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Drought effects on the early development stages of *Panicum virgatum* L.: Cultivar differences

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ABSTRACT

We studied the response to moderate drought and re-watering in 55-day-old plants of the lowland cv. Kanlow and the upland cv. Greenville. Plants were grown in a growth chamber; drought treatment was applied by water suppression until moderate stress was reached. The optimal harvest time was determined when the relative water content (RWC) in plants reached an average of 60%. The effects of drought and recovering on growth (i.e. plant height and leaf number), antioxidant enzymes (SOD and CAT), abscisic, salicylic and jasmonic acids levels (ABA, SA, JA), and water stress related genes were analyzed. Drought induced a decrease in growth in cv. Greenville but not in Kanlow. Drought tolerance in cv. Kanlow was correlated with higher antioxidant activities both in stress and recovery. Thus, we investigated stress hormones known to induce reactive oxygen species (ROS). ABA was the main hormone under stress, and increased higher in cv. Kanlow than in cv. Greenville. SA and JA basal levels were higher in well watered plants of cv. Kanlow. At gene levels, *RAB18* and *RD22* overexpressed after 24 h of re-watering in cv. Kanlow, resembling a "stress memory" mechanism described in other species. Further molecular markers of hormone effects (*PR2*) or drought (*DREB2*) well known in dicots seem not to be specific for *Panicum virgatum*. Overall, we found that cultivar Kanlow has an effective machinery of stress response, which makes it promising to be grown successfully in semi-arid regions.

1. Introduction

The interest in using *Panicum virgatum* L. (switchgrass) as feedstock for the production of bio-ethanol is rapidly growing. However, basic agronomic properties of *P. virgatum* are not thoroughly understood and the environmental effects have not been fully investigated yet.

P. virgatum is a high C4 perennial warm-season grass native to North America and member of the Poaceae family.

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The Poaceae is the fourth largest plant family in the world – with over 10.000 species widely distributed – which includes the top four agricultural commodities by quantity (sugarcane, maize, rice, wheat). In addition to livestock feed, biomass production by several large perennial grasses, such as switchgrass, shows potential for utilization as energy crops.

Switchgrass is used in a large portion of the USA, in part due to its broad adaptation to marginal, highly erodible, and droughty soils [1]. In Argentina, efforts have been made to introduce this grass to low production soils from semi-arid regions, which are 25% of the arable surface of the country [2]. One of the most persistent issues in producing switchgrass has been the difficulty to rapidly and consistently establish strong stands of plants, especially in semi-arid regions where recurrent drought occurs during implantation. Switchgrass is a small-seeded species that initially allocates a large amount of energy to develop a strong root system. As such, it usually reaches only 33–66% of its maximum production capacity during the first and second years, and reaches its full capacity during the third year after planting. Protocols of management to establish strong stands of plants include studies to improve seed germination, evaluate a range of herbicide treatments for weed control, define tolerance limits for acidity, temperature, and water, and alter seedling vigor through breeding; however a proper establishment of plants is still a problem [3]. To develop genetic material with background of drought tolerance would contribute to the diffusion of this species of increasing economic importance for bio-ethanol production.

The study at cellular and molecular level of switchgrass under water deprivation may lead to new approaches to enhance plant establishment and improve yield in marginal areas, thus refining the economies of biomass production that have in the past limited its utilization. In the mid-term, increases in drought tolerance could be introgressed from tolerant genotypes using a marker-assisted breeding approach. As well, a better understanding of drought tolerance in switchgrass will help to understand other grasses, since the Poaceae family has an extensive synteny among the genomes of its members. Hence, what we learn about one member of the family can enhance our understanding of the entire group contributing to the improvement of grass crops in meeting the challenge for developing renewable supplies of fuel and industrial products [4].

Drought effects are often manifested as inhibition of growth [5], accumulation of compatible organic solutes [6], increase of the antioxidant response [7], changes in phytohormones contents [8], modifications in stress-responsive-genes expression [8], among others. Some of these responses are triggered by a change in the water status while others are brought about by plant hormones [9]. In this sense, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) are involved in a complex signal-transduction network that coordinates growth and development with plant responses to adverse environment [10,11].

Increased levels of endogenous ABA have been reported in several species under abiotic stresses such as water deficit [12]. ABA increments under water stress have been observed in *Arabidopsis* as well as in cereals and forage crops, such as maize [13,14], sorghum [15], wheat [16], fescue [17], barley [18] and alfalfa [19]. In turn, ABA controls many dehydration-responsive genes that participate in drought tolerance. ABA-induced genes include late embryogenesis abundant (LEA), such as RAB18 (Responsive to ABA 18) and RD22 (which are widely distributed among monocots and dicots [20,21]). In addition to ABA-dependent genes, there are a number of dehydration-responsive genes that are ABA-independent. One of the most studied is the family of DRE (dehydration-responsive element) transcription factor that controls a stress signaling pathway. Within this family, the subgroup 2 (DREB2) is the more involved in the response to drought [22]. DREB2-type genes were investigated in species of the grass family, such as rice [23], maize [24], *Penisetum glaucum* [25] and orthologues and have also been reported in other plant species [26], showing the wide distribution of the family within the plant kingdom.

In the same line, SA has been recognized as a regulatory signal mediating plant response to abiotic stresses such as drought [27], low and high temperatures [28], heavy metals [29], and osmotic stress [30]. Recent results show that most abiotic stresses altered *in planta* SA contents [31]. Munné-Bosch and Peñuelas [27] reported in *Phillyrea angustifolia* L. plants that the SA level increased progressively up to 5-fold during drought stress, and showed a strong negative correlation with the relative water content. Similarly, Bandurska and Stroiński [32] found an SA increase in barley roots under water stress. High levels of SA induce the expression of pathogenesis-related (PR) genes, which were originally associated with biotic stress [33], but more recent evidence links them to the response to abiotic stress [34,35], including water stress [36].

