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Leandro Cáceres
Centro de Investigaciones Agropecuarias, Argentina

Edith Taleisnik
Centro de Investigaciones Agropecuarias, Argentina

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Leandro Cáceres and Edith Taleisnik

CONICET Instituto de Fisiología y Recursos Genéticos Vegetales - Centro de Investigaciones Agropecuarias (IFRGV-CIAP), INTA, Camino a 60 Cuadras, Km 5.5, X5020ICA Córdoba, Argentina

Contact email: etaleisnik@conicet.gov.ar

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Introduction

Soil salinity is a growing constraint to crop and forage production worldwide and has driven the quest for salt-tolerant germplasm. Perennial forage grasses are regarded as choice alternative for the productive use of saline areas as well as for mitigating salinity in these areas (Ridley and Pannell 2005). *Panicum coloratum* L. is a C4 perennial grass native to tropical Africa, adapted to a wide range of rainfall conditions which makes it attractive as a forage for semiarid areas (Jones 1985). In forage grasses, yield is directly related to leaf area expansion and duration and the purpose of this work was to analyse the underlying cellular causes for reduced leaf growth under saline conditions. Kinematic studies on the spatial distribution of cell lengths, along with information on leaf elongation rates, can be used to calculate the contribution of cell expansion and division to leaf growth (Silk et al. 1989) and provide a first insight to the causes of stress-associated reductions in leaf expansion (Rymen *et al.* 2010). While kinematic analyses have been performed in several grass species, including perennial grasses (Volenec and Nelson 1981, Schnyder *et al.* 1987, Fiorani *et al.* 2000), to determine, for example, the association between leaf growth, meristematic activity and cell expansion, however, the contribution of alterations in cell division and expansion to salt-associated reductions in leaf size of a perennial forage grass had not been explored before.

Methods

Plant growth

P. coloratum cv Klein plants obtained from an established pasture growing in a salt-affected area, were brought to a greenhouse, transplanted to pots containing peat-enriched soil and subsequently vegetatively multiplied to obtain clonal families. Tillers with 2-3 leaves, from one of such clones, were transplanted to PVC pots (5.5 cm diameter x 33 cm height), containing a mixture of perlite and washed river sand (2:1), and pots (60) were in turn placed into 59.5 x 40 x 18.5 cm plastic trays. Pots were automatically sub-irrigated every 2 h, from 8 to 18 h, with half-strength Hoagland solution. Salinity treatments were provided by gradually adding NaCl to the nutrient solution until 150 mM was reached.

Leaf growth analyses and flow cytometry

Leaf 4 elongation was analysed in tillers that developed after plants reached the final salinity level, and similar tillers in non-salinized plants. Leaf and sheath length was measured with a ruler once a day, at the same hour, from emergence until final length was attained. The increase of leaf length with time was linear for at least 4 d. Leaves for kinematic analyses were harvested within 24 h of emergence, during the linear elongation phase, fixed with FAA (formaldehyde: acetic acid: ethanol, 2:1:10 v/v) for 24 h, and then transferred to 70% v/v ethanol until processed. To visualize cell walls, tissues were stained with Calcofluor (1 mg/mL, Sigma) for 30 min and observed with an inverted Nikon Eclipse Cs1 Spectral Confocal microscope. Moving distally from the leaf base, in the abaxial epidermis, epidermal cells length were measured in cell files adjacent to the mid-vein, until length was constant. An additional subset of leaves was used to estimate the length of the division zone. Tissues were stained with 4',6-diamidino-2-phenylindole (DAPI; 2 µg/ml) for 30 min according to (Rymen, Fiorani *et al.* 2007) and nuclei were observed with Confocal microscope, as was mentioned above. Mitotic figures in cell files adjacent to mid-vein were identified and counted moving distally from the ligule. Measurements of leaf elongation rate, cell length distribution, and length of the meristem, were used for a kinematic analysis performed according to (Rymen *et al.* 2010), number and time of residence of cells in each zone, cell production, cell division rate, and cell cycle duration.

The effects of salinity on the cell cycle were analyzed by flow cytometry, in nuclei suspensions treated with RNAse and propidium iodide (both at 50 µg/ml final concentration). Fluorescence intensity of 10,000 nuclei (linked to DNA content) was measured with a FACSCAN argon laser flow cytometer (Becton Dickinson, 488 nm, 15 mW).

Results

Leaves were shorter in salinized plants and leaf elongation rates were always lower under salinity. However, mature cells attained the same length (Table 1). Cells in leaves from salt-treated plants remained longer in the elongation-only zone, which may have contributed to the similar mature cell size attained in both

Table 1. Effect of salinity (150 mM NaCl) on the elongation rate and cellular characteristics of leaf 4 of *P. coloratum*. Different letters indicate significant differences at P<0.05.

Parameter	Control		Salt-treated	
Leaf elongation rate (cm.ºC/d)	0.05	A	0.03	B
Mature cell length (µm)	115.83	A	98.75	A
Length of the meristem (mm)	5.6	A	3.1	B
Length of the elongation-only zone (mm)	11.93	A	12.28	A
Length of the total growth zone (mm)	17.53	A	15.39	A
No. of cells in the meristem	258.84	A	150.6	B
No. of cells in the elongation-only zone	126.31	A	174.87	A
No. of cells in the growth zone	385.15	A	325.47	A
Average cell division rate (cells/cell h)	0.03	A	0.02	A
Res. time in the cell division zone (h)	186.62	A	221.42	A
Cell cycle duration (h)	23.23	A	30.39	A
Cell flux in the mature zone (cells/h)	7.67	A	3.47	B
Res. time in the elongation-only zone (h)	16.48	A	51.5	B
Average cell relative elongation rate (/h)	0.04	A	0.02	B

treatments. The length of the division zone was reduced in salt-treated plants, which also had less cells in that zone, but on average, cells remained in the division zone the same time in salt-treated and control samples. This suggested salinity may have affected the number of dividing cells in that zone.

Mitotic figures were observed in the first 5 mm from the ligule in leaves from control plants, but declined closer to the ligule in salinized plants, resulting in an overall lower number of dividing cells in that zone in salt-affected plants, with no significant variations in cell cycle duration and cell division rates due to salinity. Cell cycle analysis indicated a significantly lower proportion of cells in G2/M.

Conclusion

Leaves in salinized *P. coloratum* plants were shorter than in non-salinized controls essentially because the number of cells in the meristem was negatively affected. This was partially due to a lower mitosis rate in that zone, and, additionally, perhaps fewer cells may have left the basal meristem to conform the leaf primordium. Though leaf elongation rates were lower in salt treated plants, cells remained longer in the expansion-only zone, which may have buffered in part the reduced leaf expansion rate. Mechanisms underlying the control of cell divisions and the prolongation of cell expansion duration are implied in the effects of soil salinity on leaf expansion in this species.

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