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Effect of H_2O_2 pretreatment on the response of two seashore paspalum (*Paspalum vaginatum* Sw.) cultivars (Salam and Seaspray) to cold stress

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Abstract: Seashore paspalum is a warm season grass that requires few maintenance inputs. Expanded use of seashore paspalum could play a key role in making recreational sites more sustainable and environmentally. However, one key barrier to widespread Seashore paspalum use is a relative lack of winter hardiness. Under severe stress conditions, the antioxidant capacity may not be sufficient to minimize the harmful effect of oxidative injury. The search for signal molecules that mediate the stress tolerance is an important step in better understanding how plants acclimate to the adverse environment. This study aims to screen the responses of two *Paspalum vaginatum* cultivars (Salam and Seaspray) to local weather conditions and to study how to enhance its cold tolerance by a foliar pretreatment by hydrogen peroxide at low concentrations of 10 mM under controlled conditions. The current study provides evidence that exogenous H_2O_2 decreases the endogenous content of H_2O_2 and malondialdehyde in the first three days of exposure to cold stress in pretreated 'Seaspray' plants. in comparison to their control and pretreated 'Salam'. These results indicate that pretreatment with 10 mM H_2O_2 could improve the tolerance of seashore paspalum to cold stress, especially Seaspray cultivar which showed better response to cold stress compared to 'Salam'. Exogenous H_2O_2 could constitute a signaling molecule that significantly increases peroxidase relative density, and decreases MDA and H_2O_2 content.

1. Introduction

Seashore paspalum is among compatible warm-season turfgrasses used for recreational sites, such as golf courses, which requires low insecticide and fertilizer applications and is tolerant to salt (Duncan, 1997). Seashore paspalum is found between 35° N-S latitudes in the Americas and expands to several islands of the Caribbean-Atlantic-Pacific rim and the Mediterranean-African coastal areas. This species lacks winter hardiness (Duncan, 1997); it is sensitive to freezing or chilling due to its tropical and subtropical origins (Allen and Ort, 2001). A remarkable physiological distruption known as chilling injury is exihibited when plants of this species are exposed to temperatures below about 10 to 12°C (Lyons, 1973).

Low temperature may disrupt major components of photosynthesis including thylakoid electron transport, the carbon reduction cycle and controls stomatal conductance (Allen and Ort, 2001). It constitutes one main issue for the management of warm-season turfgrasses in tropical areas, usually causing yellowing and withering during the winter season (Xia *et al*., 2000). Exposure of plants to unfavorable growing conditions such as high temperature, heavy metals, drought, water availability, air pollutants, nutrient deficiency, or salt stress increases the production of reactive oxygen species (ROS) such as superoxide (O2.-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH) and singlet oxygen (1O2). Usually ROS production can be coupled with development of oxidative injury and disruption of metabolic functions in plants (Mittler, 2002). The major generation site of reactive oxygen species (ROS) are reaction centers of PSI and PSII in chloroplast thylakoids (Asada *et al*., 1999). ROS may affect several cellular functions by damaging nucleic acids, oxidizing proteins, and causing lipid

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peroxidation (LPO) (Foyer and Noctor, 2005). Depending on the delicate equilibrium between ROS production and scavenging at the proper site and time, ROS will act as damaging, or as signaling molecules (Gratão *et al*., 2005). Oxidative damage occurs when production of ROS exceeds the capacity of these scavenging systems. Components of the antioxidant defense system can be divided into enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GD), monodehydroascobate reductase (MDHAR), glutathione reductase (GR), and dehydroascorbate reductase (DHAR). Non-enzymatic antioxidants are ascorbate (AsA), glutathione (GSH) (both water soluble), carotenoids and tocopherols (lipid soluble) (Munné-Bosch and Falk, 2004). Among ROS, H_2O_2 is considered to be the best suited signaling molecule due to its stability and longer half-life. It is able to cross biological membranes and diffuses from cell to cell or can be transported long distances from its sites of origin in plants depending on the availability of environmental stimuli. Prasad *et al*. (1994) and Wahid *et al*. (2007) found that pre-treated H_2O_2 at the appropriate concentrations improved salt-tolerance and chilling-tolerance in wheat and maize seedlings respectively (Neto *et al*., 2005). These authors suggest that H_2O_2 signals the activation of antioxidants in seed. Hydrogen peroxide is thus hypothesized to be implicated in enhancing the chilling resistance of plants associated with the function of ROS scavenging systems.

