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Individual and combined effects of drought and heat on antioxidant parameters and growth performance in Buffel grass (*Cenchrus ciliaris* L.) genotypes

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ABSTRACT

Drought and heat stress are two critical threats to crop growth and sustainable agriculture worldwide. In the last decade, many studies focused on the response of crops to a single stress, nevertheless studying the response of plants to a combination stress may be critical to our understanding of stress tolerance in plants and the development of tolerant genotypes. Buffel grass (*Cenchrus ciliaris* L.) is a warm-season grass known in arid and semiarid regions for its tolerance to heat and drought stress, productivity, and forage quality. However, in our previous works, several accessions have exhibited different responses to abiotic stresses. Therefore, the objective of this work was to evaluate the effects of combination of drought and heat stresses on biochemical parameters and plant growth and to compare the impacts of the stresses separately and when combined. We found that sensitive genotype exhibited higher lipid peroxidation content, lower total reducing power values and reduced catalase and superoxide dismutase activities than tolerant under drought or heat stress or combination stress. In this study, heat stress had a predominant effect on buffel grass genotypes over drought stress, which explained why simultaneous application of heat and drought revealed similar biochemical and growth responses to the heat stress. Antioxidant metabolism seems to be critical for tolerate abiotic stress. This study may provide useful information to perform a rapid and low-cost characterization in new buffel grass germplasm and to identify genotypes with better growth performance under drought and heat conditions.

1. Introduction

Drought and heat stress are known as major threats to growth and development of agricultural crops. Abiotic stresses frequency, duration and severity are anticipated to be increased in the future, which might have severe effects for crop and forage productivity and subsequently livestock production (Zhou et al., 2017). In the last decade, many investigations have been performed to figure out the response of plant species to a unique abiotic stress, whilst in ecosystems, plants might simultaneously be exposed to multiple abiotic stresses (Mittler, 2006; Pandey et al., 2015).

Plants have evolved various morphological, cellular, physiological, biochemical and molecular adaptations to preserve themselves in abiotic stress situations (Pandey et al., 2015). Reactive oxygen species (ROS) including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH), are one of the earliest known biochemical responses of eukaryotic cells to abiotic stresses. ROS are produced mostly in chloroplasts, mitochondria, and peroxisomes (Apel and Hirt, 2004). Drought and heat stresses dramatically increase ROS levels which lead in oxidative damage of proteins, DNA and lipids (Apel and Hirt, 2004; Farooq et al., 2009; Mittler, 2002; Gill and Tuteja, 2010). Particularly, when ROS directly attack membrane lipids, the malondialdehyde (MDA), a product of peroxidation of unsaturated fatty acids, increases their content (Gill and Tuteja, 2010). MDA has been considered as an indicator of oxidative damage in various crops including forage grass species (Luna et al., 2002; Moller et al., 2007; Bi et al., 2016) and it has been used as a suitable indicator for tolerant genotype selection (Luna et al., 2002; Lanza Castelli et al., 2010; Tommasino et al., 2012). ROS also acts as signaling molecules in many biological

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processes such as stomatal closure, growth, development, and stress signaling (Suzuki et al., 2012).

Due to the dual roles of ROS, plants are able to fine-tune ROS concentrations between certain thresholds by means of production and scavenging mechanisms (Sekmen et al., 2014). Since ROS homeostasis is disrupted under stress, induced enzymatic antioxidant defenses are considered as an important factor of plant stress tolerance (Mittler et al., 2011; Suzuki et al., 2011, 2012; Sekmen et al., 2014; You and Chan, 2015). Higher plant species generally apply a defense system, which is involved with antioxidative enzymes and non-enzymatic compounds to protect plants against ROS. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) among others (Ashraf, 2009; Gill and Tuteja, 2010; Sharma et al., 2012; You and Chan, 2015).

Knowledge regarding the response of plants to multiple abiotic stresses is limited and also its required be improved for a better understanding of mechanisms underlying stress tolerance in plants species (Rizhsky et al., 2002). When plants deal with multiple abiotic stresses-induced factors concurrently, their adaptation strategy will be governed by the interaction of abiotic stress factors, which means a new level of stress (Mittler, 2006). In general, plant responses to multiple stresses are majorly determined by the more severe stress (dominant stressor) (Pandey et al., 2015). However, it depends largely on the age of plant, the genotype, the stress-susceptibility or tolerance behaviour of plant, and severity of multiple factors involved with abiotic stress (Silva et al., 2010). Several authors underline the need to develop crops with better performance and resilience to abiotic stress combination and indicate the complex interaction between drought and heat stress (Mittler, 2006; Feller and Vaseva, 2014; Pandey et al., 2015).