JA participation during abiotic stress conditions has also been proved in drought [8] and salinity [37]. For example, treatment of barley leaves with sorbitol or mannitol (solutes to simulate water stress) increased JAs contents, followed by synthesis of jasmonate-induced proteins (JIPs) [38]. Other study showed that sorbitol treatment enhanced octadecanoids and JAs content, and this threshold was necessary and sufficient to initiate JA-responsive gene expression [39]. In addition, under water stress JA content increased in maize root cells [13] and this compound was able to elicit betaine accumulation in pear leaves [40].

In response to environmental stresses, there is an increment in the reactive oxygen species (ROS). This ROS overproduction is harmful for biological systems, thus plants have evolved mechanisms that limit ROS formation and promote its removal [41]. Plant enzymatic defenses include antioxidant enzymes such as the phenol peroxidase (POX), ascorbate peroxidase (APX), glutathione peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT), and high activity of these enzymes are potential defense mechanism of stress tolerance [42]. Numerous studies propose that increasing antioxidant activity correlates with improved stress tolerance. For instance, SOD and CAT activity was significantly higher in leaves of stressed wheat plants compared to well-watered plants [43]. ROS signaling is connected to ABA, flux of Ca^{+2} and sugars, and it is possible that they participate both up and downstream of ABA-dependent pathways in drought conditions [44]. Similarly, ROS production has been related to SA since exogenous SA alleviates the stress damage and increases antioxidant enzyme activity [45].

In the present study, we investigated to what extent drought affect growth parameters, the antioxidant machinery, stress hormone levels and responsive genes in two

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cultivars of contrasting behavior to water stress. Accordingly, we quantified and compared the effects of moderate drought and recovering on growth, antioxidant enzymes such as SOD and CAT, abscisic, salicylic and jasmonic acids levels and water stress marker genes.

2. Material and methods

2.1. Plant material, growth conditions and treatments

P. virgatum L. cultivars of contrasting water stress tolerance were used: tolerant cv. Kanlow and sensitive cv. Greenville [46,47]. Seed were collected from 10 year old experimental plots and provided by the Germoplasm Bank of the EEA G. Covas (36°31′S; 64°01′W). Seeds were harvested from the whole plant, threshed by hand, dried with silica gel and stored at 7° in trilaminate aluminum envelopes. Experiments were conducted in growth chamber located in the greenhouse of the Facultad de Agronomía, Universidad Nacional de La Pampa, La Pampa, Argentina (36°46′S; 64°16′W). Plants were grown in pots of 220 cm^3 containing ground substrate (1 part soil and 3 parts sand) in a growth chamber with a cycle of 16 h light (300 $\mu E\ m^2\ s^{-1}$), 29 °C – 8 h dark, 20 °C. When plants were 55 day-old, drought treatment was applied by water suppression until moderate stress was reached. Controls plants were grown continuously with normal irrigation. The optimal harvest time was determined when the relative water content (RWC) reached an average of 60% and visual symptoms were observed (drought treatment – D).

To evaluate plant recovering after drought, plants were re-watered and measurements were performed 12 and 24 h after re-watering (RW 12 h and RW 24 h). At the end of experiment, leaves of control and treated pants were collected in liquid N_2 and stored at −80 °C for RNA extraction and enzyme activity and. For hormone quantification leaves were first lyophilized and then stored at −80 °C. Plant height and leaf number were measured during the drought treatment, and after 12 and 24 h of re-watering.

2.2. SOD and CAT activities

Antioxidant enzyme activities were assayed separately in samples obtained from the leaves. 100 mg fresh weight of frozen leaf were ground up into a fine powder with liquid nitrogen, and homogenized in 1 cm^3 of potassium phosphate buffer 5 mol m^{-3}, pH 7.5 supplemented with EDTA (0.1 mol m^{-3}).

The homogenates were centrifuged (16,000 *g*, 4 °C, 25 min), and the supernatant was used as crude extract to determine protein concentration [48] and enzyme activity.

Total superoxide dismutase (SOD) activity was assayed at 560 nm by measuring the inhibition of the photochemical reduction of NBT [49]. Catalase (CAT) activity was determined at room temperature by measuring the decrease in A240 after adding 0.5 mol m^{-3} H_2O_2 to samples [50].

2.2.1. Extraction and purification of endogenous hormones

ABA, SA and JA and were extracted from 0.2 g of dry weight leaf tissues of cvs. Kanlow and Greenville according to Durgbanshi [51] with some modifications as detailed below. Plant material was ground up in a mortar and a pestle with liquid nitrogen and homogenized in IKA-ULTRATURRAX T25 basic (Staufen, Germany) with 5 cm^3 deionized water. Stable isotope-labeled compounds used as internal standards were D_6-JA, D_6-SA (Leibniz-Institute of Plant Biochemistry, Halle, Germany) and D_6-ABA (NRC-Plant Biotechnology Institute, Saskatoon, Canada), and 50 ng of each compound was placed in each sample. The samples were transferred to 50-cm^3 tubes and centrifuged at 1540 *g* for 15 min. The supernatant was collected, adjusted to pH 2.8 with 2620 mol m^{-3} acetic acid and extracted twice with an equal volume of diethyl ether. The aqueous phase was discarded and the organic fraction was evaporated by vacuum. Dried extracts were dissolved in 1 cm^3 methanol. Samples were filtered through a syringe filter tip on a vacuum manifold at flow rate less than 1 $cm^3\ min^{-1}$, and the eluate was evaporated at 35 °C under vacuum in a SpeedVac SC110 (Savant Instruments, Inc., New York, USA).

