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Salt tolerance variability among stress-selected *Panicum coloratum* cv. Klein plants

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Abstract

This work assessed intracultivar variability for salt tolerance within *Panicum coloratum* cv. Klein, explored some physiological parameters potentially associated with it and evaluated the contribution of cell division and expansion to the decreased leaf length observed under salinity. Individual plants that had survived severe stress environments in an established pasture were collected and clonal families were obtained by vegetative propagation. These were evaluated in a greenhouse, in pots with an inert substrate irrigated with nutrient solution containing 0, 200 or 400 mM NaCl. Salt tolerance was assessed from growth variables expressed as a percentage of non-salinized controls. Changes induced by salinity in carbon fixation, soluble sugars and compatible solutes were also measured. The selected plants showed 33% higher salt tolerance than plants from the same cultivar obtained from seeds, and variability for salt tolerance was detected within the group, suggesting these plants could be valuable germplasm for breeding programmes for saline areas. All selected plants accumulated low leaf blade Na concentrations (< 0·1 mM g^{-1} dry weight on average), and K concentrations tended to remain high under salinity. A kinematic analysis indicated a reduction in the number of cells in the division-only zone was the main cause of shorter leaves under stress. Although plants showed some differences in all these traits, they were not related to salt-tolerance variability within this group of stress-tolerant plants.

Keywords: *Panicum coloratum*, perennial grasses, phenotyping, salt tolerance, cell division, cell expansion

Introduction

Salt-affected soils are naturally present in more than 100 countries of the world. Many regions are also affected by irrigation-induced salinization, and the global area of salt-affected soils, including saline and sodic soils, is estimated to be 831 million hectares (Rengasamy, 2006). Soil salinity is a growing constraint to crop and forage production worldwide and has driven the quest for salt-tolerant germplasm. *Panicum coloratum* L. is a C4 perennial grass native to tropical Africa (Jones, 1985), and it is adapted to a wide range of soil and rainfall conditions. It is considered drought and frost tolerant (Tischler and Ocumpaugh, 2004), yet information on salt tolerance for this species is relatively scant and more knowledge on this aspect and its physiological determinants would be useful to enable breeding of this species for salt-affected areas.

Panicum coloratum is a cross-pollinated species (Pritchard and De Lacy, 1974), with some degree of apomictic reproduction (Rotar and Kretschmer, 1985), and it comprises two distinct botanical varieties: *P. coloratum* var makarikariense and *P. coloratum* var coloratum (Bogdan, 1977). Several cultivars have been released for each variety. Cultivars Bambasti, Pollock and Burnet belong to var. makarikariense, while Selection 75, Verde Kleingrass, Tamidori, Tayutaka and Solai were bred from var. coloratum (Armando *et al.*, 2013). Members of the complex have been

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introduced as forage grasses into several countries, including Argentina, Australia, Japan, Mexico and USA (Tischler and Ocumpaugh, 2004). While *P. coloratum* cv. Bambatsi has been mentioned as more salt tolerant than several tropical pasture legumes (Keating *et al.*, 1986), a comparison of salt tolerance between cultivars Klein and Bambatsi showed that cv. Klein accumulated more dry matter than Bambatsi in a saline field (Taleisnik *et al.*, 1998) and was also more salt tolerant in greenhouse experiments. Cultivar Klein was, therefore, considered suitable material for further investigation of variability in salt tolerance in this species.

Intraspecific selection for salt tolerance has been used to develop salt-tolerant cultivars (Flowers, 2004). Environmental constraints may disclose intraspecific variability in stress tolerance, as observed in *Elymus scabrifolius* populations established in areas with different stress intensity (Zabala *et al.*, 2011), where lines coming from saline habitats exhibited salt tolerance traits. As in many forage grasses, *P. coloratum* cultivars are highly heterozygous and represent panmictic populations where internal genotypic variability offers the possibility of selecting individuals with improved performance in specific traits (Walter *et al.*, 2012). The research reported in this study was based on the hypotheses that *P. coloratum* plants from an established pasture and that had survived fluctuating soil salinity, severe droughts and frost periods would exhibit greater salt tolerance than non-selected plants and that variability in salt tolerance could be found within the group. Therefore, the first two objectives of this study were to test these hypotheses. Collected plants were cloned by vegetative propagation, and several trials using a supported hydroponics system with potted plants were conducted in a greenhouse. Salt tolerance variability assessment based on one or several growth variables was compared. The convenience of including an internal control in all trials was also addressed.

As closely located plants in an established pasture could have resulted from vegetative propagation of a single mother plant, it was essential to verify whether collected plants were actually different. An intersimple sequence repeat (ISSR) analysis was performed with this purpose. This technique has been used to study genetic diversity in a number of crops including rice, beans, lupins, bananas and potatoes (Reddy *et al.*, 2002). ISSR combines the advantages of AFLP and microsatellite (SSR) analysis but, unlike the latter, prior sequence information for primer design is not required (Reddy *et al.*, 2002) and it is regarded as a simple and inexpensive, yet reliable technique for the assessment of genetic diversity in grasses (Paäakinskiene *et al.*, 2000).

The mechanisms of salinity tolerance have been extensively reviewed (Munns and Tester, 2008). Coincidences between salt tolerance and salinity-induced changes in several physiological traits, such as soluble sugars and compatible solutes concentration, and alterations in ion balance, have been reported in many species, and it is generally agreed that physiological traits which relate to salt tolerance may provide indirect selection tools for breeding purposes (Ashraf and Harris, 2004). The relation between physiological attributes and salt tolerance has not been explored in *P. coloratum*. In grasses, salt tolerance may be related to either Na exclusion or accumulation (Vasquez *et al.*, 2006). In species that rely on the first mechanism, adjustment to low substrate water potential usually involves K as the main cation for osmotic adjustment, and thus, high K/Na ratios are observed. The most intensively studied example of this response is wheat, where salt tolerance is highly related to leaf blade Na exclusion and high K/Na ratios (Munns *et al.*, 2012). As Na is considered to be toxic for metabolism (Maathuiss and Amtmann, 1999), Na-accumulating grass species regulate the cellular compartmentation of this cation (Apse and Blumwald, 2002) or have effective mechanisms to extrude it (Céccoli *et al.*, 2015), and these mechanisms are also related to salt tolerance. Therefore, as the third objective of this work, salinity-induced changes in leaf blade concentrations of K and Na, soluble sugars, proline and glycine betaine were assessed, and their relatedness to relative salt tolerance of the selected *P. coloratum* plants was considered.

The fourth objective was to gain insight on the cellular basis of reduced yield of this species under saline conditions. In forage grasses, yield is directly related to leaf area expansion and duration, aspects which are most sensitive to salinity (Taleisnik *et al.*, 2009). An examination of the underlying causes for reduced leaf growth may provide functional information on effects of salinity on grass yield (De Smet *et al.*, 2013). The contribution of cell expansion and division to leaf growth can be estimated from kinematic studies that use information on the spatial distribution of cell lengths and leaf elongation rates to calculate various growth parameters (Silk *et al.*, 1989). This type of analysis has been performed in several grass species to examine the association between meristematic activity, cell expansion and leaf growth (Volenec and Nelson, 1981; Schnyder *et al.*, 1987; Fiorani *et al.*, 2000), providing a first insight onto the causes of stress-induced growth alterations (Bernstein *et al.*, 1993; Kavanová *et al.*, 2006, 2008; Ortega *et al.*, 2006; Hu and Schmidhalter, 2008; Rymen *et al.*, 2010). However, it has not been previously applied to assess the cellular basis for reduced leaf growth under salinity in

P. coloratum. It was expected that this type of analysis would shed light on the basis for the suspected variability in the response to this stress among the selected plants in this study, and the last objective of this research was to obtain this information.

