 2g–i), when, in general, the values increased in comparison with previous harvests for most of the genotypes and were negatively correlated with A_{sat} (r = −0.704). The values for WUE_{inst}, calculated as the ratio of the assimilation rate divided by the transpiration rate, generally decreased with plant age (Figure 2j–l). At 135 DAP, WUE_{inst} was significantly reduced at the two highest salinity treatments for all genotypes, and this was coupled with a significant impact of salinity (F = 10.779, $p < 0.001$) and genotype (F = 12.317, $p < 0.001$). Significantly lower Fv/Fm values were generally found for all genotypes at 180 DAP with respect to previous harvests measurements (Table 3), but they were not significantly affected by the salinity treatments.

Table 3. The maximum quantum efficiency of PSII (Fv/Fm) for the control, 50, 100, 200 and 300 mM NaCl treatments for Alamo, Kanlow and Trailblazer genotypes of *Panicum virgatum*.

		Fv/Fm			
	Treatment	**DAP 60**	**DAP 90**	**DAP 135**	**DAP 180**
Alamo	Control	0.827a	0.813a	0.821a	-
	50 mM	-	0.835a	0.813a	0.724a
	100 mM	-	0.828a	0.798a	0.651b
	200 mM	-	-	0.799a	0.645b
	300 mM	-	-	0.806a	0.611b
Kanlow	Control	0.837a	0.824a	0.799a	0.685ab
	50 mM	-	0.818a	0.801a	0.766a
	100 mM	-	0.838a	0.826a	0.741a
	200 mM	-	-	0.814a	0.701ab
	300 mM	-	-	0.800a	0.617b
Trailblazer	Control	0.711a	0.814a	0.812a	0.620b
	50 mM	-	0.825a	0.789a	0.699ab
	100 mM	-	0.809a	0.817a	0.621b
	200 mM	-	-	0.796a	-
	300 mM	-	-	0.818a	-
Salinity		-	ns	ns	ns
Genotype		-	ns	ns	*
Interaction		-	ns	ns	ns

The values are the mean ± SE (n = 3–6). Within each DAP, the statistical differences are indicated by different letters ($p < 0.05$). ns, *, indicate, respectively, non-significant or significant at 5% levels for the results of the two-way ANOVA with salt stress and genotype as the main effects.

3.3. Leaf Water Status

The leaf relative water content was significantly lower after the first 30 days of salinity exposure in the higher salinity treatment for Alamo and Trailblazer (90 DAP) (Table 4). At later stages, the RWC values were slightly lower, especially at 180 DAP, but there were no significant differences between the treatments for any of the genotypes. In general, the Ψ_{pd} values were significantly reduced by salinity for all harvests (Table 4), although they were less negative in the Alamo genotype at DAP 180.

Table 4. The leaf relative water content (RWC) and pre-dawn leaf water potential (Ψ_{pd}) for the control, 50, 100, 200 and 300 mM NaCl treatments for Alamo, Kanlow and Trailblazer genotypes of *Panicum virgatum*.

		RWC (%)				Ψ_{pd} (MPa)			
	Treatment	**DAP 60**	**DAP 90**	**DAP 135**	**DAP 180**	**DAP 60**	**DAP 90**	**DAP 135**	**DAP 180**
Alamo	Control	98.51a	96.13a	94.93a	-	−0.024a	−0.167ab	−0.168a	-
	50 mM	-	94.18ab	95.14a	86.10a	-	−0.222bc	−0.309ab	−0.473c
	100 mM	-	92.45bc	93.81a	88.80a	-	−0.198ab	−0.488bc	−0.489c
	200 mM	-	-	-	63.80ab	-	-	-	−0.707d
	300 mM	-	-	-	93.37a	-	-	-	−0.785e
Kanlow	Control	97.51a	95.89a	95.83a	72.65ab	−0.034a	−0.097a	−0.174a	−0.211b
	50 mM	-	95.82a	93.18a	86.88a	-	−0.306c	−0.422ab	−0.448c
	100 mM	-	95.03a	92.46ab	88.80a	-	−0.484d	−0.506bc	−0.683d
	200 mM	-	-	-	87.01a	-	-	-	−0.844efg
	300 mM	-	-	-	52.12ab	-	-	-	−0.925g
Trailblazer	Control	94.79a	95.56a	91.87ab	52.96ab	−0.092b	−0.109a	−0.211a	−0.194ab
	50 mM	-	92.46bc	82.73b	56.58ab	-	−0.217bc	−0.703cd	−0.522c
	100 mM	-	91.94c	91.92ab	62.3ab	-	−0.173ab	−0.759d	−0.752de
	200 mM	-	-	-	92.25a	-	-	-	−0.814ef
	300 mM	-	-	-	39.2b	-	-	-	−0.917fg
Salinity		-	***	ns	*	-	***	***	***
Genotype		-	**	*	ns	-	***	***	***
Interaction		-	ns	ns	*	-	***	ns	***