2.2.2. Liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI MS/MS) analysis for hormonal identification and quantification

Mass spectrometric analysis for simultaneous measurements of hormones was performed on a quadruple tandem mass spectrometer (MS–MS, Quattro Ultima, Micromass, Manchester, UK) fitted with an electrospray ion source (ESI). A mixture of all unlabeled compounds and internal standards was separated by reversed-phase high-performance liquid chromatography (HPLC), and analyzed by tandem mass spectrometry with multiple reactions monitoring (MRM) to determine retention times for all compounds. The spectrometer software used was MassLynx™ v. 4.1 (Micromass, Manchester, UK). Response was calculated as product ion peak area × (IS concentration/IS product ion peak area), where IS concentration is the amount of internal standard added. ABA, SA and JA were separated from tissues by HPLC. An Alliance 2695 separation module (Waters, Milford, MA, USA) equipped with a 100 mm × 2.1 mm, 3-μm RESTEK C_{18} column was used to maintain performance of the analytical column. Fractions were separated using a gradient of increasing methanol concentration, constant glacial acetic acid concentration 33 mol cm^{-3} in water, and initial flow rate 0.2 $cm^3\ min^{-1}$. The gradient was increased linearly from two parts methanol – three parts water–acetic acid at 25 min, to four parts methanol – one part water–acetic acid. After 1 min, initial conditions were restored and the system was allowed to equilibrate for 7 min. The identification and quantification of all hormones were performed in MRM using precursor ion and its transitions (*m/z*) to ABA (*m/z* 263/153) and D_6-ABA (*m/z* 269/159), SA (*m/z* 137/93), D_3-SA (*m/z* 141/97), JA (*m/z* 209/59), D_6-JA (*m/z* 215/59), with retention times of 9.80, 4.50 and 13.90 min respectively. Collision energies used were 20 electron volts (eV) and cone voltage was 35 V.

2.3. Gene expression analysis

Total RNA was isolated from leaf tissue using TRIZOL (Reagent® Invitrogen™ Life Technologies) according to the manufacturer's instructions and quantified using a UV–Visible spectrophotometer (Ocean Optics CHEM 2000). The RNA quality was checked using 1 1000 g m^{-3} agarose gel

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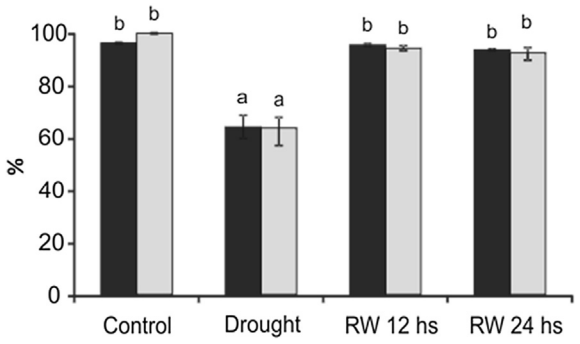

Fig. 1 – Relative water content (RWC) of *Panicum virgatum*; cvs. Kanlow (black bars) and Greenville (gray bars) grown under well-watered conditions (Control), drought (Drought), after 12 h of re-watering (RW 12 h), and after 24 h of re-watering (RW 24 h). Data represented are the means of four replicates with SEs, and values with the same letter are no significantly different at $p \leq 0.05$.

electrophoresis. 1 μg RNA was reverse transcribed using oligo (dT) primer and reverse transcriptase (Promega M1701) according to the manufacturer's instructions. The *P. virgatum*-specific elongation factor-1 (housekeeping gene) was amplified in parallel with the target transcripts for gene expression normalization. The primers used to find the differential expression of genes were designed with Primer3 program ([73]): EF-1-alpha left primer CCAAGAGGCCTTCAGACAAG, right primer TGAGATCCTTCACAGCAACG; RAB18 left primer TCACACTGCACACACAGGTG, right primer AGAAGGGCATCATGGACAAG; RD22 left primer ACCGTCCAGTTCACCAACAC, right primer AGGACTTCTTCTCGCCCTTG; DREB2a left primer TCTTGCAGCAACAACCTCTG, right primer GTGCCTTCACAGTGCTTGAA; PR2 right primer GGCTGTCGGGGAAGTAGAC, left primer ACAATGGCGAGGCAACAAG.

Level of expression was analyzed by semi-quantitative PCR (MJ-Research model PTC 100) and the programs were 29 cycles for RAB18, RD22 and DREB2a and 35 cycles for PR2 (95 °C 1 min, 55 °C 30 s and 72 °C 1 min), for each particular primer. Images of PCR products were processed using ImageJ 1.29 [52]

2.4. Experimental design and statistical analysis

The experimental design was completely randomized. For statistical analysis of plant height and number of leaves each plant was considered a replica (24 replicates per treatment). For statistical analysis of hormone, SOD and CAT activities and gene transcripts, three replicates per treatment (each consisting of a pool of six plants) were considered. All results were analyzed by ANOVA and Least Significant Difference (LSD) *a posteriori* using InfoStat program [53].

3. Results

3.1. Growth parameters

The relative water content (RWC) during the drought treatment reached 60% in both cultivars. After 12 h of re-watering, plants of both cultivars recovered the control value (Fig. 1). In parallel, plant height of the tolerant cv. Kanlow under drought did not show significant differences in comparison to controls, indicating a superior performance of this cultivar. In contrast, this parameter was significantly decreased by water stress in the sensitive cv. Greenville compared to control (Fig. 2A). After 12 h of re-watering, no differences were observed in Kanlow whereas a reduced plant height was observed in Greenville (Fig. 2B). After 24 h of re-watering plant height reached the control level in Greenville.