Materials and methods

Plant material

Sixteen individual plants were obtained in 2010 from a 2500 m^2 plot of *Panicum coloratum* cv. Klein plants that had been established 5 years earlier in Chascomús, province of Buenos Aires, Argentina (35°25′S, 57°43′W), on a typical Natracualf soil. The collected plants had survived fluctuating soil salinity and severe droughts and had remained green among all other, dry plants. They were transplanted to pots (10 L, 35 cm height) containing peat-enriched soil and each plant was subsequently propagated to obtain clonal families, herewith termed P1 to P16. All trials were performed in a naturally illuminated greenhouse, with plants grown in a supported hydroponics system using pots with an inert substrate (a mixture of perlite and washed river sand, 2:1 volume ratio) irrigated with nutrient solution. Air temperature and incident photosynthetically active radiation (PAR) in the greenhouse were measured and recorded every hour with a digital datalogger (Cavadevices SA™; Buenos Aires, Argentina). Supplemental illumination was provided with Powerstar HQI-T 400W/D (OSRAM) lamps set to a 16-h photoperiod with a mean irradiance of 197 μmol m^{-2} s^{-1} PAR.

Comparison of salt tolerance in stress-selected and non-selected plants

As all studies on the selected plants were performed in clonal families, to compare salt tolerance between selected plants and the original cultivar, plants obtained from *P. coloratum* cv. Klein seeds were propagated. Tillers with 2–3 leaves, from both selected and non-selected plants, were transplanted to PVC pots (5·5 cm diameter × 33 cm height), containing a mixture of perlite and washed river sand (2:1), which were placed in 59·5 × 40 × 18·5 cm plastic trays, 60 per tray. Pots were gradually salinized with half-strength Hoagland solution (Hoagland and Arnon, 1950) containing increasingly higher NaCl concentrations, supplied in steps (50, 100, 200 mM) every other day. Pots were subsequently irrigated manually, with 1·5 times the void pot volume provided once or twice a week to completely flush out any previously remaining solution. Tap water was added to replace water loss during the rest of the week. Conductivity in the drainage solution was monitored to assess the real salinity level to which roots were exposed. Salt tolerance was assessed from relative growth rate (RGR) measurements that were calculated as $[\ln(FW_f) - \ln(FW_i)]*(°Cd_i - °Cd_f)^{-1}$, where FW indicates fresh weight, and the subindices *i* and *f* indicate the initiation and harvest of the trial (Hunt, 1978).

Genetic characterization of the clones

To make sure that collected plants were not identical, they were genetically characterized by intersimple sequence repeat analysis (ISSR). DNA was extracted from live, mature leaf tissues and processed essentially as described by Assefa *et al.* (2003). After a preliminary test, 12 Invitrogen™ primers (Life Technologies Argentina, Invitrogen Argentina S.A., Buenos Aires, Argentina) were selected for further analyses based on their clear and reproducible patterns (Table 1). Amplification reactions were performed in a thermal cycler L series, Model MGL96G, version 2.0 (LongGene Scientific Instruments Co., Ltd., Hangzhou, China). Amplification products were separated in 1·5% agarose gels. Electrophoresis was performed at 80 V for 90 min and gels were stained with ethidium bromide, exposed to ultraviolet light and photographed. A 100-base pair ladder (PB-L Productos Bio-Lógicos, Benal, Argentina) was loaded on either side of the gel as a standard for the estimation of band size.

Each ISSR band was considered as an independent character or locus, and polymorphic bands were scored visually as absent (0) or present (1). Only reproducible, well-defined bands were considered. The banding pattern was used to calculate the genetic distances between the 16 clones using the square root of the complement of Dice's similarity coefficient, which

Table 1 Primers used in ISSR polymorphism analysis, total number of bands generated and number of polymorphic bands.

	Number of bands generated	
Primer sequence	**Total**	**Polymorphic**
$(CAC)_4GC$	10	9
$(GA)_9T$	10	10
$(GTG)_3C$	8	7
$(CAA)_5$	11	11
$(GACA)_4$	9	9
$(ACTG)_2$ ACCG (ACTG)	11	11
$(ACTG)_4$	11	10
$(CT)_8TG$	8	8
$(ACC)_5$	9	8

takes into account the presence or absence of bands. This method excludes negative co-occurrences. The coefficient can be interpreted as the proportion of coincidences in relation to the total number of bands obtained. Then, genetic distances based on banding patterns were subject to multivariate conglomerate analysis with Info-Gen (Balzarini and Di Rienzo, 2013) using the unweighed pair-group arithmetic average method (UPGMA).

Salt-tolerance variability among stress-selected plants

To accommodate sufficient replicates of each clonal family and treatment in the greenhouse, clones had to be characterized in successive trials; therefore, six trials were run to determine relative salt tolerance among these plants, using the set-up described earlier. Irrigation was manual in all trials but Trial 5, where pots were automatically irrigated and kept at field capacity. Two common clones, P4 and P15, were included in every trial to serve as internal controls. Table 2 indicates trial conditions and clones included.

It had previously been shown that this species can grow at 200 mM NaCl under greenhouse conditions (Taleisnik *et al.*, 1998), but to provide a stringent test for salt tolerance, in the current experiments, they were challenged with 200 and 400 mM NaCl. These concentrations are far higher than those expected to be found in the field, but were chosen to disclose variability within germplasm that was expected to exhibit a certain degree of salt tolerance.

Leaves (L) and tillers (T) per plant were periodically counted. Monocot leaf growth is often directly related to air temperature (Walter *et al.*, 2009). Hourly temperature records were used to calculate thermal time (°Cd) as the daily integral of the difference between recorded temperatures and a base temperature (Granier and Tardieu, 1998) of 10°C, following Ferri *et al.* (2006). Leaf emergence (LR) and tillering (TR) rates were calculated as a function of thermal time as $\times*(°Cd)^{-1}$, where $\times$ is either number of leaves (in LR) or tillers (in TR). At harvest (at about 600 °D), plants were separated into shoots and roots and fresh and dry weights were determined (SDW: shoot dry weight, RDW: root dry weight).

In every trial, relative-to-control growth data were generated for every variable (V), per clone (C_x) and salt treatment (S). They were calculated as follows: for each variable, results from every salinized plant from a given clone were divided by the average value of V (for the same clone), obtained under non-salinized (control) conditions (V_{C0}). This generated, per C_x relative values for every variable in each plant, which could then be statistically analysed.

Variability for salt tolerance among clones was calculated by three procedures (subsequently termed A, B and C). Input data in all three procedures were relative-to-control values for the various growth variables, described in the previous paragraphs. The procedures differed in two aspects: whether tolerance estimation was based on one (in A) or several growth variables (in B and C), and whether clone tolerance was based only on its own relative growth values (in A and B), or, alternatively, if each clone was compared to one of the common clones in every trial (in C).