The values are the mean ± SE (n = 3–6). Within each DAP, the statistical differences are indicated by different letters ($p < 0.05$). ns, *, **, and *** indicate, respectively, non-significant or significant at 5%, 1% and 0.1% levels for the results of the two-way ANOVA with salt stress and genotype as the main effects.

3.4. Proline, Total Soluble Sugars and Cation Analysis

At 90 DAP, both Alamo and Trailblazer had significantly higher proline concentrations due to the effects of salinity (Table 5; F = 20.545, $p < 0.001$). Significantly higher values were also observed for Kanlow and Trailblazer at 180 DAP but not for Alamo. The concentrations of TSS increased with plant age for all the genotypes and to a similar extent until 240 DAP.

Salinity influenced the accumulation of Na in leaves, stems and roots (Table 6) at 180 DAP, and this was significant ($p < 0.001$). Significantly higher values were found in the leaves of Kanlow and Trailblazer compared to Alamo at 300 mM NaCl. Increases in Na concentration were linked to small increases in K in leaves, although the values at 300 mM NaCl were similar to the control ones (Table 6). Despite the fact that there was evidence of a reduction in K in stems, this was quite variable, whilst a more consistent salinity-related reduction in K was found in roots. For Mg and Ca, salinity-related changes were minor, although consistently higher values for Mg and Ca were found in leaves compared to stems and roots. Significantly higher Ca concentrations were also found in the leaves of Trailblazer when compared to the Alamo and Kanlow genotypes. The cation balance (Table 7) was significantly lower in plant tissues exposed to salinity, particularly for Kanlow and Trailblazer, with the greatest effects in leaves (>47-fold reduction in Kanlow; 29-fold reduction in Trailblazer). In contrast, the effects on the cation balance of roots were much lower for all the genotypes (~3–6-fold reduction).

Table 5. The proline and total soluble sugar (TSS) concentrations for the control, 50, 100, 200 and 300 mM NaCl treatments in Alamo, Kanlow and Trailblazer genotypes of *Panicum virgatum*.

		Proline (μmol g^{-1} DM)					TSS (mg g^{-1} DM)				
	Treatment	**DAP 60**	**DAP 90**	**DAP 135**	**DAP 180**	**DAP 240**	**DAP 60**	**DAP 90**	**DAP 135**	**DAP 180**	**DAP 240**
Alamo	Control	7.89b	0.78c	1.27a	3.69bc	28.88ab	34.68a	38.36bc	71.09ab	144.81a	39.85ab
	50 mM	-	2.78bc	1.51a	1.15c	16.99b	-	33.61c	72.64ab	99.70a	56.90ab
	100 mM	-	8.07ab	1.11a	3.30bc	37.57ab	-	54.64ab	98.84a	101.64a	55.60ab
	200 mM	-	-		2.35c	30.37ab	-	-	-	105.60a	54.54ab
	300 mM	-	-		1.34c	-	-	-	-	142.00a	58.64ab
Kanlow	Control	11.08a	1.45c	2.11a	1.62c	25.62ab	45.96a	52.86bc	60.48b	119.67a	47.11ab
	50 mM	-	3.51bc	0.84a	1.26c	24.24ab	-	63.62a	66.90ab	98.67a	41.63ab
	100 mM	-	4.35bc	1.23a	1.23c	28.17ab	-	58.41ab	70.77ab	81.65a	38.81b
	200 mM	-	-	-	0.60c	68.51a	-	-	-	94.25a	60.21ab
	300 mM	-	-	-	8.60b	26.45ab	-	-	-	123.86a	38.46b
Trailblazer	Control	8.42b	1.32c	0.70a	3.22bc	25.47ab	47.65a	59.07ab	66.80ab	150.90a	64.57a
	50 mM	-	1.94c	1.31a	1.60c	33.81ab	-	66.37a	61.98b	84.01a	39.31ab
	100 mM	-	10.46a	1.64a	3.70bc	52.31a	-	55.42ab	74.54ab	151.9a	60.53ab
	200 mM	-	-	-	6.04bc	17.56ab	-	-	-	124.50a	45.04ab
	300 mM	-	-	-	15.60a	21.64ab	-	-	-	154.4a	76.70a
Salinity		-	***	ns	***	ns	-	ns	*	ns	ns
Genotype		-	ns	ns	***	ns	-	***	*	*	ns
Interaction		-	*	ns	***	ns	-	*	ns	ns	ns