Buffel grass (*Cenchrus ciliaris* L. syn. *Pennisetum ciliare* (L.) Link) is an apomictic, polyploid warm-season grass (Ozias-Akins, 2006) used for cattle and sheep grazing in arid and semiarid regions worldwide (Saini et al., 2007). Buffel grass is known to be tolerant to heat and drought stress and easy to establish with high productivity and quality (Hacker and Waite, 2001; Kharrat-Souissi et al., 2010; Marshall et al., 2012). However, genetic variability was found when several accessions were exposed to abiotic stresses (Mansoor et al., 2002; Kharrat-Souissi et al., 2012; Al-Dakheel et al., 2015; Al-Dakheel and Iftikhar Hussain, 2016). In Argentina, the species is cultivated mainly in the Northwestern region (De León, 2003; Griffa et al., 2010), in areas with a considerable dry season during a long part of the year, and strong sunshine in the summer (Guevara et al., 2009). In our previous work, the effect of heat stress on biochemical parameters was investigated in buffel grass genotypes (Tommasino et al., 2012). However, understanding of combined effect of heat and drought on biochemical parameters, biomass production and the relationship between the biochemical responses to single and combined stress remained unclear. Therefore, the objective of this work was to evaluate the effects of combination of drought and heat stresses on biochemical parameters and plant growth in different genotypes of *Cenchrus ciliaris* and to compare the impact of the stresses separately and when combined.

2. Materials and methods

2.1. Plant material

In this study, two genotypes (Register Number: RN51 and RN1) of buffel grass (*Cenchrus ciliaris* L.) were used in all experiments because they showed different responses (tolerant and sensitive genotypes, respectively) to salt and heat stress as observed in our previous studies (Lanza Castelli et al., 2010; Tommasino et al., 2012). In addition, a widespread genotype (RN49) and two somaclonal mutants, named as J20 and S6 were used in a combined stress assay under controlled conditions. J20 was obtained through mutation and in vitro selection assay for drought tolerance (López Colomba et al., 2011). While S6 is a somaclonal variant that is already determined as salt tolerant genotype (López Colomba et al., 2013).

2.2. General growth conditions and treatments application

For all assays, including treatments and control, 0.2 g seeds of individual genotypes were sown in pots (25 cm in diameter × 15 cm in depth) containing 2.76 kg sand and soil substrate (1:1) previously dried in stove at 105 °C for 48 h. After sowing the seeds, the surface of all pots were covered with 200 g of sieved soil substrate. The pots were watered and the soil water content (SWC) was calculated after the complete drainage. This value was considered as the maximum amount of water capable to be retained by the substrate (100% SWC). The SWC was determined via gravimetric method. Afterwards, the pots were transferred to a growth chamber, under following conditions: temperature (28 °C ± 2 °C), photoperiod (16/8 h light/dark), humidity (40%) and photosynthetic photon flux density (PAR) (250 μmol m^{-2} s^{-1}). Pots were watered daily until SWC reached to 80%. Seedlings emerged after 15 days past sown and we kept 35 small seedlings in each pot.

2.2.1. Drought stress evaluation

Water stress assays were performed using the RN1 and RN51 genotypes following the protocol described by Tommasino (2013). Briefly, 30 days after sowing the seeds, drought treatment was carried out by interrupting irrigation until 30% SWC was obtained. This 30% SWC value was used for this study because buffel grass genotypes have been previously reported to showing water stress symptoms under drought conditions (Tommasino, 2013). Pots with 80% of SWC were considered as control treatment. A completely randomized design was performed with six repetitions (pots) per genotype and treatment. Five plants of control and treatment were collected to evaluate biochemical parameters after 24, 48 and 72 h when pots obtained 30% of SWC. Whilst, growth performance was measured after 54 days after sowing (DAS).

2.2.2. Heat stress evaluation

RN1 and RN51 seeds were grown in pots as previously described. Thirty DAS, half of the pots, were exposed to heat-treatment (H), in a growth chamber already set for 16 h day length, 45% RH, 250 μmol m^{-2} s^{1} light intensity and with constant 45 ± 1 °C day/night temperature during 72 h constantly. The other half pots were considered as control (C) and were kept in a growth chamber under normal conditions (28 °C, at 16 h day length, 45% RH and 250 μmol m^{-2} s^{-1} light intensity). All treatments were watered regularly (80% SWC) to avoid drought stress. Leaf samples from five plants of heat treated and control pots were collected at 24, 48 and 72 h of 45 °C to evaluate biochemical parameters. At the end of 72 h of heat treatment, C and H pots were kept under normal growth conditions (28 °C, at 16 h day length, 45% RH and 250 μmol m^{-2} s^{-1} light intensity). The growth performance was measured after 54 DAS, through the same characters as mentioned above.

2.2.3. Combined drought and heat stress evaluation

RN1 and RN51 seeds were grown as previously described. Thirty DAS, the drought treatment was carried out by interrupting irrigation until 30% SWC was obtained. Then, the temperature of the chamber was raised to 45 ± 1 °C day/night temperature during 72 h to provoke heat stress treatment and the pots were watered regularly to keep 30% SWC. A completely randomized design was performed with six repetitions (pots) per genotype and per treatment. Leaf samples from five plants of each pot were harvested at 24, 48 and 72 h of combined drought and heat stress treatment for evaluation of biochemical parameters. Then, pots were transferred to growth chamber at 28 °C, at 16 h day length, 45% RH under 250 μmol m^{-2} s^{-1} light intensity, via 30%

SWC. Subsequently, the growth performance was measured after 54 DAS, through the same characters as mentioned above.