Salt tolerance estimates in A were based solely on relative SDW values from all trials. The procedures termed B and C were based on several growth variables (SDW, T, L, TR, LR), all expressed as proportions of control. Data for all individual variables were subject to ANOVA, and several variables were simultaneously analysed with a principal component (PC) multivariate analysis (PCA) (Di Rienzo *et al.*, 2014). For B, the value of the first PC was then averaged per clone, across all trials, and used for ranking the clones.

Tolerance estimation by the third method (C) took into account that successive trials included different groups of clones and two common clones (Table 2). The comparison of results from every clone with those from P15 (one of the common clones), in each trial, provided a uniform basis for assessing all the trials. In every trial, after PCA, the first PC of each clone was

Table 2 Details of the six trials performed in this study.

Trial	Salinity levels (mM NaCl)	Clones included and number of replicates (*n*)	Average Temp. (°C)	Maximum PAR (μmol m^{-2} s^{-1})	Trial duration °Cd (d)
1	0, 200, 400	4, 5, 8, 9, 13, 14, 15, 16 (7)	29·75	379·33	567 (28)
2	0, 200, 400	1, 2, 3, 4, 7, 10, 11, 15 (7)	26·37	234·54	550 (35)
3	0, 200, 400	4, 6, 12, 15 (7)	22·23	nd	550 (45)
4	0, 200, 400	2, 3, 4, 5, 8, 9, 12, 14, 15, 16 (7)	26·60	213·56	683 (41)
5	0, 200	3, 4, 5, 9, 12, 14, 15, 16 (6)	25·40	nd	616 (40)
6	0, 200	2, 3, 4, 14, 15, 16 (7)	26·32	478·35	474 (30)

Temp., temperature; PAR, photosynthetically active radiation.Trial duration is indicated in °Cd and in calendar days (d).

compared to the corresponding value of P15 in the same trial [$(PC1_x - PC1_{P15})/PC1_{P15}$], where the subindex $_{P15}$ indicates the P15, and × indicates a given clone. Those results were averaged per clone, across trials, to provide an estimate of each clone's tolerance in comparison with P15.

Na and K concentration

In trials 1, 2 and 3 (Table 2), samples for Na and K determination were obtained from mature leaf blades, at harvest time. Tissues were suspended in 0·1N HNO_3. Ions in the suspension medium were determined by flame photometry. Samples were subsequently dried and weighed, and Na and K concentrations were calculated on a dry weight (DW) basis.

Carbon fixation and chlorophyll fluorescence

Carbon fixation was measured with a LI-6400 portable photosynthesis system (LICOR, Inc., Lincoln, NE, USA) in the most recently fully expanded leaf in plants that had been exposed to the final salinity level for at least 3 weeks. Chlorophyll fluorescence was measured in the same leaves with a FSM_2 fluorometer (Hansatech Inc., Norfolk, UK).

Sucrose, proline and glycine betaine determination

These compounds were measured in the expanding zone of leaf blades from newly formed tillers obtained from plants that had been exposed to salinity for at least 4 weeks (ca. 500°D). Proline was assessed in 100–150 mg leaf tissue samples and measured spectrophotometrically (Bates, 1973). Glycine betaine was determined according to Grieve and Grattan (1983) in 25 mg samples of dry leaf tissues.

Kinematic analysis of leaf growth

Plants from clones P2, P3, P4, P5 and P14 intended for kinematic analyses were propagated and grown in vermiculite as detailed above. After 2 weeks, they were transferred to the PVC pots and automatically subirrigated with half-strength Hoagland solution every 2 h, from 8:00 to 18:00 h. Salinity treatments (150 mM NaCl) were provided by gradually adding NaCl to the nutrient solution. Salt concentration was lower than in the previous trials because 200 mM NaCl was a too severe stress to obtain sufficient leaf samples.

Leaf length was measured with a ruler, from emergence until final length was attained. In a preliminary experiment, length was recorded in leaves 3, 4 and 5 in tillers initiated at least 2 weeks after plants had reached the final salt concentration in salinized plants. The increase in leaf length was linear for at least 4 d. The duration of leaf elongation was determined for each individual leaf. Sheath elongation was also followed in leaf 4, tillers were sampled at the same hour every day for 9 d, dissected to expose the growth zone of leaf 4, and the length of the sheath was measured under a stereoscopic microscope (4× lens). At least 10 plants were measured at every time point.

Kinematic analyses were performed in leaf 4 according to Rymen *et al.* (2010). Plants were harvested within 24 h of leaf 4 emergence, during the linear elongation phase, immediately fixed in FAA solution (2:1:10 v/v, 40% v/v formaldehyde: glacial acetic acid: 95% v/v ethanol) for 24 h, and then transferred to 70% v/v ethanol until processed. Leaf 4 was isolated, stained with calcofluor (1 mg mL^{-1}; Sigma) for 30 min, and the growth zone was observed with an inverted Nikon Eclipse Cs1 spectral confocal microscope (Nikon Corporation, Tokyo, Japan) to visualize cell walls. Samples were illuminated with a 405 nm diode laser beam and cell wall autofluorescence observed through 475/25 BP and 515/30 BP filters (blue and green channels respectively). Moving distally from the leaf base in the abaxial side, epidermal cell lengths photographed in files adjacent to the stomata until length was constant. Images were acquired using 20 ms pixel dwelling time and 1024 × 1024 dpi resolution. Cell lengths were measured using the Cell-o-Tape macro (French *et al.*, 2012) developed for the ImageJ open source software.

Estimation of the leaf meristem length

The length of the cell division zone was estimated in an additional subset of control and salinized plants, harvested and fixed as above. The growth zone of leaf 4 was exposed and tissues were stained with 4′,6-diamidino-2-phenylindole (DAPI; 2 μg mL^{-1}; Sigma) for 30 min according to Rymen *et al.* (2007). Nuclei were observed by confocal microscopy. Mitotic figures in epidermal cell files adjacent to main vein were identified and counted, moving distally from the ligule to the apex. The size of the meristem was calculated as the distance from the ligule to where mitotic figures were initially absent. The number of cells and residence time in the division and the elongation zones, cell production, cell division rates and cell cycle duration were calculated based on the measurements of leaf elongation rate, cell length distribution and length of the meristem, according to Rymen *et al.* (2010).

Shoot apical dome dimensions

Tillers of *P. coloratum* were collected when leaf 4 was growing linearly. Whole tillers were fixed (FAA, as described above). Subsequently, shoot apical meristems were dissected under a stereoscopic microscope and placed on a microscope slide with a drop of 50% glycerol. Slides were observed with the confocal microscope and settings mentioned above. Z-stack images were acquired with a 20× apochromat objective using a 30 mm pinhole, 10 ms pixel dwelling time and 512 × 512 dpi resolution. Between 60 and 120 frames, spanning the whole dome were acquired and a 3D reconstruction was performed in every case. The virtual manipulation of the 3D deconvolved image, allowed examining the point of primordial differentiation.