The values are the mean ± SE (n = 3–6). Within each DAP, the statistical differences are indicated by different letters ($p < 0.05$). ns, * and *** indicate, respectively, non-significant or significant at 5% and 0.1% levels for the results of the two-way ANOVA with salt stress and genotype as the main effects. DM: dry matter.

Table 6. The sodium (Na), potassium (K), magnesium (Mg) and calcium (Ca) concentration in the leaves, stems and roots of Alamo, Kanlow and Traiblazer genotypes of *Panicum virgatum* at 180 DAP and subjected to the control, 50, 100, 200 and 300 mM NaCl treatments.

		Na (g kg^{-1} DM)			K (g kg^{-1} DM)			Mg (g kg^{-1} DM)			Ca (g kg^{-1} DM)		
	Treatment	**Leaves**	**Stems**	**Roots**	**Leaves**	**Stems**	**Roots**	**Leaves**	**Stems**	**Roots**	**Leaves**	**Stems**	**Roots**
Alamo	Control	0.49d	0.80c	2.62e	10.27cd	14.33abc	10.14ab	12.96a	3.42ab	2.46c	10.68d	1.62cde	1.62bcd
	50 mM	2.05cd	2.67c	6.88bcde	10.87bcd	18.27ab	8.44abcd	11.64ab	4.37a	3.02abc	14.05bcd	1.87bcde	1.41bcd
	100 mM	2.19cd	3.10c	4.81cde	11.97abcd	17.36abc	4.71d	7.61b	3.07ab	2.42c	11.84cd	1.37de	1.01cd
	200 mM	4.53bc	5.39c	9.16bc	12.24abcd	16.11abc	5.92bcd	9.53ab	2.80ab	3.06abc	10.04d	1.25de	1.41bcd
	300 mM	3.17cd	2.18c	6.53bcde	14.04abcd	14.97abc	5.09cd	8.01b	2.88ab	2.77abc	8.99d	1.15e	1.29bcd
Kanlow	Control	0.29d	1.45c	3.60de	12.39abcd	20.92a	7.95abcd	9.64ab	3.26ab	2.64bc	11.25cd	2.26abcde	1.36bcd
	50 mM	1.13d	1.54c	5.34cde	9.53cd	17.27abc	10.53a	7.54b	4.10a	2.93abc	14.08bcd	2.84ab	1.27bcd
	100 mM	0.80d	2.32c	8.20bcd	10.51cd	20.57a	8.00abcd	8.28b	3.19ab	3.21abc	12.51bcd	2.14abcde	1.21bcd
	200 mM	1.03d	0.98c	3.57de	11.66abcd	19.50a	7.03abcd	7.93b	3.13ab	2.84abc	10.03d	1.08e	1.12bcd
	300 mM	16.23a	13.55b	10.37ab	15.10abc	10.90bcd	4.94cd	8.62b	2.10b	3.47abc	13.00bcd	1.68bcde	1.38bcd
Trailblazer	Control	1.23d	1.92c	2.68e	16.52ab	18.27ab	9.38abc	9.78ab	3.45ab	3.60abc	18.40ab	2.73abc	2.66a
	50 mM	1.30d	2.63c	4.87cde	12.13abcd	18.46ab	7.66abcd	9.72ab	2.81ab	4.45a	20.59a	2.43abcd	2.02ab
	100 mM	2.56cd	2.95c	6.78bcde	8.88d	9.84cd	7.73abcd	11.23ab	2.26b	2.76abc	20.32a	1.33de	0.95d
	200 mM	6.66b	6.14c	7.77bcd	11.89abcd	7.04d	6.42abcd	9.90ab	2.78ab	2.95abc	20.36a	1.65cde	1.24bcd
	300 mM	15.47a	23.39a	14.11a	16.97a	10.07cd	6.78abcd	8.21b	3.26ab	4.21ab	17.30abc	3.11a	1.87bc
Salinity		***	***	***	***	***	***	*	**	*	*	***	***
Genotype		***	***	ns	ns	***	ns	*	ns	**	***	***	***
Interaction		***	***	***	*	***	*	*	*	ns	ns	**	**

The values are the means ± SE (n = 3). Within each treatment, the values that are significantly different are identified with asterisks ($p < 0.05$). ns, *, **, and *** indicate, respectively, non-significant or significant differences at 5%, 1% and 0.1% levels for the results of the two-way ANOVA with salt stress and genotype as the main effects. DM: dry matter.