An additional assay was performed using five genotypes in combined drought and heat stress (RN1, RN51, J20, S6 and RN49). A completely randomized design was performed with three repetitions per each genotype and treatment.

2.3. Evaluation of biochemical measurements

2.3.1. Determination of malondialdehyde content (MDA)

Lipid peroxidation in leaves was evaluated by measuring MDA, as described by Heath and Packer (1968) with minor modifications (Tommasino et al., 2012). Briefly, about 100 mg of the frozen material was ground in 1.5 ml of 0.1% trichloroacetic acid (TCA) followed by centrifugation at 12,000 rpm for 10 min at 4 °C. An aliquot of 0.5 ml of the supernatant was mixed with 0.5 ml 20% TCA containing 0.5% thiobarbituric acid (TBA) and incubated for 20 min at 90 °C. After that, the resulting mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. 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 nmol MDA per g fresh weight.

2.3.2. Total reducing power by ferric reducing ability of plasma assay (FRAP)

The FRAP method was used to determine the total reducing power via measuring the reduction of ferric ion to ferrous form in presence of antioxidant components (Benzie and Strain, 1996). The fresh FRAP reagent consisted of 525 ml of acetate buffer (300 mmol/l pH 3.6), 50 ml of 2,4,6-Tris (2-pyridyl)-s-triazin (10 mmol/l), and 25 ml of $FeCl_3$•6 H_2O (200 mmol/l). About 100 mg of the frozen material was ground in 1 ml of 95% ethyl alcohol followed by centrifugation at 12,000 rpm for 10 min at 4 °C. An aliquot of 30 μl of the supernatant was mixed with 270 μl of FRAP reagent, and then optical density was recorded after 40 min at 600 nm. Results were expressed as μmol Fe (II)/g per g fresh weight.

2.3.3. Determination of catalase (CAT) activity

CAT activity was measured through the consumption of H_2O_2 at 240 nm (Aebi, 1984). The samples were processed following the protocol in López Colomba et al. (2013). Briefly, 100 mg of frozen leaf samples were ground to obtain a fine powder in liquid nitrogen and homogenized in 1.5 ml of 50 mM potassium phosphate buffer (pH 7.4), containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% (25 mg) polyvinylpolypyrrolidone (PVPP). This was followed by centrifugation of homogenates 12,000 rpm at 4 °C for 30 min. Then, the supernatant was used to determine protein concentration and antioxidant enzyme activity. One unit of CAT activity was defined as the amount of enzyme required for catalyzing the conversion of 1 μmol H_2O_2 into water per minute. Results were expressed as μmol H_2O_2 extinct per minute per mg of protein. Protein content in the enzyme extracts was determined according to the method described by Bradford (1976).

2.3.4. Determination of superoxide dismutase (SOD) activity

SOD activity was estimated according to the method described by Beauchamp and Fridovich (1973), which is based on the ability of this enzyme to inhibit nitro blue tetrazolium (NBT) reduction. The samples were processed following the protocol in López Colomba et al. (2013). Briefly, the reaction mixture (1 ml) consisted of 30 μl of enzymatic extract and a phosphate buffer solution (50 mM, pH 7.4) that included 13 mM methionine, 75 μM NBT and 1 μM EDTA. The reaction was started by adding 4 μM riboflavin to the mixture and placing it under UV lamps for 9 min. A complete reaction mixture without enzymatic extract, which gave the maximal color, served as the control of the reaction. The reaction was ceased by keeping the tubes in dark room. Absorbance of the reaction mixture was recorded at 560 nm, and one unit of enzyme activity was considered as the amount of enzyme that reduces the optical density to 50% of control (reaction mixture lacking enzyme). Specific enzymatic activity was expressed as SOD units (USOD) per mg of protein. Protein content in the enzyme extracts was determined according to the method of Bradford (1976).

2.4. Evaluation of growth performance

Based on preliminary experiments, growth performance of five randomly selected plants per each pot and genotype was measured after 54 DAS. The following characters were measured: height, fresh and dry weight of aerial part (He, AFW and ADW, respectively). The plants were dried in a forced air oven at 60 °C until constant dry weight was obtained (72 h).

2.5. Statistical analysis

For comparisons between means of biochemical parameters, general linear mixed models were used. ANOVA was applied for a three-factor model with interaction between the factors genotype × treatment × sampling time, in a completely randomized design. For comparisons between means in growth characters, general linear mixed models were used and ANOVA was applied for a two-factor model with interaction between the factors genotype × treatment in a completely randomized design. The means were compared by Di Rienzo, Guzman and Casanoves (DGC) test (Di Rienzo et al., 2002) at 5% level of significance ($p \leq 0.05$) using InfoStat software (Di Rienzo et al., 2016). The standard error is plotted in all figures. The percentage values shown in all figures represent the increase or decrease in the average value of stress treatment in comparison to the average value of control treatment, which was calculated using the following formula: Value % = [(Xs * 100/Xc)]–100. Where, Xs and Xc are the mean values for each genotype (MDA or FRAP or CAT or SOD or ADW) obtained from the stress and control treatments, respectively.