The aim of the present study is to investigate whether H_2O_2 supply enhances low temperature resistance of seashore paspalum turfgrass. For this purpose, we used two cultivars of *Paspalum vaginatum*, Salam and Seaspray, the latter having better response to cold stress than the former (Arbaoui *et al*., 2010).

2. Materials and Methods

Field study

The study was carried out in Tunisia at INAT Tunisia (National Agronomy Institute) (36°82' N; 10°17' E; 13 m) in 2011-2012 on two mature turfs of seashore paspalum (*Paspalum vaginatum* Swartz cv. Salam and Seaspray). The swards were established in a semiarid area on slit-loam soil (40% clay, 30% silt and 25% sand) with 40 g of P_2O_5 45%, 40 g of K_2SO_4 as basic mineral fertilizer and pH 7.9. During the trial period, regular mowing and irrigation were applied to maintain a healthy turf. The experimental design was randomized block for each species with four replicates each of 1 m^2 area (Fig. 1). The conditions of relative humidity, total rainfall, and air temperature are reported in Table 1 from the beginning of the vegetative period to the end of the trial.

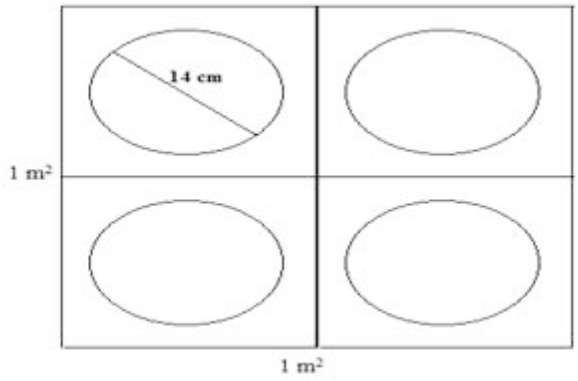

Fig. 1 - Schematic representation of one of the three experimental units with four replicates per species.

Table 1 - Total rainfall, relative humidity, and air temperature from the beginning of the vegetative period to the end of the trial (Centre for Water Resources Management, INAT Tunisia)

Period	Total rain fall (mm)	Average of RH (%)	Temperature Max (°C)	Temperature Min. (°C)	Average of air temperature (°C)
Septembre	11.5	64.2	26.0	16.6	20.6
Octobre	3.80	62.8	21.5	12.1	16.8
Novembre	6.50	67.4	16.7	9.8	12.3
Decembre	3.80	54.5	13.3	6.3	10.1
Janury	9.20	58.7	10.9	3.8	6.3
February	8.40	68.7	10.7	3.8	7.3
March	3.20	65.6	13.4	4.6	9.0
April	11.2	64.8	17.4	8.7	13.0

Each plot was photographed in winter on minimum green coverage and after winter on spring green up with a digital camera; the images were taken at a height of 1.20 m with an angle of 90°. Spring green up was the date when new stolon growth started to occur (recovery) in ecotypes. Paspalum coverage was determined using Environment for Visualizing Images (ENVI version 4.7, ITT Corporation, NY) (Fig. 2). Photochemical efficiency (Fv/Fm) was evaluated during winter, subsequent low temperature, and recovery. Leaf water content (LWC) and Dry weight (DW) were also measured at the end of the trial.