Table 7. The cation balance ((K + Ca + Mg)/Na) in the leaves, stems and roots of Alamo, Kanlow and Trailblazer genotypes of *Panicum virgatum* subjected to the control, 50, 100, 200 and 300 mM NaCl treatments at 180 DAP.

		Cation Balance		
	Treatment	**Leaves**	**Stems**	**Roots**
Alamo	Control	80.81bc	23.38abc	6.14a
	50 mM	19.66efg	9.05bcde	1.97cd
	100 mM	20.14efg	8.42cde	1.86cd
	200 mM	7.10fg	3.28e	1.17cd
	300 mM	19.06efg	12.56abcde	1.45cd
Kanlow	Control	142.87a	22.26abc	4.15b
	50 mM	34.50defg	16.09abcd	2.94bc
	100 mM	55.79cd	27.69a	1.75cd
	200 mM	36.00def	24.55ab	3.00bc
	300 mM	2.99g	2.52e	1.27cd
Trailblazer	Control	88.43b	20.34abcd	6.20a
	50 mM	38.73de	10.85bcde	2.80bcd
	100 mM	14.31efg	4.97de	1.72cd
	200 mM	6.35fg	1.73e	1.37cd
	300 mM	3.03g	0.73e	0.96d
Salinity		***	***	***
Genotype		***	***	ns
Interaction		***	***	**

The values are the means ± SE (n = 3). Within each plant tissue, the statistical differences are indicated by different letters ($p < 0.05$). ns, **, and *** indicate, respectively, non-significant or significant effects at 1% and 0.1% levels for the results of the two-way ANOVA with salt stress and genotype as the main effects.

3.5. Stomata and Salt Glands

In general, the stomatal density was significantly higher on the adaxial surface (Figure 3a,c,e) of leaves for all genotypes and was associated with a significantly higher stomatal size on the abaxial surface (Figure 3b,d,f). Overall, the lowland genotypes had a higher stomatal density on the adaxial surface, which was related to genotypic variations and not to the salinity treatments (Table 8). Further study of the leaf surfaces showed the presence of salt glands in some genotypes at 90 (Figure 3d,f) and 180 DAP (Table 8). Some salt glands were also found in Trailblazer and Alamo but generally in low numbers. However, Kanlow had salt glands in all saline treatments on both the adaxial and abaxial surfaces at 180 DAP (Table 8).