3. Results

3.1. Evaluation of biochemical measurements

3.1.1. Determination of malondialdehyde content (MDA)

The MDA values were significant for three-way interaction (genotype * treatment * sampling time) ($p \leq 0.0001$). In both genotypes (RN1 and RN51), no significant differences were found for MDA content under control conditions at 24, 48 and 72 h. However, under drought, heat, and combined stress, MDA content was significantly higher in RN1 comparing with RN51 in all time tested (24, 48 and 72 h). The most significant differences between both genotypes were observed at 72 h of stresses were applied separately or combined. Accordingly, RN1 showed 60, 73 and 86%, increases in MDA content when drought, heat and combination of both stresses exposed, respectively. Whilst RN51 only revealed 30, 33 and 21% growing MDA content in drought, heat and combined stress conditions, respectively (Fig. 1A, B, C, respectively). In addition, for RN1 genotype, the combined stress treatment resulted in an increase of MDA content than when drought and heat were applied separately. Nevertheless, RN51 showed similar MDA content increase percentages in the three treatments (drought, heat and combined stress).

When the combined stress (drought + heat treatment) evaluation was performed for all five genotypes, MDA values were significant for three-way interaction (genotype * treatment * sampling time) ($p \leq 0.0001$). The most significant differences could be observed between all genotypes when they were remained under combined stress condition for 72 h. At this period of stress treatment (72 h), RN51 and J20 genotypes exhibited the lowest value of MDA content,