As a leaf primordium develops and differentiates from the apical dome, a swirling groove appears around it that can be used to define the base of the dome (Figure 7a). Thus, apex diameter was defined at the length of the line joining the base of the developing primordium and the opposite side of the apex, at the groove (white line, Figure 7b). Apex height was defined as the length of the line connecting the midpoint of the width and the highest point of the dome (broken white line, in Figure 7b). The line measuring tool of the image acquisition program Nikon EZ-C1 (version 3.09) with manufacturer calibration was used to determine basal diameter and height of the apices.

Results

Salt tolerance in stress-selected and non-selected *P. coloratum* plants

When growth of stress-selected and non-selected *P. coloratum* plants was compared, it was observed that while relative growth rates were similar in both types of plants in the absence of saline stress, they were more negatively affected by salinity in non-selected plants, suggesting selected plants were more salt tolerant (Figure 1).

Genetic characterization of stress-selected *P. coloratum* plants

The ISSR analysis performed to determine whether the collected plants were actually different showed all plants had different banding patterns, confirming that they were different. A multivariate conglomerate analysis of ISSR bands with a cofenetic correlation of 0·764 suggested plants could be grouped into three clusters (Figure 2), indicating genetic proximity, where P15 was in a cluster by itself, another included P1, P10, P16, P4, P5, P11, P7 and P2, and the remaining plants were in the third cluster.

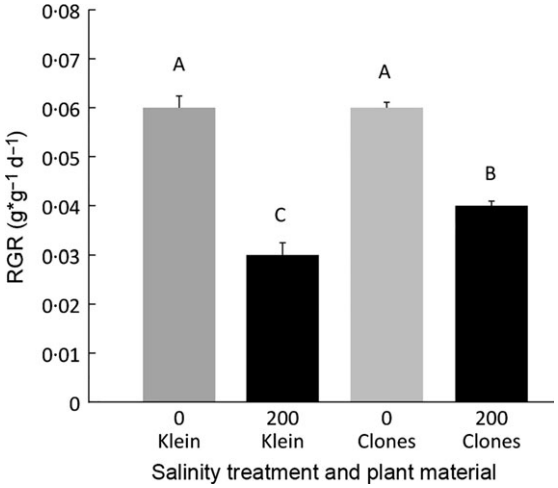

Figure 1 Relative growth rates (RGRs) of unselected (Klein) or stress-selected (clones) of *Panicum coloratum* plants grown under 0 or 200 mM NaCl. Results are means of a total of 113 plants grown for 23 d under those conditions. Different letters indicate significant differences at $P < 0{\cdot}05$ (DGC).

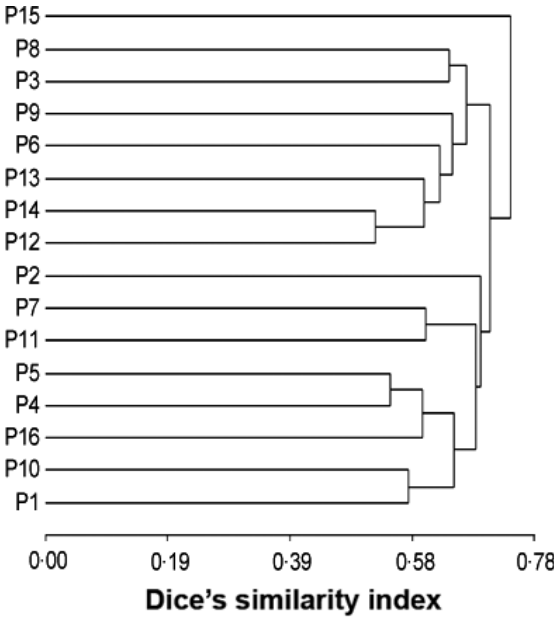

Figure 2 UPGMA cluster analysis of 16 clones of *Panicum coloratum* based on Dice's similarity coefficients.

Salt tolerance variability within the stress-selected *P. coloratum* plants

Salt tolerance assessment in the stress-selected plants was based on growth data collected in six trials. In

the first trial, it was observed that clones P4 and P15 had contrasting growth under non-saline conditions (Figure 3) and different sensitivity to the salt treatments. On account of these features, they were chosen as internal controls in the subsequent trials, and also because they were relatively easy to propagate and sufficient replicates could be readily obtained.

As expected, most growth variables in all clones were negatively affected by the salt treatments and effects were more intense at 400 mM NaCl, the highest salinity level tested. At this concentration, effects on growth were intense but did not contribute further to distinguish salt tolerance among the clones, so results from 400 mM were not considered for this purpose. Multivariate growth analysis including SDW, T, TR, L and LR were run on the results of every trial (Table 3). The first two PCs explained more than 70% of the variability among clones in all trials. Autovector values varied among trials and salinity treatments; nonetheless, T and SDW had the highest weights to distinguish among clones.

When the first trial was harvested, it was noticed that some plant material was lost in the process of separating roots from the substrate, so an accurate estimation of root weight was not feasible, although it is potentially useful trait for estimating salt tolerance, as reported in *Agrostis stolonifera*, another perennial grass (Kik, 1989). Therefore, this variable and associated ones (whole plant weight, shoot/root ratios) were not included in the analyses.

Salt tolerance was calculated by three procedures (A, B and C), using 'relative-to-control' values for the various growth variables as input data, either as such (as in A and B) or compared, in every trial, to those in one of the common clones (in C). For C, one common clone was chosen as a basis for comparisons. The decision was based on the relative salt tolerance of these clones, observed in Trial 1 (Figure 3). P15 was intermediate in salt tolerance, so it was chosen as the basis for comparisons, rather than the salt-sensitive P4.

The results of salt tolerance assessments by the three procedures are shown in Figure 4. In Figure 4a, clones are ordered according to increasing values of the % of SDW attained at 200 mM NaCl as compared to the corresponding values under non-saline conditions (procedure A). Two groups were distinguished by the DGC (test of Di Rienzo, Guzmán and Casanoves) statistic of InfoStat (Di Rienzo *et al.*, 2014), clone P15 was in the limit between them, the most tolerant clones were P8 and P5, while the most susceptible was P4. Figure 4b depicts clones ordered according to increasing values of PC1 (white columns), the synthetic variable generated after multivariate analysis of several growth variables, all expressed as % of their values under non-saline conditions (procedure B). The grey columns in the same figure indicate results of comparisons with a common clone, that is procedure C. It can be seen that all procedures detected variability in salt tolerance and that the ranking in relative salt tolerance among clones was, overall, very similar. According to all three evaluations, and considering only clones that were included in more than one trial (all, except P1, P6, P7 and P10), P15 was always intermediate in tolerance, the most salt-tolerant clones were P8, P5 and P3, while the most salt-susceptible clone was P4, followed by P13, P14 and P16.

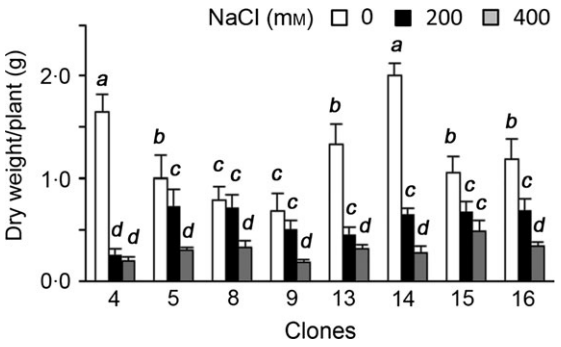

Figure 3 Shoot dry weight and tillers per plant in clones of *Panicum coloratum* grown under 0, 200 and 400 mM NaCl salinity for 23 d. Bars are means ± s.e. of six samples, Trial 1. Different letters above each column indicate significant differences at $P < 0{\cdot}05$ (DGC).