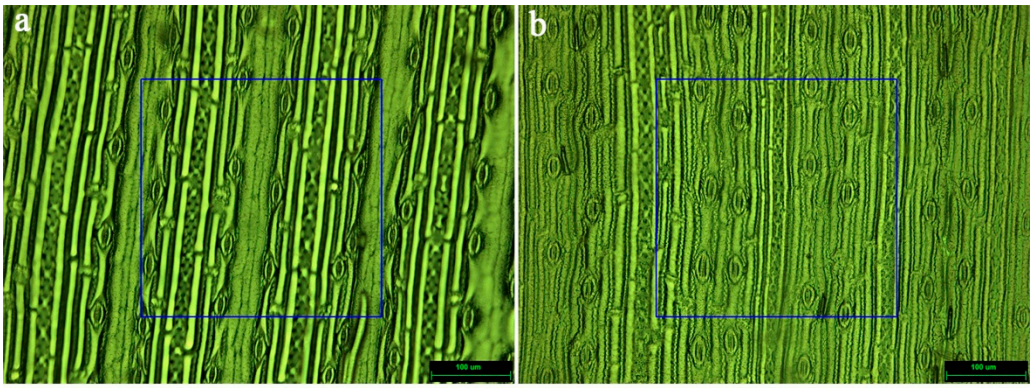

Figure 3. *Cont.*

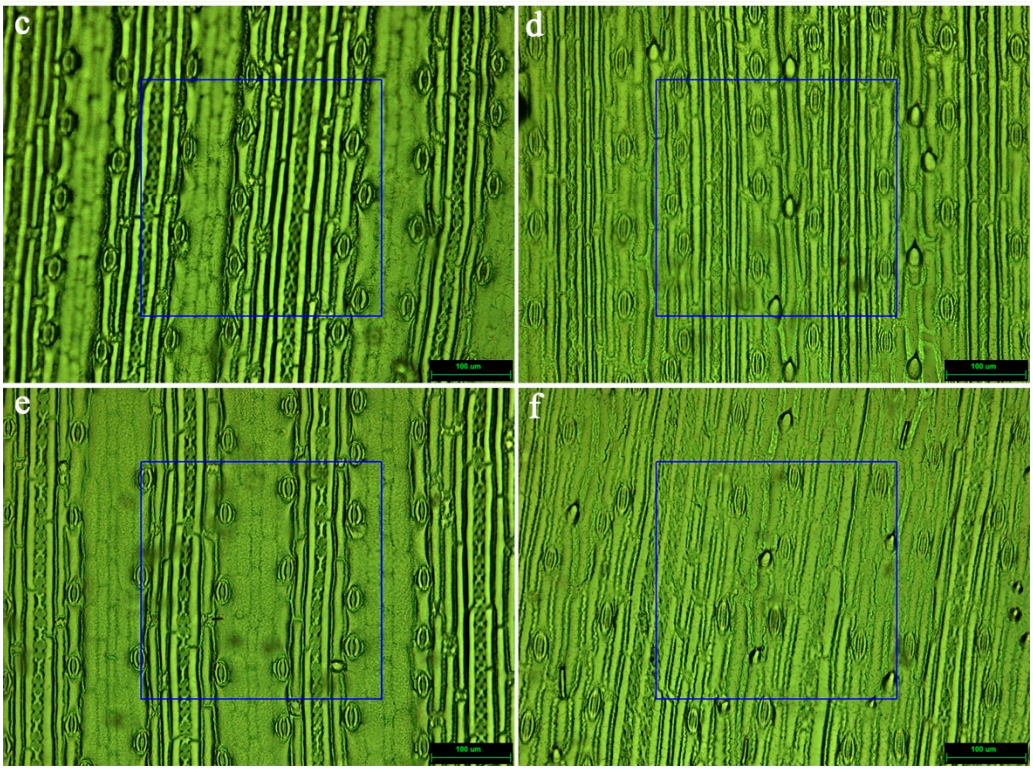

Figure 3. Stomatal impressions for the Kanlow genotype of *Panicum virgatum* at DAP 90 from the (**a**,**c**,**e**) adaxial and (**b**,**d**,**f**) abaxial surfaces. The images show different treatments—(**a**,**b**) control, (**c**,**d**) 50 mM and (**e**,**f**) 100 mM of NaCl. The data within the blue squares represent an area of 0.09 mm^2.

Table 8. The stomatal density and number of salt glands on the adaxial and abaxial leaf surfaces of *Panicum virgatum* (Alamo, Kanlow and Traiblazer) subjected to the control, 50, 100, 200 and 300 mM NaCl treatments at 180 DAP.

		Stomatal Density (Number mm^{-2})		Salt Glands (Number mm^{-2})	
	Treatment	**Adaxial**	**Abaxial**	**Adaxial**	**Abaxial**
Alamo	Control	141.19abc	105.40ab	-	-
	50 mM	169.99abc	177.78abc	-	-
	100 mM	149.70abc	174.07ab	-	-
	200 mM	119.51abc	182.72a	-	1.23d
	300 mM	157.70abc	133.33abc	-	-
Kanlow	Control	160.49abc	114.81abc	3.70d	1.23d
	50 mM	141.06abc	119.51abc	20.99ab	16.04abcd
	100 mM	177.62ab	113.09abc	18.92abc	14.32d
	200 mM	172.38abc	124.48abc	10.80bcd	1.21d
	300 mM	148.81abc	98.50c	28.29a	4.69cd
Trailblazer	Control	125.26abc	110.61abc	-	-
	50 mM	145.97abc	104.48bc	2.46d	-
	100 mM	114.28abc	107.34abc	-	-
	200 mM	116.05abc	113.58abc	-	-
	300 mM	120.19abc	99.66c	-	-
Salinity		ns		-	
Genotype		***		-	
Interaction		ns		-	

The values are the means ± SE (n = 6 leaves × 3 fields). Within each parameter, the statistical differences are indicated by different letters ($p < 0.05$). ns and *** indicate, respectively, non-significant or significant effects at 0.1% levels for the results of the two-way ANOVA with salt stress and genotype as the main effects.