In addition, a significant decrease in the leaf number was observed in the cv. Greenville compared to control in response to water stress (Fig. 3A), in a similar fashion that plant height (Fig. 2A). After 12 h of re-watering, leaf number of both cultivars were similar to that of controls (Fig. 3B). However, at 24 h of re-watering a reduction in leaf number was observed in cv. Greenville, perhaps as a negative effect of stress (Fig. 3C). Notably, cv. Kanlow maintained almost the same number of leaves under all treatments.

3.2. Activity of SOD and CAT enzymes

Activity of SOD and CAT enzymes was evaluated in order to know if the antioxidant machinery was active during moderate water stress and recovery period, as reported in some

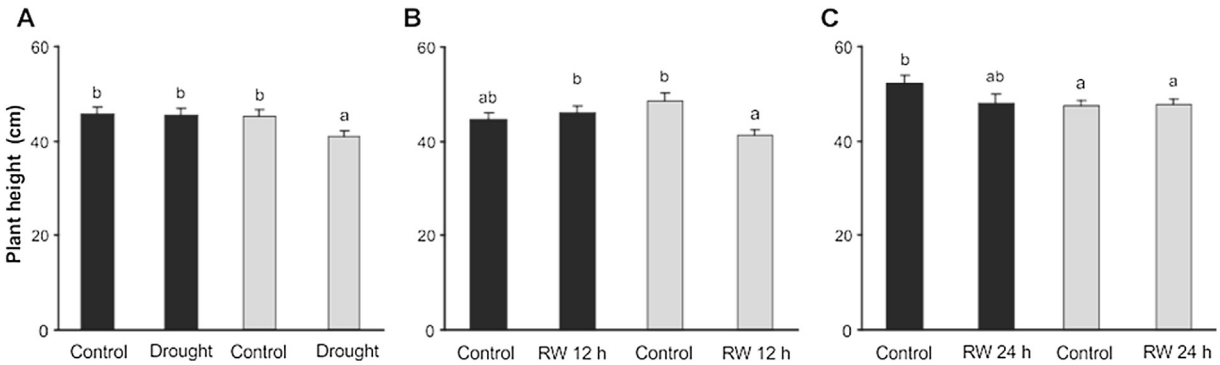

Fig. 2 – Plant height of *Panicum virgatum*; cvs. Kanlow (black bars) and Greenville (gray bars) grown under: A) drought, B) after 12 h of re-watering (RW 12 h), and C) after 24 h of re-watering (RW 24 h). Data represented in Figs. 1 and 2 are means of twenty four replicates with SEs, and values with the same letter are not significantly different at $p \leq 0.05$.

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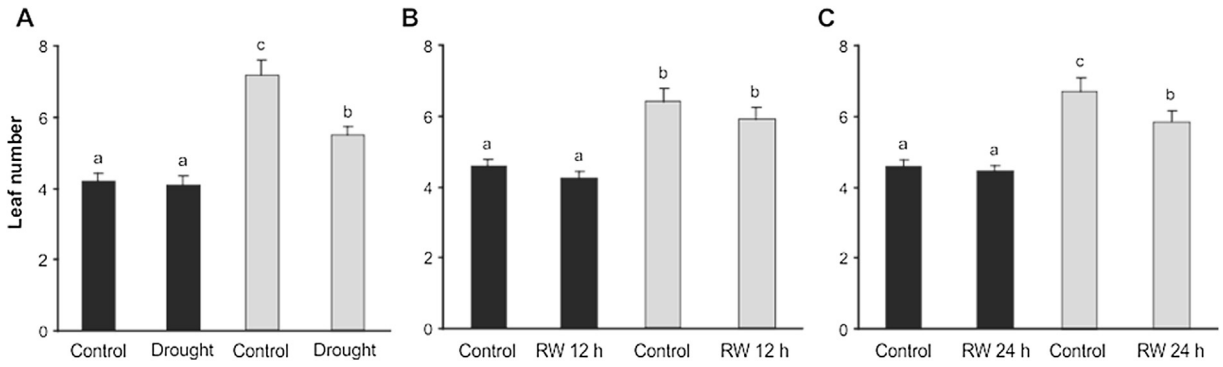

Fig. 3 – Leaf number of *Panicum virgatum*; cvs. Kanlow (black bars) and Greenville (gray bars) grown under: A) drought, B) after 12 h of re-watering (RW 12 h), and C) after 24 h of re-watering (RW 24 h).

species. In leaves of plants from both cultivars, SOD was more abundant than CAT, although both enzymes showed a similar kinetic.

In control condition, both cultivars showed the same SOD and CAT basal level. In response to drought cv. Kanlow increased 2- fold SOD activity compared to the control, and a high level of SOD was maintained during the recovery period. The same trend was observed for cv. Greenville, although SOD level was lower than that of Kanlow (Fig. 4A). CAT activity also increased under water stress in both cultivars. Again, an increasing trend was registered after 12 and 24 h of re-watering in the two cultivars (Fig. 4B).

3.3. ABA, SA and JA under drought and re-watering

In control plants, endogenous ABA levels of cvs. Kanlow and Greenville were similar. However, water stress triggered abrupt peaks of ABA in both cultivars. In Kanlow, ABA increased 11-fold under stress and returned to control values after 12 and 24 h of re-watering. In Greenville, instead, ABA increase during stress was 4.5-fold and decreased 1.5-fold after 12 h of re-watering to finally reach the control level at 24 h (Fig. 5A).

SA was the most abundant compound. Basal level of SA in control leaves was significantly higher in Kanlow than in Greenville. The response of both cultivars to water stress and re-watering was different. In Kanlow, endogenous SA level was not modified by water stress; however, a significant decreased was observed at 12 and 24 h of re-watering compared to control leaves. In Greenville, on the other hand, there was a decrease in SA in response to water stress, and then a significant increase occurred at 24 h of re-watering (Fig. 5B).