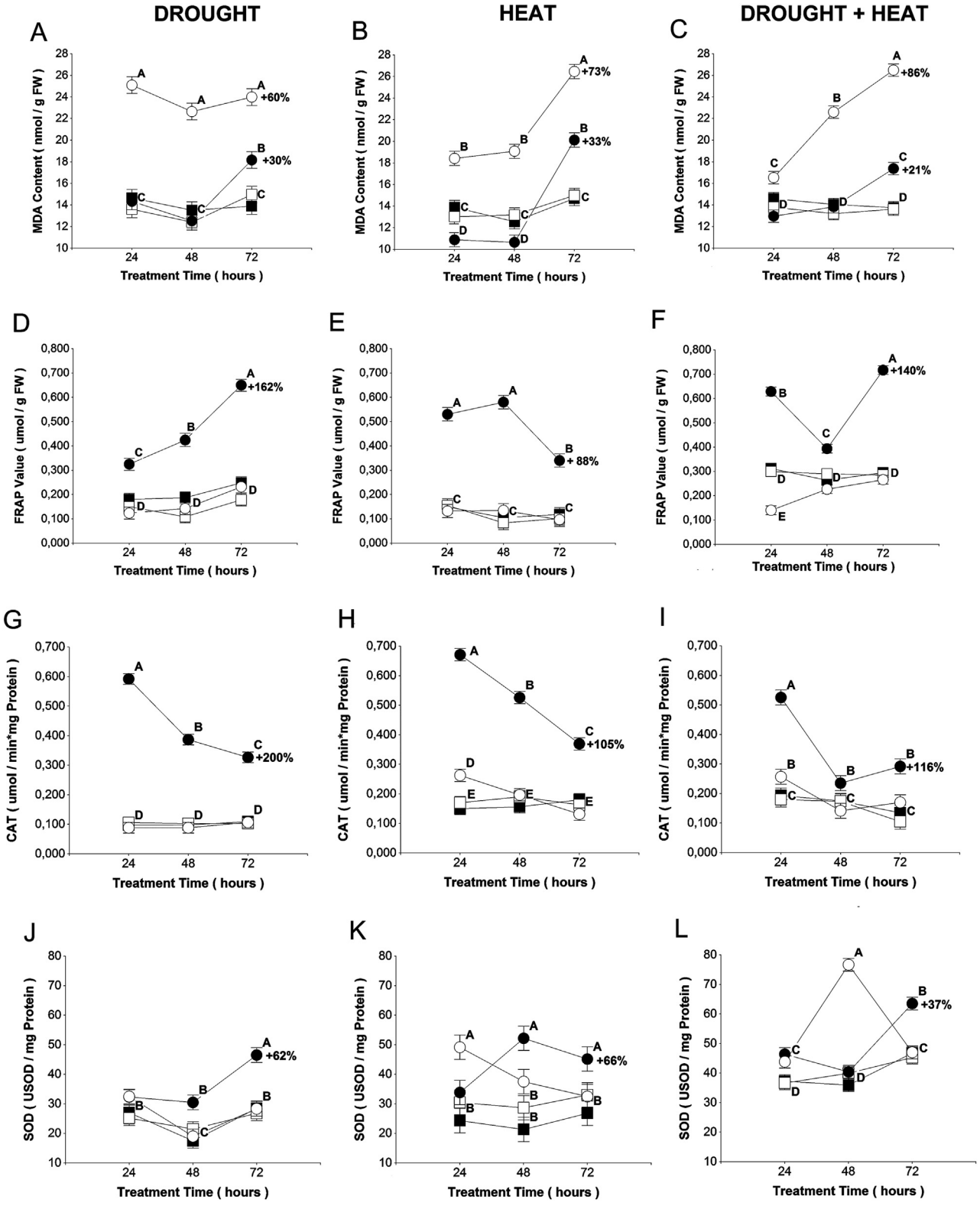

Fig. 1. Effect of drought (A, D, G, J), heat (B, E, H, K), and combined stress (C, F, I, L) on malondialdehyde (MDA) content, total reducing power quantified by FRAP assay, catalase (CAT) and superoxide dismutase (SOD) enzyme activities evaluated at 24, 48 and 72 h in RN51 and RN1 buffel grass genotypes. RN51 Control: (black square); RN51 Stress Treatment: (black circle); RN1 Control: (white square); RN1 Stress Treatment: (white circle). DGC test $\alpha = 0.05$. Different letters denote significant differences ($p \leq 0.05$). Error bars indicate standard error. The percentage values in bold represent the increase in the average value of treatment respect to the average value of control.

which was an increase of only 15 33%. While RN1, showed the highest MDA value (83% higher than control) and MDA content in RN49 and S6 genotypes were moderate via only 38 and 54% changes, respectively (Fig. 2A).

3.1.2. Total reducing power measured by ferric reducing ability of plasma assay (FRAP)

Total reducing power was significant for three-way interaction (genotype * treatment * sampling time) ($p \leq 0.0001$). RN51 had higher value of total reducing power ($p \leq 0.05$) than RN1 under drought, heat, and combined stress in all sampling times (24, 48 and 72 h) (Fig. 1D, E, F). FRAP value increase for RN51 genotype ranged 88 162% when the all three stress treatments applied for 72 h. However, no significant increases were observed in RN1 at the same conditions (Fig. 1D, E, F). Similar FRAP value increase was observed in RN51 upon exposure to drought and combined stresses.

When the combined stress (drought + heat treatment) evaluation was performed for all five genotypes, FRAP value was significant for three-way interaction (genotype* treatment * sampling time) ($p \leq 0.0001$) (Fig. 2B). In general, the analysis of experiment when studied for 72 h showed four different groups with significant differences in FRAP value. The first group including RN51 and J20 exhibited the highest increase with 143 and 166%, respectively. The second and third groups containing RN49 genotype and S6 genotype, respectively, that showed intermediate values of FRAP with only 103 and 67% increases. The last group was the control of this experiment and RN1 genotype with no significant increase ($p > 0.05$).

3.1.3. Catalase (CAT) activity measurement

In general, the genotypes RN1 and RN51 under drought, heat and the combination of the two stresses displayed different patterns of the CAT activities (Fig. 1G, H, I) and SOD activities (Fig. 1J, K, L) comparing with the control treatments. The CAT enzymatic activity decreased via increasing the treatment time, while SOD activity showed fluctuations in all sampling times (24, 48, 72 h).

Significant differences could be observed for three-way interaction (genotype * treatment * sampling time) ($p \leq 0.0001$) with higher CAT activity under drought, heat, and combined stress in RN51 as compared with RN1 for all tested treatment times (24, 48 and 72 h). At 72 h, the CAT activity increases 105 200% for RN51 genotype (Fig. 1G, H, I). Similar CAT activity increase was observed in this genotype upon exposure to heat and combined stress whereas the highest CAT activity increase was showed under drought stress applied separately. In the opposite trend, RN1 genotype not showed changes in CAT activity for all stress treatments at 72 h.

When CAT activity was evaluated for all five genotypes, it was significant for three-way interaction (genotype * treatment * sampling time) ($p \leq 0.0001$). CAT activity was significantly different at all sampling times. When combined stress (drought + heat) was imposed to all genotypes at 72 h, two different groups could be observed. The first group includes RN51, J20 and RN49 genotypes, which showed CAT activity increases ranging from 93 to 137%. While, the second group, including RN1 and S6 genotypes and control treatments, did not show any increase in CAT activity level (Fig. 2C).

3.1.4. Superoxide dismutase (SOD) activity measurement

SOD activity showed significant differences for three-way interaction ($p \leq 0.0001$) with higher SOD level under drought, heat and combined stress in RN51 as compared with RN1 only 72 h of exposing treatments for each genotype. Accordingly, the SOD activity increased about 37 66% for RN51 (Fig. 1J, K, L). For this genotype, SOD activity increase was lower in combined stress than single drought and heat stress. In the opposite trend, RN1 genotype not showed changes in SOD activity for all stress treatments at 72 h.

