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Short communication

Freeze tolerance differs between two ecotypes of *Paspalum vaginatum* (Poaceae)

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ABSTRACT

Morphological and physiological responses to freezing were evaluated in two ecotypes of the perennial turfgrass *Paspalum vaginatum*. Leaf extension rate, number of active meristems, leaf water potential and net photosynthesis were measured on plants of both a commercial cultivar, 'Sea Isle 2000', and a wild ecotype from the Flooding Pampa grasslands of Argentina. Plants were propagated by cloning, cultivated in pots, and examined during 18 consecutive days under two treatments: a non-frozen control treatment (15.5±7 ºC) and a frozen treatment with two stages: Stage 1 with four hours of freezing stress for 10 nights (-5ºC), and Stage 2 with 12 hours of freezing stress for eight nights (five nights at -5ºC and three nights at -8ºC). After these treatments, plants were returned to the outside environment to evaluate shoot injury and post-freezing recovery. Leaf water potential, net photosynthesis and leaf extension rate were significantly higher in the wild ecotype than in the commercial cultivar. Meristem density was reduced after freezing in both ecotypes, but was more pronounced in the commercial cultivar (98.5%) than in the wild ecotype (80%). Thus, the two ecotypes coming from different environments, exhibited different morphological and physiological responses to exposure to freezing temperatures.

Keywords: freezing tolerance, leaf water potential, meristem injury, *Paspalum vaginatum,* photosynthesis, seashore paspalum, turfgrass

Paspalum vaginatum Sw is one of the most important C_4 grasses, since it plays different ecological roles and has different uses. It is propagated by cloning during spring and summer through stolons and rhizomes, and is highly recommended for coastal sports fields and golf courses due to its tolerance to multiple sources of abiotic stresses such as salinity, drought, and waterlogging (Duncan & Carrow 2000). *Paspalum vaginatum* is very useful for dune stabilization and erosion control because its initial establishment is very fast in these environments (Shaw & Allen 2003). However, it may behave as a weed and compete with other native species when it is introduced into natural ecosystems (Campos *et al.* 2004; Riefner *et al.* 2010).

Commonly known as 'seashore paspalum', this species is native to tropical and subtropical regions worldwide, between 30-35º N-S latitude (Zuloaga & Morrone 2001). It occupies coastal salt and brackish water marshes and other wet habitats worldwide, and usually occurs in areas subjected to different environmental disturbances (Burkart *et al.* 1990). Freezing is the principal abiotic stress constraint involved in the geographic distribution of warm season turfgrasses. Freezing tolerance has been reported for few cultivars of *P. vaginatum*, which have a tolerance

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range between -4 and -7ºC under field conditions, and between -8 and -9ºC in acclimated plants (Cardona *et al*. 1997). Therefore, differences in freezing injuries between wild ecotypes would be also expected as a consequence of a genetic adaptation process, although no information is currently available.

The aim of this paper is to compare the effects of freezing on morphological and physiological traits in two ecotypes of *P. vaginatum* from different geographical origins. Our hypothesis was that both ecotypes differ in morphological and physiological traits when they are growing at temperatures below 0ºC and we predicted that the wild ecotype has better freezing tolerance than the commercial cultivar due to adaptation to its natural environment, subjected to freezing episodes in winter and early spring.

For this purpose an experiment was carried out during spring 2008 using cloned plants of two individuals (*genets*) with fine-textured leaves from two respective ecotypes of *P. vaginatum*: (1) a wild ecotype (WEc) collected from the Flooding Pampa grassland (Argentina: Prov. Buenos Aires) and (2) a commercial cultivar (CEc) originated in Florida (USA), patented as 'Sea Isle 2000' by the University of Georgia Research Foundation were cultivated in the experimental garden of the School of Agriculture, University of Buenos Aires, Argentina (34°35'S, 58°29'W).

While the CEc cultivar is only propagated vegetatively, the WEc diploid population probably originated from sexual reproduction as is usual among *Paspalum* diploids (Quarín 1992; Echarte & Clausen 1993). Since in this experiment the two collected specimens have different reproductive origins, we used cloned plants in order to prevent intraspecific expression differences and to standardize criteria.