Carbon fixation, chlorophyll fluorescence, organic osmolyte accumulation and changes in K and Na concentration under salinity

Photosynthetic carbon fixation and chlorophyll fluorescence were not affected by the saline treatments, and there were also no differences among clones (not shown). Proline concentration significantly increased in salt-treated plants of all the clones, from an average of 0·76 μg g^{-1} DW to nearly 19 μg g^{-1} DW (Table 4), salinity induced significant increases in sucrose in clones P3 (100%) and 14 (50%), whereas glycine betaine significantly increased (122%) in salt-treated plants of P3, 4,15 and 16. While significant increases in all solutes were registered in salt-treated plants of clone P3, which was rated within the most salt-tolerant group, this was not observed in clone 2, which was also rated as salt tolerant. Conversely, significant increases in some of these compounds were also observed in clones P4 and P14, rated as relatively salt

Table 3 Principal components analysis (PCA) results from six successive trials where *Panicum coloratum* clones were phenotyped for growth responses to three salinity levels (0, 200 and 400 mM NaCl). The proportion of variance among observations explained by the first (C1) and second (C2) components is shown.

			Prop. of variance		Autovector values				
Trial	Included clones	Salinity (mM NaCl)	C1	C2	SDW	T	TR	L	LR
1	4, 5, 8, 9, 13, 14, 15, 16	0	0·45	0·31	0·64	0·61	0·26	−0·19	−0·33
		200	0·59	0·2	0·51	0·51	0·44	0·48	0·22
2	1, 2, 3, 4, 7, 10, 11, 15	0	0·78	0·13	0·47	0·48	0·43	0·4	0·44
		200	0·49	0·35	0·52	0·58	0·59	0·21	−0·08
3	4, 6, 12, 15	0	0·72	0·26	0·52	0·28	0·5	0·47	0·44
		200	0·46	0·38	0·54	0·66	−0·02	−0·38	−0·36
4	2, 3, 4, 5, 8, 9, 12,14,15,16	0	0·41	0·33	0·44	0·55	0·47	−0·18	−0·05
		200	0·46	0·30	0·44	0·56	0·47	−0·1	0·05
5	3, 4, 5, 9, 12, 14, 15, 16	0	0·48	0·31	0·5	0·49	0·43	0·39	0·21
		200	0·68	0·16	0·46	0·48	0·45	0·17	−0·36
6	2, 3, 4, 14, 15, 16	0	0·57	0·24	0·49	0·51	0·46	0·17	−0·16
		200	0·53	0·31	0·46	0·38	0·44	−0·18	0·39

SDW, shoot dry weight; T, tillers per plant; TR, tillering rate; L, leaves per plant; LR, leaf appearance rate.Autovector values are for C1.

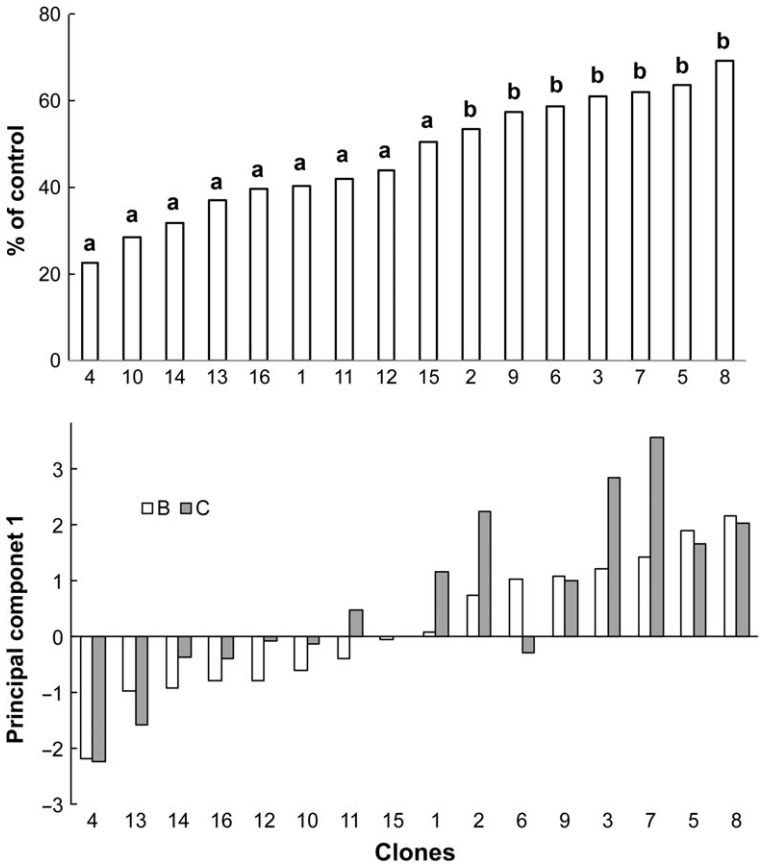

Figure 4 Salt tolerance of stress-selected *Panicum coloratum* clones. (a) Shoot dry weight under 200 mM salinity as percentage of weight under non-saline conditions (% of control). Shoot cuttings were grown under the mentioned conditions for 23 d. For each clone, bars indicate averages of all plants in all trials. Different letters indicate significant differences among clones at $P < 0{\cdot}05$. (b) Principal component 1 (PC1) scores from a principal components analysis of vegetative growth variables determined under salinity (200 mM NaCl), expressed as proportions of the corresponding values under non-saline conditions in 16 clones of *P. coloratum*. Columns B: Scores of PC1. Columns C: Scores of the same variable, normalized to clone P15 in every trial. Clones are arranged according to increasing values of B.

susceptible within this group of genotypes. Therefore, a clear correlation could not be drawn between changes in these solutes and variability in salt tolerance.

While clones showed different K concentrations in non-saline conditions, none showed significant changes under salinity (Figure 5a). Leaf blade Na concentrations at 200 mM NaCl (Figure 5b) were very low in comparison with K concentrations at the same salinity level, resulting in K/Na ratios higher than 35. Na concentrations were higher at 400 mM NaCl treatments; however, no significant differences among clones in Na accumulation were observed at either salinity level.

Table 4 Proline, sucrose and glycine betaine (GB) concentration in the growth zone of young leaves of *Panicum coloratum* clones grown under non-salinized (C) and salinized (S: 200 mM NaCl) conditions. Each number is the average of 3 independent determinations.

Clone	Condition	Proline ($\mu g\ g^{-1}$ DW)	Sucrose ($\mu g\ g^{-1}$ DW)	GB ($\mu g\ g^{-1}$ DW)
2	C	0·33 C	9·91 C	394·87 AB
	S	14·13 B	15·91 C	547·07 AB
3	C	0·58 C	15·3 C	328·9 BC
	S	23·84 A	29·65 A	647·69 A
4	C	0·64 C	10·83 C	538·26 BC
	S	19·08 AB	17·12 C	1011·86 A
14	C	0·42 C	16·12 C	357·72 BC
	S	19·3 A	23·63 B	583·4 AB
15	C	1·83 C	13·71 C	367·1 BC
	S	18·13 AB	15·08 C	920·77 A
16	C	nd	nd C	333·52 BC
	S	nd	nd C	900·46 A

Different letters in each column indicate significant differences ($P < 0·05$) among averages.

