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Differential salt-stress response during germination and vegetative growth in *in vitro* selected somaclonal mutants of *Cenchrus ciliaris* L.

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ABSTRACT

Four somaclonal mutants (S1, S4, S6 and M10) and their parental *Cenchrus ciliaris* L. cultivar Biloela were characterized under salinity conditions at germination and vegetative growth stages. Seeds of all somaclonal mutants had higher germination percentages than cv. Biloela seeds in the control and salt treatments. At 150 mM, germination was significantly higher in M10, S6 and S4 (72.3%, 66.3% and 61.8%, respectively) than in cv. Biloela (35.5%). Mutants grown under salinity along with cv. Biloela for 35 days had a different relative growth rate. S6 had the highest growth rate, indicating its potential tolerance to salt stress, whereas M10 was the most sensitive, with Bi, S4 and S1 being intermediate tolerant genotypes. Catalase enzyme activity (CAT) in M10 decreased in response to salt stress and was significantly associated with malondialdehide content, suggesting salt injury, whereas higher levels of CAT activity in S6 during salt stress were associated with increased salinity tolerance. The present results indicate that somaclonal variation and *in vitro* mutagenesis offer an effective tool for improvement of *C. ciliaris* because the somaclonal mutants showed differential tolerance to salt stress with respect to their parental and could be a better choice for use in a breeding program.

1. Introduction

In Argentina, crop expansion is forcing pastoral livestock production systems to relocate in drier lands, such as north-western Argentina (Guevara et al., 2009). *Cenchrus ciliaris* L. Syn. *Pennisetum ciliare* Link (Buffel grass), an apomictic, polyploid warm-season forage grass (Ozias-Akins, 2006; Snyder et al., 1955), is known worldwide for its tolerance to high temperatures (45 °C) and water stress, ease of establishment, productivity, and forage quality (Hacker and Waite, 2001; Kharrat-Souissi et al., 2011; Marshall et al., 2012). Hence, this species has been introduced as a forage resource to enhance the productivity of these marginal lands. In these environments, salinity, along with drought, is one of most important abiotic factors that contribute to severe declines of forage grass production and persistence (Griffa et al., 2010). Therefore, identifying and using plants adapted to saline soils is of increasing importance (Ashraf, 2009; Flowers and Flowers, 2005; Krishnamurthy et al., 2007; Nichols et al., 2009).

With the aim of obtaining new salt-tolerant *C. ciliaris* L. genotypes, a genetic improvement program is currently being conducted at the Institute of Physiology and Plant Genetic Resources (IFRGV-INTA), Córdoba, Argentina (López Colomba, 2009; López Colomba et al., 2011). Despite considerable efforts made to increase salt tolerance in *C. ciliaris* L. through conventional breeding, progress has been slow (López Colomba et al., 2011). *In vitro* mutation and selection techniques offer an alternative and effective tool for crop improvement programs through the generation of biotic and abiotic resistant plants (Jain, 2001, 2005; Maluszynski, 2001); nevertheless, forage grass species, including *C. ciliaris*, have been poorly studied. A protocol for somatic embryogenesis, plant regeneration and *in vitro* mutagenesis of *C. ciliaris* L. cultivar Biloela has been established by our group and several somaclonal mutants have been selected and already characterized using morphological and molecular markers (López Colomba, 2009; López Colomba et al., 2011). As a result of this work, four mutants, named M10, S1, S4 and S6, were identified for their differential behaviour in the field (López Colomba, 2009). However, these mutants have still not been characterized under salinity conditions.

Salt stress affects plants at various stages of development, including germination and establishment, vegetative growth, and finally reproduction and yield (Munns and Tester, 2008). Several authors reported that plant ability to germinate and establish seedlings on saline land is particularly important in perennial grasses (Abogadallah and Quick, 2009). Tolerance to salinity stress has been often associated with oxidative stress, since one of the consequences of exposure to salinity is the production of reactive oxygen species (ROS), such as superoxide radicals ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH) (Apel and Hirt, 2004). Antioxidant defense system plays an important role in salt tolerance in various plant species (Abogadallah and Quick, 2009; Hasegawa et al., 2000). Increases in

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the activity of the enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) under salt stress conditions have been reported for leaves of tolerant genotypes of *Triticum aestivum*, *Chloris gayana*, *Oryza sativa*, *C. ciliaris* and *Setaria viridis* (Kim et al., 2004; Lanza Castelli et al., 2010; Luna et al., 2002; Sairam et al., 2002, 2005; Vaidyanathan et al., 2003). Most of these studies suggest a correlation between tolerance to salinity stress and the presence of an efficient antioxidant system (Ashraf, 2009; Gossett et al., 1994; Lanza Castelli et al., 2010; Luna et al., 2002; Mittova et al., 2004). In a previous work we measured oxidative stress characters, such as malondialdehide content (MDA), and found some antioxidant key enzymes to be promising indicators of *C. ciliaris* salt tolerant genotypes (Lanza Castelli et al., 2010).

In this work we evaluated the behaviour of somaclonal mutants of *C. ciliaris* L. under salt conditions at germination and vegetative stages to investigate if somaclonal variation and *in vitro* mutagenesis tools were effective to develop new salt-tolerant genotypes. Oxidative stress regulation, measured as MDA content and CAT activity, was evaluated during early vegetative stage to characterize tolerance of somaclonal mutants to salinity stress.

2. Materials and methods

2.1. Plant material

R3 seeds of somaclonal mutants of *C. ciliaris* L. (identified as M10, S1, S4 and S6) (López Colomba, 2009; López Colomba et al., 2011) and seeds of cv Biloela (Bi) were collected from plots established at IFRGV-INTA, Córdoba, Argentina (600 m a.s.l., 31° 24′S; 61° 11′W) in January–March 2011. Bi, which was used for the development of the mutant lines, was used here as the parental control. Seeds were stored in paper bags at 6 °C to prevent loss of viability.