Clones were obtained in May from 16 tillers of each ecotype. All tillers were similar in diameter and stage of development and bore three to four fully expanded leaves. They were placed in volcanic perlite for seven days in order to induce rooting. Then, clones were transferred to plastic pots (30 x 20 x 10 cm depth) containing a mixture of organic soil and sand (9:1 v/v) and established in a glasshouse (16.2 ±5 ºC) during two months in the winter season. Photoperiod, radiation and humidity were not controlled in the glasshouse, and 25% of the total radiation was intercepted by the polyethylene cover (PAR inside the glasshouse: 225±101 mol m^2 day^{-1}, averaged over 60 days). In August, plants were grown under outdoor conditions (13±6 ºC) until stolons covered the soil surface (Tab. 1). The experiment began in September and was carried out following a completely randomized design with eight replications per treatment and ecotype (eight rows x four columns). During 18 consecutive days, plants were assigned to two treatments: (1) control plants were maintained outside, where temperatures never dropped below 3ºC and (2) freeze treatment plants were subjected to two consecutive stages (Stage 1 and Stage 2, Tab. 1). During Stage 1 plants were exposed to a short-time freeze stress (STF) for four hours at -5ºC during 10 consecutive nights (cycles) in order to mimic natural spring conditions characterized by night freezing events preceded and followed by warm temperatures. For each cycle, eight pots of each ecotype were re-randomized at the end of a cycle inside a climate chamber (Kent S. A., Four stars tropical freezer, Argentina); and temperature stabilized at -5°C. Following the four hours of freezing exposure, plants were returned to their original location outdoors (13.5±5 ºC) until the onset of the following Stage 1 cycle. Stage 2 was performed during eight consecutive nights (cycles) immediately after completion of Stage 1 following a similar procedure. A long-time freeze stress (LTF) of 12 h per night was added, reaching -5ºC during the first five cycles (15±5 ºC outdoors) and -8ºC for the remaining three cycles (18±5 ºC outdoors).

In order to evaluate freeze damage and recovery of stolons and rhizomes, a period of 30 days of re-acclimation outdoors (19.5±6 ºC) was applied to freeze treatment plants of both ecotypes. Leaf water potential (Ψ_l) was measured in each plant (eight per ecotype) on the distally and fully expanded leaf of a stolon using a Schölander-type pressure chamber (Bio-Control, Buenos Aires, Argentina). Simultaneously, net photosynthesis (P_n) was calculated as net CO_2 exchange rate using a LI-6200 portable closed system (LI-COR Inc., Lincoln, Nebraska). For each plant, P_n was measured for 30 seconds on a portion of a young expanded leaf placed into a chamber ($25x10^{-5}m^3$), with additional lighting of 1500 µmol $m^{-2}s^{-1}$ (PPFD) light source (QB1 205LI-670, Quantum Devices Inc., Barneveld, WI). At the beginning of each measurement, concentrations of both carbon dioxide and water vapor did not substantially differ from those of the surrounding environment, and the water vapor concentration was maintained constant by regulation with a desiccant valve. These measurements took place daily at midday on sunny days during the first five days of Stage 1.

Leaf elongation rate was recorded daily in both eight control and eight frozen plants of each ecotype over the last four days of Stage 1. Measurements of leaf length (mm day^{-1}) were carried out early in the morning, using a ruler, on two tillers per plant. The elongation of each leaf was followed since its emergence until the ligule became exposed.

Meristem injury was evaluated above (stolons) and below ground (rhizomes) on a square area of the pots ($1x10^{-2}$ m^2). For this purpose, meristem density (defined as the number of viable meristems per unit area) was estimated at the end of Stage 2, after the 30 day re-acclimation period, in both ecotypes and both treatments. Meristems were considered viable when re-growth was visible at the nodes of all living rhizomes and stolons sampled.

Repeated observations of leaf Ψ_l and P_n were analyzed using a linear mixed model for repeated measures (SAS 1990). Leaf extension rate was analyzed using a one way ANOVA and Tukey test for comparisons between

means. Meristem number analysis was performed by a non-parametric Wald-Wolfowitz test using the Infostat software (Di Rienzo *et al*. 2011). Statistical significance of the adjusted models was $P<0.05$.

A general decreasing pattern of P_n was observed over time, although with a clear difference between ecotypes due to the effect of freezing (Fig. 1A). In fact, while in WEc plants P_n decreased slightly in both treatments, P_n decreased significantly in CEc plants (93% lower than controls, five days after the onset of Stage 1, $P<0.001$) under freezing conditions, remaining constant in control plants. Further, at sub-zero temperatures, WEc P_n surpassed CEc ($P<0.0004$), while in control plants P_n rates did not differ between ecotypes. These patterns clearly revealed a higher tolerance of WEc plants to freezing events, in agreement with reports made on other C_4 species which may acclimate their photosynthetic apparatus to survive occasional frosts (Sage & Kubien 2007; Long & Spence 2013; Głowacka *et al*. 2015). Although differences are certainly attributed to a genotypic adaptation of WEc to the cooler environments of Argentina, further research is needed in order to elucidate if the higher tolerance of WEc is due to a constitutive frost adaptation or a greater ability to develop cold acclimation.