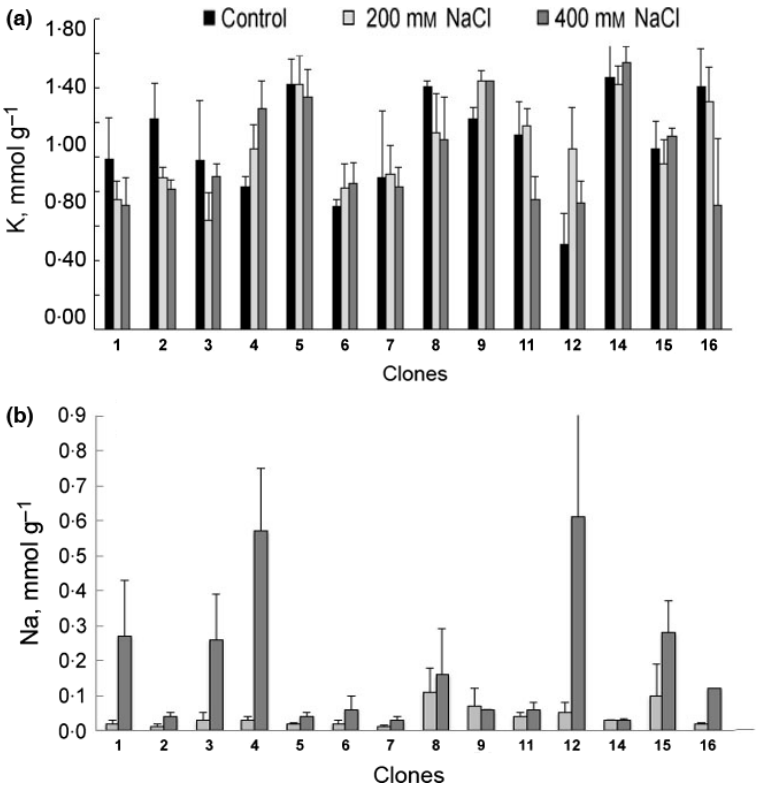

Figure 5 Potassium (a) and sodium (b) concentration in leaf tissues from clones of *Panicum coloratum* grown under 0, 200 and 400 mM NaCl salinity for 23 d. Bars are means ± s.e. of three samples.

Effects of salinity on leaf growth, cell divisions and expansion

Clone P3 was used for calibrating sampling conditions. All leaves in all tillers were measured under control and saline conditions (150 mM NaCl), but only leaves 3, 4 and 5 had enough replicates to be subject to statistical analysis. No significant tiller × treatment interactions in final leaf length or elongation rates (LER) were detected (Table S1) for every leaf, which allowed pooling, per treatment, homologous leaves from different tillers for growth analyses.

Kinematic analyses were subsequently performed in leaf 4 from clones P2, P3, P4, P5 and P14. As shown above, P2, P3 and P5 were characterized as relatively more salt tolerant, while P4 and P14 were less tolerant. For this purpose, leaf 4 was harvested on the second day after emergence (approximately 20°Cd from emergence), when expansion rates were linear (Figure 6a). At this stage, sheath lengths were 0·15 ± 0·04 mm in control and 0·12 ± 0·02 mm in salt-treated plants, about 1% of total leaf length, and therefore did not contribute significantly to it (Figure 6b). In all clones, the studied leaves were shorter in salinized plants and leaf elongation rates were lower under salinity (Table 5); however, elongation rates were affected similarly by this condition in all the clones, regardless of their relative salt tolerance.

Mitotic figures were observed in the first 5 mm from the ligule in leaves from control plants, as seen in P3, but declined closer to the ligule in salinized plants, and salinity exerted a significant effect on the length of the division zone, shortening the length of the meristem and the number of cells within it (Figure S1). The cell cycle became slower in salt-treated plants, which was then reflected in a tendency to prolong the residence time of cells in this region. A slower cell cycle resulted in reducing the average number of cell cycles in the meristem. It was also determined whether shoot apical domes generating leaf primordia were smaller in salinized plants and could thus provide lower number of cells entering the leaf primordium. However, apex dimensions were not affected by the salt treatment (Figure 7c).

Although salinity did not affect the length of the elongation-only zone nor the number of cells in it, in this case, the residence time was prolonged in this zone (Table 5). This could contribute to buffer the effects of salinity on the final length of cells in salinized plants, as mature cells in salinized plants reached the same length as those in controls.

In sum, leaves in salinized plants were shorter essentially because the number of cells in the meristem was negatively affected, and fewer mitosis events were observed in that zone. However, none of these parameters were different among clones that had different sensitivity to salinity.

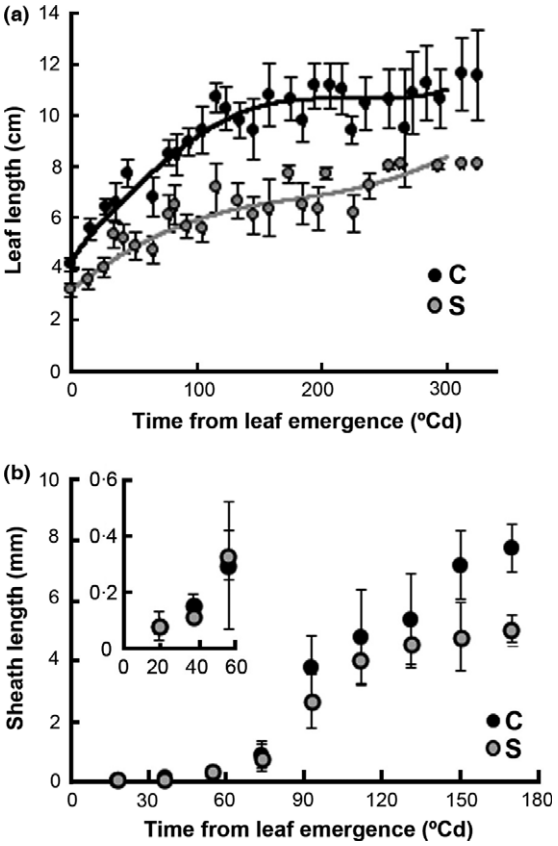

Figure 6 Whole leaf and sheath length in leaf 4 of *Panicum coloratum* tillers from plants treated with 0 (C) or 150 mM NaCl (S), measured on successive days after emergence. (a) Total length of leaf 4 (blade plus sheath). (b) Detail of sheath length on days 1–3, when leaves were collected for the experiments detailed in the text. Dark symbols, control (C); light symbols, salinized (S). Each point is the mean ± s.d. of 10 leaves from different tillers and independent plants. Inset in B: detail of sheath length from leaf emergence to 60°Cd.