JA, contrarily to ABA and SA, was the least abundant compound in both *Panicum* cultivars. Endogenous JA in control leaves was higher in cv. Kanlow compared to cv. Greenville and one more time the response of both cultivars was not similar. Kanlow showed a decreasing trend in JA level under stress which became significant at 12 h of re-watering, recovering then the control value at 24 h. In Greenville, JA levels were not affected by water stress. However, after watering was restored, level of JA increased and significantly overcame the control at 24 h (Fig. 5C).

3.4. Gene expression under drought and recovering

The expression of genes involved in water stress and related hormones were evaluated. In this sense, expression of ABA-dependent (RAB18 and RD22) and independent genes (DREB2a), and SA dependent (PR2) were measured.

In cv. Kanlow, the expression of RAB18 increased 3- fold under water stress and at 12 h of re-watering its expression

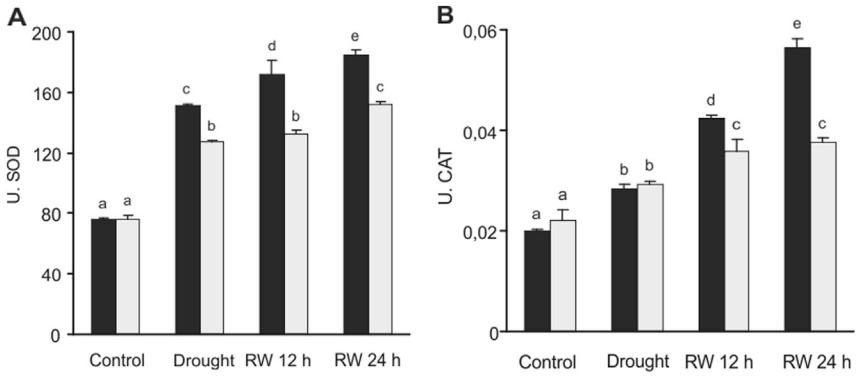

Fig. 4 – Activity of antioxidant enzymes isolated from leaves of *Panicum virgatum*; cvs. Kanlow (black bars) and Greenville (gray bars) grown under drought and after 12 h and 24 h of re-watering (RW 12 h and RW 24 h). A) Superoxide dismutase (SOD), B) catalase (CAT). Data are means of three replicates with SEs. Values with the same letter are not significantly different at $p \leq 0.05$.

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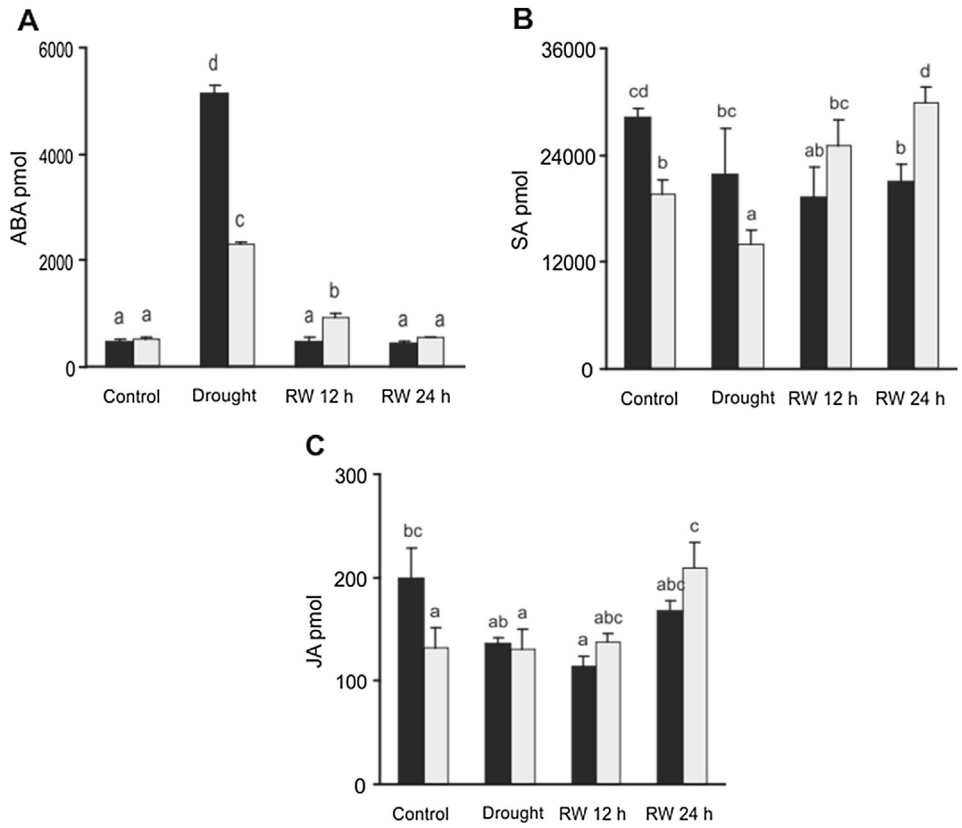

Fig. 5 – Content in leaves of *Panicum virgatum* plants: A) abscisic acid (ABA), B) salicylic acid (SA) and C) jasmonic acid (JA); cvs. Kanlow (black bars) and Greenville (gray bars) grown under drought and after 12 and 24 h of re-watering (RW 12 h and RW 24 h). Data are means of three replicates with SEs. Values with the same letter are not significantly different at $p \leq 0.05$.

was suppressed. While, at 24 h of re-watering RAB18 expression was significantly higher than that of control. In contrast, water stress treatment did not cause a significant increase in the expression of RAB18 gene in cv. Greenville. After 12 h of re-watering, gene transcripts were not observed and finally recovered control level at 24 h of re-watering (Fig. 6A).

In cv. Kanlow, the expression of RD22 did not change under water stress or 12 h of the recovery; however, a significant increase occurred after 24 h of re-watering. In cv. Greenville, RD22 expression showed not major differences during water stress and both re-watering periods (Fig. 6B).