2.2. Response to salt tolerance at the germination stage

Three independent experiments were conducted during September–December 2011. In germination tests five replicates of 20 seeds per treatment and per genotype were used. Seeds were disinfected for 15 min in 10% commercial bleach (NaClO 55 g L^{-1}) and placed on Whatman filter paper in 50-mm diameter Petri dishes moistened with 10 mL of test solution. Solutions included distilled water (0 mM), 50, 100, and 150 mM of NaCl, corresponding to 0, 0.2, 0.4 and 0.6 MPa osmotic potential, respectively. Seed incubation was performed in a growth chamber under the following conditions: 16 h light/8 h dark photoperiod (55 μmol m^{-2} s^{-1}) (4100 lux) at alternating temperatures of 25 °C (16 h) and 20 °C (8 h). Germinated seeds were counted and discarded every 2 days for 20 days. Seeds were considered to have germinated when radicals were at least 5 mm long. Rate of germination was estimated using a modified Timson's index of germination velocity: $GR = (\Sigma\ Gi)/t$, where Gi is the percentage of seed germination at 2-day intervals and t is the total germination period (Khan and Ungar, 1984). The maximum value possible using this index was 50.

In recovery experiments, seeds that did not germinate under high salt concentrations were transferred to distilled water to study germination recovery. Recovery percentage was calculated with the formula: $[(a - b)/(c - b)] \times 100$, where a is the number of seeds germinated in salt solutions plus those that recovered germination in distilled water, b is the number of seeds germinated in saline solution, and c is the total number of seeds tested (Khan and Ungar, 1984).

2.3. Measurement of relative growth rate (RGR)

Seeds of *C. ciliaris* L. somaclonal mutants and cv. Biloela (Bi) were sown in plastic trays (42 cm × 14 cm × 5 cm), with fine-mesh bottom filled with soil and vermiculite (1:1). The seeds were planted in rows approximately 2 cm apart and covered with a thin layer of soil. The trays were suspended in 10-L rectangular plastic trays (45 cm × 16 cm × 14 cm) filled with aerated Hoagland nutrient solution (Hoagland and Arnon, 1950). The salt treatment was started when seedlings had three folded leaves and was accomplished by weekly increments of 100 mM until reaching a final concentration of 500 mM. Nutrient solution without NaCl was used as control (0 mM). Evaporative conditions of nutrient solution were controlled regularly and the nutrient solution was renewed every 5 days. After 7 days of exposure to each concentration, nine plants were removed from the soil and vermiculite with their roots intact. Plants from both treatments were harvested and separated into aerial and radical part for fresh and dry weight determinations. Dry weight (DW) was determined after drying the plant parts in an oven at 65 °C for 72 h until constant weight was reached. RGR was determined as follows (Hilbert et al., 1981):

$$\mathrm{RGR} = (\ln\ \mathrm{DW}_2 - \ln\ \mathrm{DW}_1)/t_2 - t_1, \left(g\ g^{-1}\ d^{-1}\right)$$

where DW_1 is the initial total (aerial and radical part) dry weight, DW_2 is the final total dry weight and $(t_2 - t_1)$ is the difference in time interval between samplings (7 days).

2.4. Response to salt tolerance at vegetative stage

For this experiment, seeds of *C. ciliaris* L. somaclonal mutants and cv. Biloela (Bi) were grown in pots (30 cm in diameter), in greenhouse under natural light and day/night temperature of 30/15 °C and 65–75% relative humidity. Twenty days after sowing, seedlings were placed individually in holes of a Styrofoam board (20 plants per board); the boards were set on rectangular plastic trays (30 cm × 20 cm × 60 cm) filled with aerated Hoagland nutrient solution (Hoagland and Arnon, 1950). The plants were maintained under these conditions during 10 days. Salinization was accomplished by gradually adding 50 mL of 1 M NaCl per L of nutrient solution (100 mM every 48 h). Nutrient solution without NaCl was used as control. Evaporative conditions of nutrient solution were controlled regularly and the nutrient solution was renewed every 5 days. When the treatment reached 300 mM NaCl, leaf samples (five plants per treatment) were collected at different times (24 and 48 h and 15 days) for biochemical determinations.

2.4.1. Lipid peroxidation assay

Lipid peroxidation in leaves was evaluated by measuring malondialdehyde content (MDA), as described by Heath and Packer (1968). About 100 mg of the frozen material was ground in 1.5 mL of 0.1% trichloroacetic acid (TCA) and centrifuged. An aliquot of 0.5 mL of the supernatant was reacted with 0.5 mL 20% TCA containing 0.5% thiobarbituric acid (TBA) at 90 °C for 20 min, and cooled in an ice bath. The resulting mixture was centrifuged at 12,000 rpm for 10 min and the absorbance of the supernatant was measured at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. Each sample had a control without TBA (Hodges et al., 1999). MDA concentration was calculated by using extinction coefficient of 155 mM^{-1} cm^{-1}; results are expressed as mmol MDA mg fresh weight. The procedure was repeated five times using extracts from different samples. Data of MDA were expressed as percentage of control (100%).

2.4.2. Determination of antioxidant enzyme activity (CAT)

CAT activity was measured by following the consumption of H_2O_2 at 240 nm (Aebi, 1984). For antioxidant enzyme activities, 100 mg of frozen leaf samples were ground to a fine powder in liquid nitrogen and homogenized in 1.5 mL of 50 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA and 1% (25 mg) PVPP (polyvinylpolypyrrolidone). Homogenates were centrifuged at