Discussion

In natural ecosystems, environmental conditions that limit plant performance have resulted in adaptation, acclimation and speciation (Nilsen and Orcutt, 1996). Grime (1977) observed that severe stress exerts a dominant and more immediate impact upon species composition and vegetation structure. He suggested stress-adapted plants expressed a basic similarity in the mechanisms that allow them to adapt to different forms of severe stress. Salinity, among other stress

Table 5 Effect of salinity (150 mM NaCl) on the elongation rate and cellular characteristics of leaf 4 of *Panicum coloratum*.

Parameter	Control	Salt treated
Leaf elongation rate (mm h^{-1})	1·52 B	0·77 A
Mature cell length (μm)	116·08 A	116·06 A
Length, meristem (mm)	7·82 B	5·61 A
Length, elongation-only zone (mm)	13·61 A	12·60 A
Total length of the growth zone (mm)	21·53 B	18·41 A
No. of cells, meristem	411·63 B	304·04 A
No. of cells, elongation-only zone	199·68 A	188·46 A
Average cell division rate (cells cell h^{-1})	0·03 B	0·02 A
Res. time in the cell division zone (h)	192·35 B	295·01 A
Cell cycle duration (h)	22·12 A	35·77 B
Number of cell cycles per cell (n)	8·65 A	8·19 B
Cell flux in the mature zone (cells h^{-1})	13·42 B	7·42 A
Res. time, elongation-only zone (h)	15·53 A	33·38 B
Cell relative elongation rate (μ μh^{-1})	0·07 B	0·04 A

Different letters indicate significant differences at $P < 0·05$.

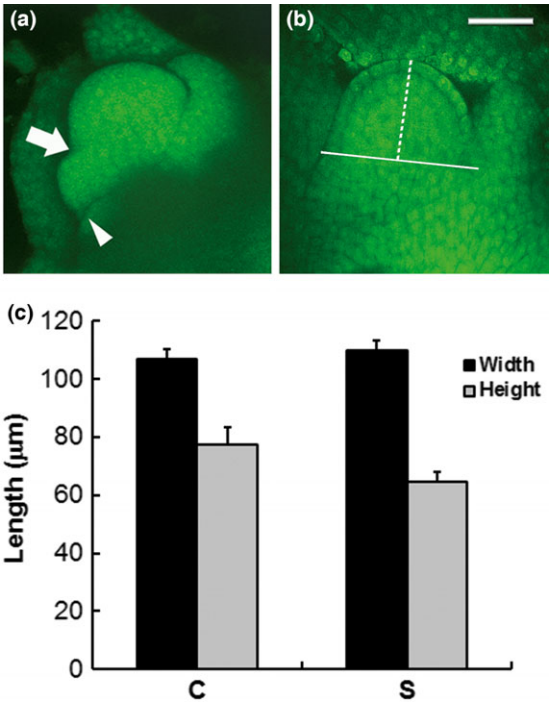

Figure 7 Shoot apical meristem (SAM) dimensions of *Panicum coloratum* tillers from plants treated with 0 (C) or 150 mM NaCl (S). (a) Apex 3D reconstruction from a z-stack series of images. The long arrow marks the upper contact point between the apex and the surrounding groove defined by the youngest primordium. The arrowhead marks the lowest observable ending point of the groove. (b) Image of the apex middle section. The white and dashed lines mark apex base (width) and height respectively. (Bar is 50 μm). (c) Width and height of apices from control and salinized plants. Each bar is the mean ± s.e. of 10–14 plants.

factors, has been regarded as one of the main environmental forces driving the increased occurrence of C4 species in Argentinean grasslands (Feldman *et al.*, 2008). Varietal differences in salt tolerance have been known since the 1930s, and intraspecific selection for salt tolerance has been used to develop salt-tolerant cultivars (Flowers, 2004). Surviving *P. coloratum* plants collected from a field plot that had been subject to extreme stress conditions expressed higher tolerance than non-selected plants of the same cultivar (Figure 1), confirming selected plants had salt tolerance mechanisms.

Salt-tolerance variability among stress-selected *P. coloratum* plants

The next step in this work was to attempt to identify variability for salt tolerance within the group. The plants in this study had been collected from a plot established several years before; thus, closely located plants could have resulted from vegetative propagation of a mother plant. It was therefore essential to verify first that the collected plants were actually different. An ISSR analysis confirmed they were all different (Figure 2), although genetic proximity was detected among them, as conglomerate analysis grouped several plants in two of the clusters.

Field trials that include yield assessment can provide the most reliable information on salt tolerance (Royo *et al.*, 2000); notwithstanding, evaluations of salt tolerance are most commonly carried out in greenhouses, under relatively controlled conditions (Munns and James, 2003) where hydroponics set-ups ensure the level of salinity to which plants are exposed. A hydroponics system was used in this work. Available greenhouse space for screening purposes is

often a constraint and encourages the use of trait-based rather than biomass information for screening purposes (Munns and James, 2003). However, in our case, available physiological information on salt tolerance of *P. coloratum* was very scant and there was none on the performance of these clones, so we opted for a controlled system that could be easily replicated to compare growth in batches of different plants. These trials always included two common clones: P4 and P15. Of these, P15 was subsequently chosen as the internal control for its intermediate response to salinity, as opposed to the higher sensitivity of P4.

Variability in salt tolerance in the *P. coloratum* clones was estimated by three procedures. In the procedure termed 'A', tolerance was based only in SDW, while in the other two it was based on several variables. The contribution of several variables to overall salt tolerance has had various resolutions in the literature. Multiple regression analysis was used by Rajendran *et al.* (2009) to determine the weighting of various mechanisms to salt tolerance indices, using the reduction in shoot area as the dependent variable. When we attempted to apply this analysis to assess salt tolerance, using SDW as dependent variables, the coefficients of the other variables differed among clones and were affected by the treatments. Therefore, this tool was not practical for our purpose. An instrument that would distinguish among clones in a single space was required. PCA and biplot graphs are statistical techniques used to reduce dimensions in order to examine all data in a space smaller than that of the original variables (Di Rienzo *et al.*, 2014). This technique had been successfully used to identify salt-tolerant genotypes of *Panicum hemitomon* and *Spartina alterniflora* (Hester *et al.*, 1996) and in the flooding response of the same species (Lessmann *et al.*, 1997). The inclusion of several growth variables (in the 'B' procedure) did not modify the clone tolerance ranking substantially, as compared to the 'A' procedure, indicating that, in our case, SDW was a strong tool to assess salt tolerance in these plants.

It was expected that comparisons based on a common clone would reduce variability in the analysis. However, the comparison of coefficients of variability (Mean/s.e.) in procedures 'B' and 'C' did not sustain this expectation. Thus, the inclusion of common clones in all trials served as internal control of consistency for the trials, but it did not improve the assessment tool. In short, it was possible, by any of the three procedures, to detect salt-tolerance variability within a group of genotypes that had already expressed a high degree of stress tolerance. Among the clones in this study, the most salt tolerant were P8, P5 and P3, the most susceptible was P4, and clone P15 was intermediate. Therefore, identified plants with higher salt tolerance are potentially useful germplasm to incorporate into a breeding programme. They expressed stress tolerance under field conditions, as they were surviving plants collected from an established pasture, but they must be tested for productivity under stress, and heritability of stress tolerance after poly-crosses should also be assessed. Such assessments are currently under way.