On the other hand, under water stress the transcript level of DREB2a significantly decreased in both cultivars in a similar fashion (Kanlow 5-fold, and Greenville 4-fold), to finally return to control level at 24 h of re-watering (Fig. 6C).

The expression of PR2 (SA dependent) was detected only at 24 h of re-watering in the cv. Kanlow (Fig. 6D).

4. Discussion

4.1. Growth response to drought

The response of the cv. Kanlow and cv. Greenville to moderate drought and re-watering show differences between them. Moderate drought was considered when young plants reached, in average, 60% of relative water content. Drought did not affect growth parameters (plant height and leaves number) in cv. Kanlow, but it affected the performance of cv. Greenville. Given the wide biological diversity of *P. virgatum*, two distinct ecotypes of this grass are recognized: lowland ecotypes, associated with hydric soils and common in floodplains, and lowland ecotypes, associated with mesic to xeric environments [54]. Earlier evidences suggest that upland ecotypes would outperform lowland types under low soil moisture availability, and vice versa under excess soil moisture. However, a more recent study compared growth and performance in upland and lowland switchgrass types to water and nitrogen stress and they found that upland cultivars showed a smaller response to drought stress [46].

Similarly, Barney [47] compared genotypes of the lowland and highland in conditions ranging from water stress (−4 MPa) to flood in different stages of development. They found that lowland ecotypes were the best performers in the entire range of water conditions imposed. These results are in agreement with our findings since cv. Kanlow belongs to the lowlands ecotypes and cv. Greenville belongs to the highlands ecotypes.

4.2. SOD and CAT activity under water stress and re-watering

A great deal of research has established that the induction of the cellular antioxidant machinery is important for protection against various stresses [42]. In both cultivars of *P. virgatum*

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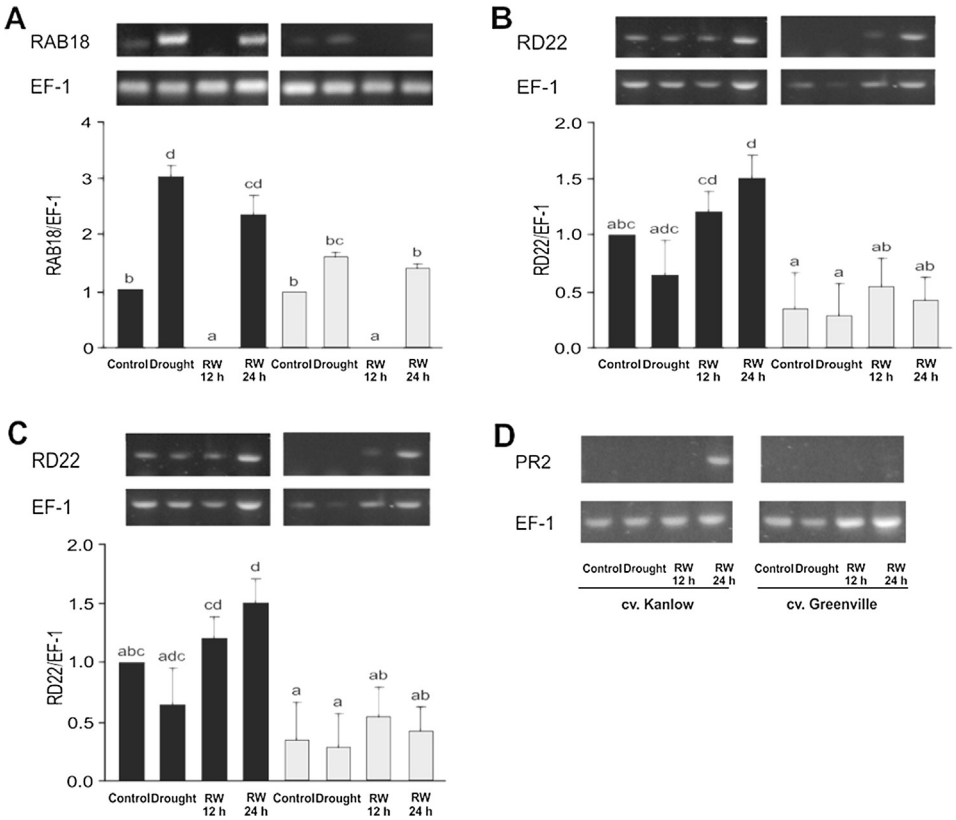

Fig. 6 – Expression analysis of: A) RAB18, B) RD22, C) DREB2a, and D) PR2 gene in leaves of *Panicum virgatum* plants in response to drought and after 12 h and 24 h of re-watering using the RT-PCR technique. Relative transcript abundance was calculated and normalized with respect to the *Panicum elongation factor-1-alpha* (EF-1-alpha) transcript level. Data are means of three replicates with SEs. Same letters indicate no difference between means $p < 0.05$. Values with the same letter are not significantly different at $p \leq 0.05$.

evaluated, we also found that SOD and CAT activities were increased under drought and these activities keep increasing after plant re-watering. This might suggest that the antioxidant defense machinery activated under stress remain operative during recovery period, something that is applied to some species during the acclimation process [55–57].

Increased levels of SOD after recovery were reported in *Kochia scoparia* [54] and *Cupressus funebris* [57]. Srivalli [55] studied the acclimation process in rice subjected to three cycles of water stress of increasing intensity. These authors proposed that the participation of enzymatic complexes (SOD, CAT, others) varied according to the stress level followed by recovering and highlighted the different role of each complex in the drought acclimation process.