In the same way, the patterns of leaf Ψ_1 were consistent with those observed for P_n (Fig. 1B). While WEc plants exhibited a similar behavior with respect to treated and control plants between -0.1 and -1.2 MPa, a significant drop to -1.4 MPa was observed in treated CEc plants at day 5 ($P<0.001$). Control CEc values were similar to those for WEc plants. The results clearly suggest that the sensitivity of P_n after freezing conditions is linked to water deficit. Although water availability was not limiting, a possible explanation is that the exposure of roots to low temperatures could have reduced absorption and root extension, negatively affecting water uptake and hence leaf water potential in a few hours (Yu *et al*. 2006; Taiz & Zeiger 2012). This possibility should be tested in further experiments with plants subjected to different temperature regimes between roots and aerial organs.

Differences in the leaf extension rate between ecotypes and treatments ($P=0.0182$) were detected. The mean comparison test revealed significantly lower leaf extension rates for CEc plants subjected to freezing. In CEc plants leaf extension was nearly 40% lower under freezing while WEc values were similar to those of WEc and CEc controls (Fig. 2). Meristem density was affected by freezing in both ecotypes and the effects persisted 30 days after the end of Stage 2 (Fig. 3). These values were consistent with the more extreme freezing conditions in Stage 2 (Tab. 1). Finally, these results also corroborated the higher tolerance of the wild ecotype (WEc) in response to an abiotic stress such as freezing. First, WEc plants showed a higher potential of meristem availability even without stress, since meristem density was 30% higher than in CEc control

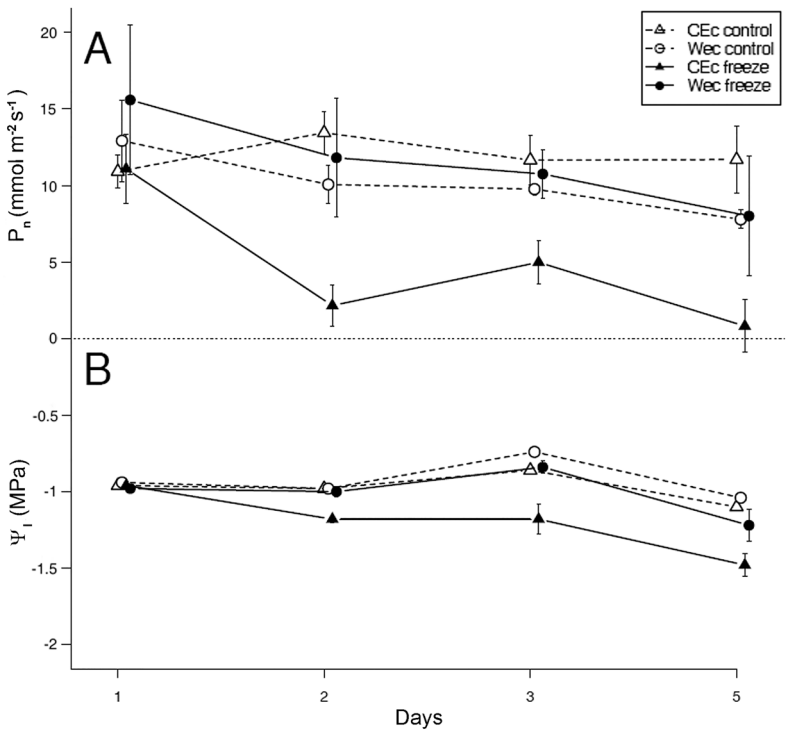

Figure 1. Changes in net photosynthesis (P_n, A) and leaf water potential (Ψ_1, B) of two *Paspalum vaginatum* ecotypes growing under control (open symbols) and freeze (closed symbols) treatments. Measurements were taken during the first five days of Stage 1 (except the fourth which was rainy), on clear days under similar photon flux density (PPFD) conditions (c. 1500 µmol m^{-2} s^{-1}, over 400-700 nm). Results represent the mean of eight plants per ecotype. Vertical bars denote standard errors (SE) and are shown only when larger than symbols.