When SOD activity was evaluated for all five genotypes, it was significant for three-way interaction (genotype * treatment * sampling time)

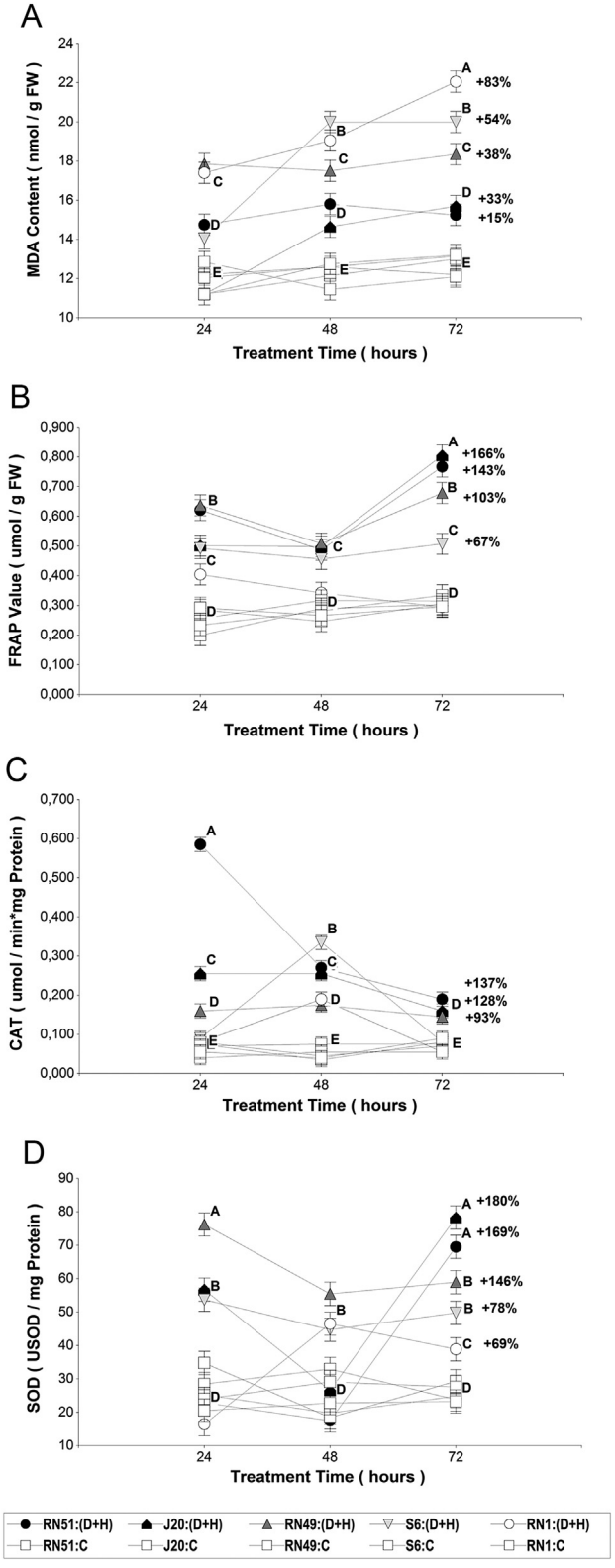

Fig. 2. Effect of combined stress on malondialdehyde (MDA) content (A), total reducing power (FRAP value) (B), catalase (CAT) (C) and superoxide dismutase (SOD) (D) enzyme activities evaluated at 24, 48 and 72 h in five buffel grass genotypes (RN51, RN1, RN49, S6 and J20). C: control, (D + H): combined stress. DGC test $\alpha = 0.05$. Different letters denote significant differences ($p \leq 0.05$). Error bars indicate standard error. The percentage values in bold represent the increase in the average value of treatment respect to the average value of control.

($p \leq 0.0001$). Also at 72 h of exposing of combined stress, SOD activity growth was significant for all tested genotypes. This contains forming four groups of studied genotypes. The first group includes RN51 and J20 genotypes with the highest activity increase by 180 and 169%, respectively. The second group containing RN49 and S6 genotypes revealed 146 and 78%, growth, respectively. The third (RN1), showed the lowest activity increases by 69% when compared with the last group which was the control treatments (Fig. 2D).

3.2. Evaluation of growth performance

After completion of drought, heat and combined stress treatments, the two buffel grass genotypes showed less growth comparing with their control treatments. The Aerial Dry Weight (ADW) values exhibited significant differences for two-way interaction ($p \leq 0.0001$), via a higher level under all stress conditions (drought, heat and combined stress) for RN51 as compared with RN1 (Fig. 3A, B, C). Weight reduction varied 31 42% and 52 69% for RN51 and RN1, respectively. A more pronounced reduction in ADW was observed in both genotypes upon exposure to heat and combined stresses than in drought stress applied separately with similar percentages. However, drought stress applied separately produced slight reduction in ADW.

The other characters measured (height and fresh weight of aerial part) showed the same pattern as observed for ADW (data not showed). When the five genotypes were evaluated in drought and heat combined stress conditions, ADW values was significantly different for two-way interaction ($p \leq 0.0001$), containing four groups (Fig. 4). The first (RN51) and second (J20, RN1, S6 and RN49) groups corresponded to control treatments. The third group included RN51, J20 and RN49 genotypes, with lower level of growth reduction by −46, −40 and − 41%, respectively. While RN1 and S6 (fourth group) showed higher level of growth reduction (−71 and − 64%, respectively) in combined stress. The other characters measured (He and AFW) showed the same pattern as described for ADW (data not showed).