In the tolerant as well as in the sensitive *P. virgatum* cultivars, SOD and CAT activities kept increasing after water stress was applied and plants were re-watered. A study with contrasting cultivars of wheat demonstrated that in the drought-sensitive cv. Adamello the activity of antioxidant enzymes returned to control level upon rehydration, in contrast to drought-tolerant cv. Ofanto, suggesting that different mechanisms are operating in both cultivars [58]. In a similar approach, Khanna-Chopra and Selote [59] reported that drought-resistant wheat genotype acclimated better than sensitive genotype by maintaining low oxidative damage through inducing well-coordinated antioxidant defense, which included an up-regulation of antioxidant enzymes. In our experiment with *P. virgatum*, SOD and CAT play a significant role under drought and recovering following water deprivation perhaps helping plants to recover growth after stress periods.

4.3. ABA, SA and JA under drought and re-watering

The role of ABA, JA and SA as primary signals in the regulation of plant defense has been well studied [11]. These hormones generate a network signal-transduction leading to a cascade of events responsible for the physiological adaptation of plants to numerous stresses.

In terms of hormonal balance, endogenous ABA increased in cv. Kanlow under water stress corresponded with no changes in SA and JA. After re-watering, ABA abruptly decreased to control value. On the other hand, basal level of SA and JA were appreciably higher in cv. Kanlow. Yang [45] compared SA endogenous levels in rice, tobacco and *Arabidopsis*. In contrast to tobacco and *Arabidopsis*, which contain low basal levels of SA, rice has two orders of magnitude higher levels of SA. They generated SA-deficient transgenic rice to determine the role of SA and they found that depletion of high levels of endogenous SA reduces the plant's capacity to detoxify reactive oxygen intermediates. SA-deficient transgenic rice contains elevated levels of superoxide and H_2O_2,

exhibits spontaneous lesion formation in an age- and light-dependent manner, and is hypersensitive to oxidative damage caused by paraquat treatment. Finally, they postulated that high SA in rice plays an important role as protective agent against biotic and abiotic stress. In abiotic stresses SA might have an acclimation-like effect, causing enhanced tolerance due to enhanced antioxidant capacity [45]. The higher SA basal levels in control leaves of cv. Kanlow than in cv. Greenville suggest that this compound has a defensive role against drought stress.

The ABA increase under moderate drought in cv. Kanlow agree with previous reports. In maize seedling leaves, Wang [14] reported that endogenous ABA contents increased dramatically after 24 h of exposure to water stress. Similarly, in alfalfa, cv. Longdong (strong drought-resistance) and cv. BL-02-329 (weak drought-resistance) ABA content increased in leaves under water stress. In response to severe drought stress, the drought-resistant cv. Longdong adjusted better to growth rate reduction to ensure surviving and avoid water deficit damage [18] as seems to occur in cv. Kanlow.

As regards to JA, Pedranzani [60] reported that in tomato amounts of JA were higher in salt-tolerant cv. Pera compared to the salt-sensitive cv. Hellfruchtfrühstamm. Moreover, studies in contrasting environments showed different basal jasmonates contents and patterns of response to water stress in two populations of *Pinus pinaster* Ait. perhaps as an adaptation to diverse ecological conditions [61]. In contrast, JA contents in cv. Kanlow did not changed significantly under drought and re-watering, suggesting a minor role of JA in the response of this cultivar. Taken together, high basal levels of SA and JA in normal conditions, and the increase of ABA in response to drought, could be important characteristics for drought tolerance in the tolerant cv. Kanlow.

In Greenville, on the other hand, ABA, SA and JA seem to play differently during drought and re-watering. Under stress, ABA peak correlated with an SA drop off, probably as a result of an antagonistic interaction between the two hormones, as it seems to occur naturally in diverse species [62]. Several studies have indicated that plant responses to environmental stresses have some effects on their response to pathogens [63]. In many cases, ABA acts as a negative regulator of disease resistance SA-mediated [64]. Antagonistic interaction between ABA-mediated abiotic stress signaling and disease resistance might suggest that plants developed strategies to produce proteins involved in abiotic stress and disease resistance. The view that the ABA-mediated abiotic stress signaling potentially takes precedence over biotic stress signaling [65] supports the notion that water stress threatens plant survival more significant than pathogen infection does [10]. This could be a conceivable explanation to the ABA-SA interplay in cv. Greenville, where the role of ABA during water stress could be predominant and antagonist to SA participation.

After re-watering (24 h), SA and JA levels were significantly higher than those of well-water controls in cv. Greenville. A similar trend was observed for JA in *Arabidopsis* plants during early stages of water and saline stress, where JA contents remained constant under stress and recovered after re-watering [66]. Thus, induced levels of SA and JA probably activate an acclimation and/or defense response during the recovery period.

Overall, the interplay and fine-tune among several hormones drives the final response of drought tolerance and recovering in these two cultivars, and it appear that a different strategy could be operating, probably as an adaptation to different ecological environments.

4.4. *Expression of ABA dependent and independent genes, and SA-dependent genes*

We studied stress marker genes that are ABA dependent (RAB18 and RD22) and independent (DREB2a) and SA dependent (PR2) to better understand the participation of ABA and SA in the drought response and re-watering of *P. virgatum*.

In cv. Kanlow under drought the ABA peak was coincident with a higher expression of the stress reporter gene RAB18. These findings agree with previous information indicating a correspondence between ABA level and RAB18 transcripts in different plant species under drought [20,21,67]. On the contrary, in the sensitive cv. Greenville a significant increase in RAB18 was not observed, a response that is plausible considering that the ABA peak was less than half compared to that in cv. Kanlow.

In both cultivars of *P. virgatum*, RAB18 transcripts were no detected after 12 h of re-watering in correspondence to previous literature showing a lack of expression in RAB18 after re-watering [67].