4. Discussion

The objective of this study was to assess the performance and genetic variability in the response of switchgrass to elevated salinity levels due to its potential importance for the wider exploitation of this genus as a bioenergy crop. Most studies have focused on the short-term effects of salinity on the performance of switchgrass, and the effects of long-term exposure have not been assessed in depth. After 75 days of salt stress exposure (135 DAP), there was evidence of a direct effect of salinity (50 and 100 mM) on shoot biomass, which was more evident in the lowland genotypes Alamo and Kanlow. Previous studies have shown that 30 days of exposure to 250 mM NaCl [26], 30 days of irrigation with 10.0 dS m^{-1} saline solution [34] or 60 days at ~180 mM NaCl [35] can impact the biomass yields in switchgrass. To our knowledge, however, there are no studies on the effect of lower concentrations (50 and 100 mM NaCl), although similar responses have been observed in *Miscanthus*, with significant yield-reductions after a 64-day salinity exposure to concentrations of 100 mM NaCl [36]. This biomass reduction was found to be linked to a reduced stomatal conductance and photosynthetic rate as result of a water deficit caused by salt stress, similar to previous research from Sánchez et al. [35]. The different salinity levels did not affect the MLA values in general, indicating that differences in LA were possibly related to an effect of salinity on the initiation/emergence of new leaves in switchgrass rather than on leaf expansion. Leaf growth has been reported to be directly affected by salinity [25,35], and the significantly positive correlations between LA and shoot yield at 135 DAP ($r = 0.744$, $p < 0.001$) and 180 DAP ($r = 0.570$, $p < 0.001$) indicated strong relationships between the two parameters in switchgrass that could be exploited for the selection of high yielding genotypes. On the other hand, switchgrass also exhibited changes in development related to salinity. The flowering times were affected in Kanlow with a one-week flowering delay in the 200 mM treatment, and anthesis was not reached in the 300 mM NaCl treatment. These developmental disorders should be further studied under field conditions to select high-yielding genotypes that also have a high establishment rate in subsequent growing seasons. In fact, Zannetti et al. [37] have recently demonstrated that Alamo has a high germination rate under saline conditions, although it shows a lower salt tolerance at a more mature stage.

Both short [15,26,35] and long-term studies [23,24,37] found general salinity-related reductions in CO_2 assimilation rates that were similar to the findings of this study. These differences in CO_2 assimilation were more pronounced during early development, and became smaller toward the end of the experiment when the assimilation rates of non-stressed plants were lower as they approached the senescence phase. Overall, the absence of an impact on Fv/Fm, coupled with largely constant values for Ci/Ca in the first two harvests, suggests that the photosynthetic/photochemical performance was not affected. However, a lower stomatal conductance under salt stress may limit CO_2 uptake with a consequent reduction of photosynthesis. Presumably, this was associated with salt-related water deficits, which is consistent with reductions in leaf Ψ and RWC. Correlated reductions in A_{sat} and g_s in response to salinity have previously been reported [6,38,39], even in halophytes [40]. Similar results have also been found in other C_4 species, in which a stomatal constraint was the main reason for the reduction in CO_2 assimilation in the early stages of exposure to osmotic stress [41]. The negative correlation between Ci/Ca and A_{sat} in all treatments, including the controls at later stages, were probably largely developmental, as the photosynthetic apparatus starts to be dismantled as plants senesce [42], and not solely as a consequence of a direct impact of salinity.

Although RWC was reduced in this experiment, consistent with other studies where plants were exposed to water deficits and salinity [43,44], switchgrass was shown to experience smaller reductions in RWC than other bioenergy crops under saline conditions [35], indicating a great capability to maintain water balance. The ability of switchgrass to maintain high values of WUE_{inst} and RWC even after more than 60 days of exposure to saline conditions also gives an indication of a relatively high tolerance to salinity.

Proline may have a positive effect on the performance of plants exposed to salinity [45,46] and, together with the accumulation of soluble sugars, is often an indication of metabolic resistance [47].