4. Discussion

In the present work, the individual and combined effects of drought and heat on antioxidant parameters and growth performance in Buffel grass genotypes were investigated. Our results showed that oxidative damage (estimated by MDA content) was also higher in the sensitive genotype in response to combined stresses, suggesting that the oxidative damage can be directly linked to susceptibility of genotype to the combination of drought and heat stress (Fig. 1A, B, C). These results are in accordance with the obtained of several studies conducted for other plant species (Sekmen et al., 2014; Bi et al., 2016; Jin et al., 2016; Zandalinas et al., 2017, 2018). A negative association was observed between MDA content and FRAP value indicating decreasing trend of MDA content and increased an upward trend of FRAP value in tolerant genotype. However, studies have not evaluated this parameter related to stress tolerance. In medicinal plants and vegetables and tree species (Cervilla et al., 2007; Dudonné et al., 2009; Nur Alam et al., 2013; Rabeta and Nur Faraniza, 2013; Popovic et al., 2016) it has been used as a tool for determination of total antioxidant content. On the other hand, Abideen et al. (2015) have reported an upward trend on reducing power of leaves genotypes under stress conditions in a halophytic grass.

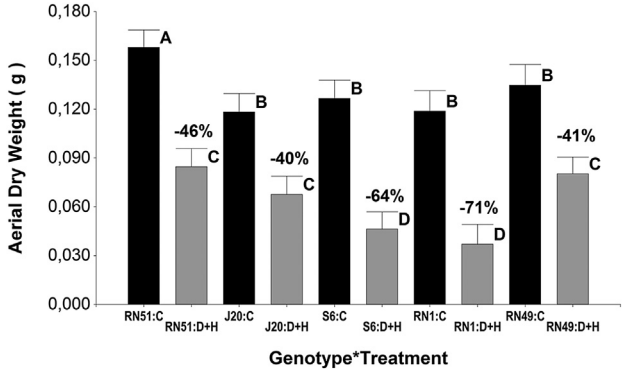

Fig. 4. Effect of combined stress on Aerial Dry Weight (ADW) evaluated at the end of stress assay in five buffel grass genotypes (RN51, RN1, RN49, S6 and J20). C: control. D + H: combined stress. DGC test $\alpha = 0.05$. Genotype*Treatment represent interaction between these two factors. Different letters denote significant differences ($p \leq 0.05$). Error bars indicate standard error. The percentage values in bold represent the decrease in the average value of treatment respect to the average value of control.

To analyze the pattern of antioxidant defenses, we measured some key antioxidant enzymes activities (Bi et al., 2016), like total SOD and CAT activities. Higher SOD activity under the drought, heat and combined stress was observed in tolerant genotype (Fig. 1J, K, L). Huseynova (2012) reported that SOD activity has been significantly decreased in sensitive wheat cultivars and remained at the control level or increased in tolerant ones. In addition, a recent study on cool-season turf grass showed the same result under drought and heat and the combined stresses (Bi et al., 2016). Based on current and previous works the tolerant genotype might have higher and longer capability to catalyze the dismutation of $O^{-\bullet 2}$ to H_2O_2 under stress conditions (Lanza Castelli et al., 2010; López Colomba et al., 2013). Different results on stresses effects on CAT activities such as induction, reduction or stable

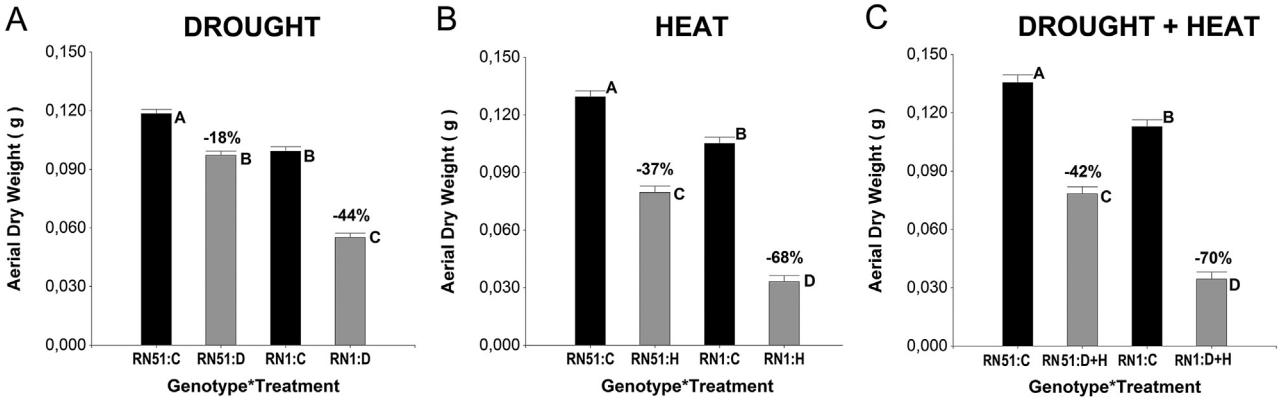

Fig. 3. Effect of drought (A), heat (B), and combined stress (C) on Aerial Dry Weight (ADW) evaluated at the end of each respective stress assay in RN51 and RN1 buffel grass genotypes. C: control; D: drought; H: heat; D + H: combined stress. DGC test $\alpha = 0.05$. Genotype*Treatment represent interaction between these two factors. Different letters denote significant differences among genotypes ($p \leq 0.05$). Error bars indicate standard error. The percentage values in bold represent the decrease in the average value of treatment respect to the average value of control.