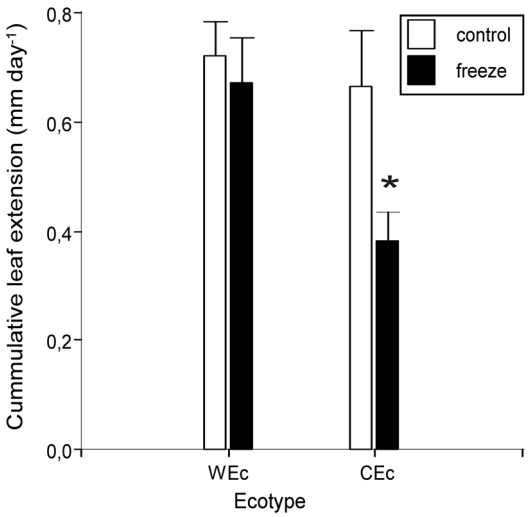

Figure 2. Cumulative leaf extension in plants of *Paspalum vaginatum* grown under control (white columns) and freeze conditions (black columns). Measures are based on the last fully expanded leaf of two tillers per plant during the last four days of Stage 1. Each value shows the mean of eight plants per ecotype. Asterisk (*) indicates statistically significant differences between means (P<0.05). Vertical bars denote SE.

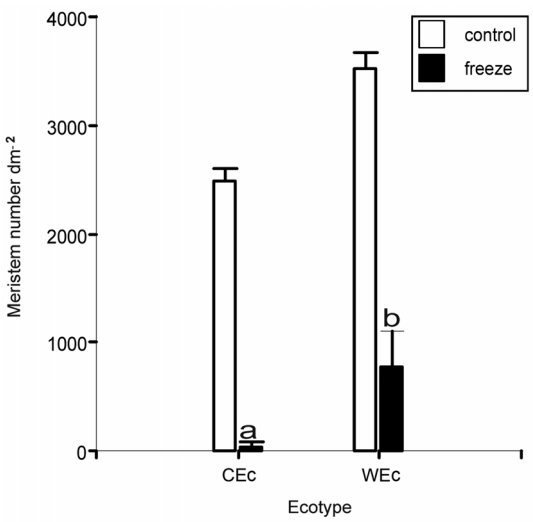

Figure 3. Final meristem density in *Paspalum vaginatum,* measured after the end of the re-acclimation period in a delimited area of the pots ($1x10^{-2}$ m^2). Each column shows the mean of eight plants per ecotype growing under control conditions (white columns) and after freeze conditions (black columns). Vertical bars denote SE. Different letters indicate statistically significant differences between ecotypes (P<0.05).

Table 1. Scheme of processing steps applied to plants under freezing treatment with their respective time-durations.

	Duration	Daily time	Place	Temperature
ACCLIMATION	30 days	24 hs	outdoor	13±6 °C
FREEZE TREATMENT Stage 1: Short-Time Freeze (STF)	10 days	4 hs	freezing chamber	-5°C (night)
		20 hs	outdoor	13.5±5 °C
FREEZE TREATMENT Stage 2: Long-Time Freeze (LTF)	5 days	12 hs	freezing chamber	-5°C (night)
		12 hs	outdoor	15±4 °C
	3 days	12 hs	freezing chamber	-8°C (night)
		12 hs	outdoor	18±4 °C
RE-ACCLIMATION	30 days	24 hs	outdoor	19.5±6 °C

plants (P=0.05). This implies a constitutive advantage of the wild material related to the perspective of surviving under an acute stress period. Second, meristem availability was reduced to 1.5% of the control in CEc frozen plants, which could imply a serious constraint for plant survival after adverse conditions. Alternatively, in WEc frozen plants meristem density was reduced to only 20% of that observed for controls (P<0.001) and was 95% higher than observed in CEc frozen plants, increasing the possibility of survival (Kalberer *et al.* 2006; Trischuk *et al.* 2006).

The present research revealed that both ecotypes of *P.vaginatum* respond differently in morphological and physiological traits when they are exposed to freezing temperatures. These preliminary results allow us to support the proposed hypothesis that the wild ecotype (WEc) expresses greater tolerance to frost and it is better adapted to freezing temperatures in winter than the commercial ecotype 'Sea Isle 2000'. Thus, *Paspalum vaginatum* ecotypes coming from natural environments subjected to frequent freezing events would promote the development of tolerant genotypes, favoring the expression of traits necessary to use in transition zones. Further studies will be necessary to determine if the different responses to freezing between genotypes are due to constitutive traits or to different abilities for acclimation.

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