At 24 h of re-watering, RAB18 and RD22 in cv. Kanlow were significantly overexpressed compared to controls plants, despite the low ABA level, resembling a “memory” mechanism described in other species [69,70]. The increased level of both gene expression, either at transcript or protein level, could act as a “sign” of stress memory. This effect has been extensively proposed to occur in animals [71,72] as well as in plants [69,70] and it has been referred to as a memory mechanism. For example, in *Arabidopsis*, the alteration of water status produced an increase in ABA content and RAB18 gene expression. A small but reproducible accumulation of RAB18 mRNA persisted after stress treatment along with expression of RAB18 protein, even after ABA contents decreased to the basal level. This exposure to water stress resulted in a 4–5 °C increase in freezing tolerance of *Arabidopsis* plants [67]. In a more recent study, Ding [72] exposed plants of *Arabidopsis* to recurring dehydration stress. They observed an increase in the rate of transcription and elevated transcript levels of RAB18 and RD29b from one stress to the next one, suggesting that these genes plays a role in transcriptional stress memory. Transcript abundance of both genes returned to basal (pre-induced) levels during recovery, showing a different mechanism for stress memory compared to the previously described by Ref. [67] under cold stress. Despite the divergence, RAB18 is proposed as a sign of stress memory in plants [68].

Expression of the ABA-independent DREB2a gene transcripts was present in control *P. virgatum* plants, although under drought a slight gene expression was evident; however, a high DREB2a expression was recovered after re-watering. In concordance, expression of ZmDREB2a in maize seedlings was detected under normal conditions in root tissue (but not in leaves) and, under water scarcity DREB2a increased its transcription [24]. In addition, DREB2a gene was expressed in

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non-stressed rice plants and, after exposure to a desiccating atmosphere, it continuously increased transcription [23].

Our findings of DREB2a expression under drought do not agree with studies performed in *Arabidopsis*, a dicot species that might have sustainable differences with grasses. *Arabidopsis* plants overexpressing DREB2a did not improve their drought tolerance compared to the wild type [22]. However, both DREB2a from maize (ZmDREB2a) constitutively overexpressed and stress inducible in *Arabidopsis* plants resulted in an improved drought tolerance [24] to a similar extent to *Arabidopsis* plants transformed with DREB2a from poplar (*Populus euphratica*, PeDREB2L) [26]. Likewise, tobacco plants overexpressing DREB2a from *P. glaucum* (PgDREB2a) enhances dehydration and salt tolerance and activates downstream stress-responsive genes [25]. These significant differences among species can account for divergences in the behavior of DREB2a from *P. virgatum* and others species. Other point to be considered is that most of the previous work has been performed in laboratory experiments with plants grown in culture media and water stress condition applied by exposure to desiccating atmosphere, or by application of osmotic agents such as PEG or mannitol. Instead, our plants were grown in soil and subjected to gradual dehydration; such difference might also explain the variation between our results and other reports.

PR-proteins of plants have been defined as proteins of a host that are induced only in response to attack by pathogens or by a related event [33]. Recently it has been shown that PR2 gene is involved in abiotic stress tolerance as drought and cold [34–36]. Tobacco plants exposed to salt and drought treatments showed a high expression of defense PR1 and PR2 genes, demonstrating that both genes play an important role in abiotic stress response [36]. On the other hand, *Arabidopsis* plants exposed to cold showed up-regulation of the genes PR1 and PR2, and increased SA accumulation [72]. We found in *P. virgatum* that the expression of PR2 appears to have some role during the re-watering in the tolerant cv. Kanlow, but not in response to drought, while in cv. Greenville PR2 expression were not evident under any condition.

Overall, the physiological, biochemical and molecular pattern of response to moderate and progressive water stress and recovering was different in both cultivars of *P. virgatum*, probably as a result to the environmental condition where they evolved. Drought tolerance in cv. Kanlow showed correlation with higher antioxidant activities both in stress and recovery. In addition, high basal levels of SA and JA in normal conditions, and the increase of ABA in response to drought could be part of the strategy that explains drought tolerance in cv. Kanlow. Based on our results, cv. Kanlow has an effective machinery of stress response, which makes it promising to be grown successfully in semi-arid regions.

5. Conclusions

This is the first comparative study that analyzes the response to drought in two different ecotypes of *P. virgatum* (i.e. lowland and upland) in terms of stress hormones, antioxidant activity and water stress induced genes. Controlled environment experiments indicate that young plant growth of the lowland cv. Kanlow is less affected by drought than the upland Greenville. Similarly, plant recovering after water stress is superior in cv. Kanlow than in cv. Greenville. ABA was the main hormone under stress, and increased more in cv. Kanlow than in cv. Greenville. As well, SA and JA basal levels were higher in well-watered plants of cv. Kanlow. In both cultivars evaluated, SOD and CAT activities went up under drought and these activities kept increasing after plant re-watering. However, cv. Kanlow showed significantly higher antioxidant activities compared to Greenville. This might suggest that the antioxidant defense machinery activated under stress remained operative during recovery period, something that is applied to some species during the acclimation process. As regards to gene expression, RAB18 and RD22 overexpressed after 24 h of re-watering in cv. Kanlow, resembling a “stress memory” mechanism described in other species. Further molecular markers of hormone effects (PR2) or drought (DREB2) well known in dicots seem not to be specific for *P. virgatum*. Overall, the physiological, biochemical and molecular pattern of response to moderate and progressive water stress and recovering was different in both cultivars, probably as a result of the environmental condition where they evolved. The results reveal, at least in part, that de mechanism of drought tolerance in cv. Kanlov comprises a suitable balance in stress response hormones and an effective antioxidant machinery that is activated under stress and maintains its activity during the recovering process. This study provides explanatory data – no reported until now – that support previous finding [46,47] reporting a better performance from the lowland ecotypes in comparison with upland ecotypes. This evidence contrasts with the classical view [54] that postulates lowland cultivars as those of best behavior in water deficit environments.

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