Salinity-related increases in proline have been widely documented in both C_3 species [48,49] and in C_4 grasses [36,50]. In our experiment, there was no strong indication of a relation between proline accumulation and salt tolerance. High proline concentrations were found at 90 and 180 DAP, but they were not consistent. However, the genotype that did not show proline accumulation at later growth stages (180 DAP) was the one that did not accumulate salt at the higher salinity levels (Alamo). The low yields achieved by Kanlow and Trailblazer at the higher salt concentrations at 180 DAP could be the result of a lack of compartmentation of salt within the cells that could have contributed to the production of proline as a stabilizer of cellular homeostasis [51]. However, other compatible solutes or ions that have not been analyzed in this study could also be involved.

There were two different patterns in the accumulation of Na in the shoots of the switchgrass genotypes. Kanlow and Trailblazer showed a significantly higher concentration of Na in the higher salinity treatments than Alamo did. This suggests the existence of different mechanisms of salt tolerance within switchgrass genotypes, as previously reported in the lowland EG1102 and the upland EG2101 genotype by Anderson et al. [23], through the exclusion from roots (Alamo) and accumulation of Na in shoots and leaves (Trailblazer and Kanlow). In roots, Alamo showed a lower Na concentration in the 300 mM treatment that could be explained by a reduction in Na influx to the roots or an efflux increase via a salt overlay sensitive (SOS) pathway [52]. Conversely, Sun et al. [34] described a higher Na concentration in Alamo compared to five other switchgrass genotypes including Kanlow. In our study, both Kanlow and Trailblazer accumulated a high concentration of Na in shoots, although the concentration of Na was almost double in the stems of Trailblazer. Previous research has suggested that the accumulation of Na in stems is greater for less saline-tolerant genotypes such as rice [53,54] and that salinity tolerance is often linked to smaller reductions in K and Ca concentrations in plant tissues [55].

In general, lower concentrations of Mg were linked to increases in salinity only in shoots. This was accompanied by lower K levels in the shoots and roots of all genotypes in the salinity treatments that were concomitant with the increase in Na. These changes in cation concentrations may reflect a differential sensitivity to saline conditions. All genotypes showed an ability to largely maintain K levels in the leaves, which has previously been documented as an indication of salinity tolerance in wheat and barley [56]. However, the major reduction in the cation balance observed in Kanlow and Trailblazer could be a consequence of reductions in the absorption of the essential cations Ca, Mg and K [23,57] or a consequence of higher concentrations of Na. Our results indicate that the ability to exclude Na from the roots, as found in Alamo, was not related to a disruption in the cation balance; in fact, these plants showed similar Ca, Mg and K levels to the other two genotypes. These results could indicate that the ability to maintain nutritional homeostasis through the exclusion of Na from the roots, as found in Alamo, could have been of benefit, as it was not associated with a cation imbalance-related yield loss that could impact on the absorption of essential nutrients [23,58].

The production of salt glands has been previously reported as an additional mechanism of salinity tolerance in switchgrass [22]. According to our results, the production of salt glands was only significant in the Kanlow genotype, although it did not contribute to a reduction of Na in the leaves. It may be possible that the excretion of salt could have reduced the total content of salt in the aboveground biomass (lower Na content in stems), as previously reported for plants that do not exclude salt from the roots [59], but these reductions seemed to be of minor significance. Our findings are in line with the results observed by Kim et al. [28], who found that the production of salt glands was unlikely to contribute significantly to salt tolerance in switchgrass.

5. Conclusions

This experiment provides evidence for the existence of different mechanisms of salt tolerance within switchgrass genotypes. These mechanisms did not appear to be related to the different ecotypes (the lowland Alamo and Kanlow ecotypes had different responses), supporting previous research on the presence of salinity tolerant genotypes in both upland and lowland types [26]. Although intracellular compartmentation and the synthesis of osmoprotectants have often been associated with plant responses

to salinity, they were unlikely to have made a major contribution to salt tolerance in two of the studied genotypes (Kanlow and Trailblazer), with no positive impact on the final plant yield. Based on the current experiments, the lowland Alamo, which showed the lowest Na accumulation, out-yielded both Kanlow and Trailblazer at every salinity concentration. This indicates that salt exclusion could be the more important mechanism for achieving high biomass yields under saline conditions, which is consistent with the proposal that salt tolerance in many species is largely based on the exclusion of Na and Cl from the roots. Further experiments would need to assess how high yields could be achieved at a high salinity under field conditions while still maintaining a good biomass quality, and these are the ultimate objectives for successful bioenergy production.

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