Association of salt-tolerance variability with physiological traits

Some physiological traits have often been reported to be associated with salt tolerance and may potentially be used as indirect selection tools. Among others, leaf blade Na exclusion could distinguish between some salt-tolerant and salt-susceptible durum wheat genotypes (Munns and James, 2003). Proline, glycine betaine and sucrose accumulation tend to increase under saline conditions and their levels may be related to salt tolerance in some species (see review by Ashraf and Harris, 2004), but not in others, such as in the perennial grasses *Panicum hemitomon*, *Spartina patens* and *S. alterniflora* (Hester *et al.*, 2001). In this study, while *P. coloratum* clones could be distinguished on the basis of growth under saline and non-saline conditions, none of the physiological measurements we performed correlated with this diversity, suggesting that the differences in growth were associated with other physiological aspects, not considered in this work. It may be possible that as all plants exhibited a certain degree of stress tolerance, physiological variables that can distinguish between salt-tolerant and salt-susceptible genotypes (Ashraf and Harris, 2004) did not reflect differences within this group of plants. As mentioned earlier, a plethora of mechanisms contribute to plant salt tolerance (Munns and Tester, 2008), and the pyramiding concept in breeding for increased salt tolerance is based on the incorporation of several different mechanisms that contribute to this end (Yeo and Flowers, 1986). Whether different salt-tolerance mechanisms exist within our group of plants could not be determined from our study, and the answer to this question requires other physiological and molecular approaches. A time-course analysis of the response to salinity could possibly reveal variability in mechanisms related to osmotic and ion-specific effects (Munns and Tester, 2008). Enzymes and non-enzymatic compounds that participate in oxidative stress balance have been associated with stress tolerance in many cases, including subtropical forage grasses (Luna *et al.*, 2000, 2002; Muscolo *et al.*, 2012), and their assessment may provide some clues to the variability in salt tolerance among stress-selected plants.

Contribution of cell division and expansion to decreased leaf length attained in salinized plants

Leaf growth reductions are among the earliest and most conspicuous effects of salinity; therefore, the effects of salinity on the cellular bases of leaf growth were examined in these plants, and it was expected that they would reflect the differences in salt tolerance detected earlier. As expected, final leaf length and leaf elongation rates were reduced in salt-treated plants. There was a positive correlation between leaf elongation rate decrease and the reduction in the number of cells in the leaf meristem of salt-treated plants, suggesting that processes related to cell division may be contributing to determine leaf length under salt stress in these plants. Similar causes for reduction of leaf elongation were reported in tall fescue under salinity (Gastal and Nelson, 1994) and in maize under phosphorus deficiency and cold nights (Assuero *et al.*, 2004; Rymen *et al.*, 2007). In those works, the reduction of leaf growth was attributed to a reduction of meristematic activity with marginal effects of cell elongation. This decrease in cell number might result from either a longer duration of single cell division cycle or a shorter developmental window for cell proliferation, but we have not explored the second alternative. Our kinematic analysis data indicate that cell division rates were significantly reduced in growing leaves of salt-treated plants, in association with a prolonged duration of the cell cycle, which resulted in a reduced flux of cells to the mature zone in salinized plants.

Variation in the number of founder cells recruited from the shoot apical meristem (SAM) to form the leaf initial could influence final leaf area, although no clear evidence for this idea has been found (Gonzalez *et al.*, 2012). Mahmoodzadeh (2008) indicated that SAM size was reduced in salinized canola plants. While no significant differences were found in shoot apex size between control and salt-treated plants in the current work, meristem activity was not measured and further studies are needed to elucidate the mechanisms involved in the reduction of the cell division zone in *P. coloratum* leaves from plants grown under salinity.

Despite reductions in final leaf length in salt-treated plants, mature cell size was not affected by the stress (Table 5). Evidence in the literature indicates reductions in final leaf size by stress conditions can be accomplished with or without effects on mature cell size. Reductions in mature cell size under stress have been reported for *Phaseolus vulgaris* L. under salt stress (Neumann *et al.*, 1988); for *Salix viminalis* L. and sunflower under nitrogen deficiency (MacDonald, 1989; Palmer and Davies, 1996); and for sunflower, maize and wheat under phosphorus deficiency (Jacob and Lawlor, 1991), while mature cell size was not reduced in other cases involving the same stresses (Gastal and Nelson, 1994; Assuero *et al.*, 2004; Rymen *et al.*, 2007; Hu and Schmidhalter, 2008), possibly implying that overall post-meristematic cell expansion was unaffected. This may be happening in the plants in this study. The tendency to prolong residence time in the elongation zone may have contributed to the attainment of mature cells with similar size in leaves from control and salt-treated plants. This prolongation may be sustained by changes in wall rheological properties that prevent cell walls from tightening (Lockhart, 1965; Cosgrove, 1997). Such compensatory mechanism has been observed in many species such as *Chloris gayana* (Ortega *et al.*, 2006) under salt stress, wheat in response to soil impedance (Beemster and Masle, 1996), Arabidopsis and sunflower under water stress (Aguirrezabal *et al.*, 2006; Pereyra-Irujo *et al.*, 2008). Mechanisms involved in the control of wall rheology under salinity may be responsible for prolonged growth under stress. In *Chloris gayana*, extractable xyloglucan endotransglycosylase was suggested to be necessary for maintaining elongation under these conditions (Ortega *et al.*, 2006), and in maize leaves, the expression of three expansin genes responded to developmental, genetic and environmental cues (Skirycz and Inzé, 2010). Alternatively, leaf growth prolongation under stress could have been due to a prolonged period of cell divisions, as reported in sunflower (Pereyra-Irujo *et al.*, 2008); however, no evidence for prolonged cell divisions was found in our work.

Conclusions

This research assessed variability for salt tolerance within *P. coloratum* cv. Klein, explored some physiological parameters potentially associated with this variability and evaluated the contribution of effects of salinity on cell division and expansion to the decreased leaf length observed in plants grown under such conditions. It is concluded that *P. coloratum* plants collected from an established field plot that had been exposed to multiple environmental stresses expressed higher salt tolerance than non-selected plants, and variability for salt tolerance was detected within the group, which suggests they could be useful for incorporation into a breeding programme for salt tolerance in this species.

All analysed plants can be considered to be Na excluders and to maintain high leaf blade K/Na ratios under salinity. Although stress-selected plants showed some differences in these traits, they were not related to salt tolerance differences, and the same can be said

for the other physiological traits explored: alterations in sugar, proline and glycine betaine accumulation under salinity. Therefore, salt-tolerance variability did not correlate with variability in several physiological traits usually related to salt tolerance, which consequently could not be suggested as indirect selection tools for salt tolerance.

Reduction in the number of cells in the leaf division-only zone was the main cause for shorter leaves under stress. This aspect did not differ among the studied plants.

Retrospectively, the lack of relation between physiological traits and salt tolerance variability perhaps should not have been altogether unexpected, given the genetic proximity among plants and their intrinsic stress tolerance, which would require more sensitive physiological and molecular tools to establish the basis for the observed stress tolerance variability.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. ANOVA probability (*P*) values for significantly different means among treatments and tillers, of growth variables measured on leaves 3, 4 and 5 of *P. coloratum* clone P3.

Figure S1. Mitotic activity in the growth zone of leaf 4 of *P. coloratum* tillers from plants treated with 0 (C) or 150 mM NaCl (S).