CAT activities under drought, heat and combined stress have been reported (Jiang and Huang, 2001; Gill and Tuteja, 2010; Anwar Hossain et al., 2013; Boaretto et al., 2014; Bi et al., 2016; Jin et al., 2016). However, in the current study, total CAT activity has been increased in tolerant genotype under drought, heat and combined stress treatment, while it has been reduced or remained unchanged in sensitive genotype (Fig. 1G, H, I). Our results are consistent with the reported by Sheikh-Mohamadi et al. (2017) who demonstrated that increased, decreased, and unchanged activities of antioxidant enzymes in the wheatgrass genotypes indicates a different forms of metabolism of antioxidant enzymes in response to drought and salinity stress. The increased CAT activities of tolerant genotype in stress conditions compared to susceptible genotype could be related to an active and efficient antioxidant strategy that might be involved in maintaining a lower MDA content (and oxidative stress) under drought and heat and the combined stress, and therefore helping buffel grass genotypes to face up to these stresses. In addition, several studies describing that tolerance to abiotic stress is associated with increasing CAT activity (Gill and Tuteja, 2010; Anwar Hossain et al., 2013; Suzuki et al., 2014; You and Chan, 2015; Choudhury et al., 2017).

When heat and drought were applied separately, the reduction in aerial dry weight was more pronounced under heat stress than drought stress whereas when stresses were applied together, the effects were similar in comparison with the injurious effects of heat stress (Fig. 3A, B, C). The lowest damage in growth characters were measured in tolerant genotype under drought, heat and combined stress. Based on our results, a significant relation could be observed between a better performance in growth characters, lower oxidative damage (lower MDA content), antioxidant defense induction and tolerance to abiotic stresses as formerly confirmed by other studies on numerous plant species (Almeselmani et al., 2006; Gill and Tuteja, 2010; Sundaram and Rathinasabapathi, 2010; Wang et al., 2010). The results showed here suggest the antioxidant metabolism seems to be critical for tolerate heat and drought stress combination and the specific molecular mechanisms underlying these parameters and the relationship with stress tolerance is our further study.

In general, when different abiotic stresses happen simultaneously, they can either urge the effect of each other or cause an antagonistic effect on crop productivity and growth performance (Pandey et al., 2015). The results showed that the heat stress had a predominant effect over drought stress on buffel grass genotypes subjected to combined stress. It could indicate that the response to combined stress not are unique and could be uncover linkage between the physiological/biochemical responses of buffel grass to heat and combined stress. Even though, emerging evidence show plant responses to simultaneous drought and heat resulted in a new profile of transcript expression that could not be predicted by the effect of the single stress applied individually (Zandalinas et al., 2018). In addition, the other factor to consider is the combined stress depend on genotype and phenological stage of plants and severity and duration of combined stress. This observation is in complete agreement with the results obtained by Silva et al. (2010).

When all five buffel grass genotypes were evaluated in combined stress, the genotype J20 showed similar response to combined stress as observed for RN51 tolerant genotype. While S6 presented a different response to stress like the case that observed for susceptible genotype RN1 (Fig. 2A, B, C, D; Fig. 4). The different responses might be due to the selection process which could be induced by mutation and somaclonal variation of these genotypes (J20 and S6) as explained by previous drought tolerance (López Colomba et al., 2011) and salt tolerance (López Colomba et al., 2013) former studies. The biochemical parameters and growth performance measured in the current study gave us a solid basis to differentiate all studied buffel grass genotypes based on their sensitivity and tolerance to individual or combined drought and heat stresses. Out of all biochemical parameters measured in this study, MDA content and the FRAP value could be important for the selection of buffel grass genotypes under single and combined stress in a breeding program of this species.

5. Conclusion

In summary, individual drought, heat, and the combined stresses induced oxidative damage in the buffel grass genotypes, as demonstrated by the reduction in antioxidant enzyme activities and increase in lipid peroxidation. We found that the sensitive genotype exhibited higher MDA content, lower FRAP values, and reduced CAT and SOD activities than tolerant under drought or heat stress or a combination of both stresses. Our results indicate that drought and heat greatly disrupt oxidative metabolism and generate biochemical and growth performance changes in buffel grass genotypes with variable responses. This can be an important tool for the characterization of genotypes in buffel grass breeding program, which allow us to develop strategies for selection of tolerant genotypes capable of producing higher biomass under stressful conditions.

Author contributions

Exequiel Tommasino, Eliana López Colomba, Magalí Carrizo and Mariana Quiroga—conducted stress assays in controlled conditions and growth and biochemical measurements in *Cenchrus ciliaris* L.; Karina Grunberg and Celina Luna—were responsible for project design; Edgardo Carloni, Sabrina Griffa and Andrea Ribotta—contributed to gardening of plants used in all assays; all authors contributed to writing the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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