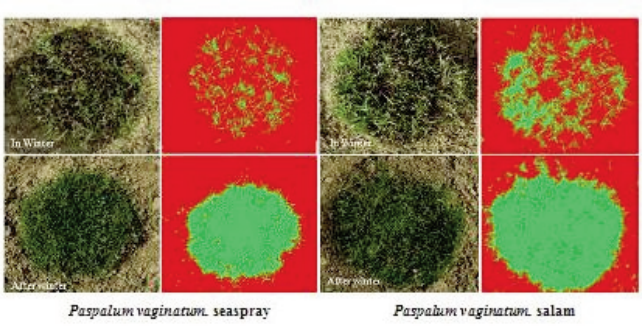

Fig. 2 - Paspalum grass coverage during and after winter. Data on minimum green coverage were collected in winter in December and spring green up data were collected in March.

Laboratory study

Plant materials and growth conditions. Seashore paspalum (*Paspalum vaginatum* cv. *S*alam and Seaspray) were collected from a two-year field plot at INAT, Tunisia. Plants were transplanted into plastic pots (0.5 L volume) filled with peat and positioned to grow for one and a half months in a greenhouse at 32°C/26°C (day/night). Before the beginning of the experiment, *Paspalum vaginatum* salam and seaspray were sprayed with 30 ml of 0, 10 mM H_2O_2 three times daily for two days and left in a normal chamber for 12 h for full absorption of the H_2O_2 solution (Fig. 3). These plants were then transferred to a growth chamber with temperature 18°C/8°C (day/night), irradiance of 2000 lux, 11 h photoperiod, and relative humidity 70%. Chlorophyll fluorescence was measured before exposure of plants to low temperature. Leaves were sampled before initiation (0 days) and after 3 and 6 days of exposure to low temperature stress. Samples from each treatment were immediately frozen in liquid nitrogen and stored at -80°C until biochemical analysis. The final harvest occurred after four months of exposure to low temperature to determine dry weight (DW), fresh weight (FW), and water content (WC).

Growth and water status in seashore Paspalum

Leaves of seashore paspalum samples were excised after four months of chilling treatment and used for fresh weight (FW) and dry weight (DW) determinations. Dry weight was obtained after drying leaves in an oven at 75 C until constant weight.

These data were used to calculate water content (WC) as follows:

$$WC= (FW-DW) \times 100 \times DW^{-1}.$$

Chlorophyll fluorescence

Chlorophyll fluorescence was measured on fully expanded young leaves by using an FIM 1500 fluorescence induction monitor (Analytical Development, Hoddesdon, England). Each leaf was excised, immediately clipped into a leaf clip (32 mm wide × 80 mm long), and the shutter plate closed to induce dark-adaptation for 30 min at room temperature. An array of six high-intensity light emitting diodes (LEDs) provided red light with a peak wavelength of 650 nm to

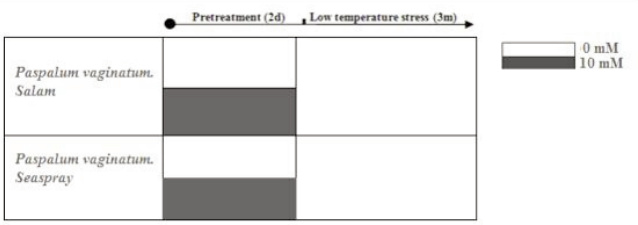

Fig. 3 - Schematic representation of the experimental design procedure. Paspalum cultivars were first sprayed then left 48 h for full absorption of different concentrations of H_2O_2 (0 mM (distilled water) and 10 mM H_2O_2). Plants appertaining to each treatment were subsequently exposed to cold stress.

illuminate the exposed leaf surface (4 mm diameter). Maximum intensity of the illuminating light was approximately 3000 µmol m^{-2} s^{-1} at leaf surface. Measured fluorescence parameters included *F*0 (initial fluorescence measured at the onset of illumination), *F*m (maximum fluorescence), *F*v (variable fluorescence = *F*m−*F*0), and *F*v/*F*m (ratio of variable to maximum fluorescence indicating the quantum yield). Measurements of three leaves from the same pot were averaged to obtain a mean.

Physiological assays to determine oxidative damage

H_2O_2 determination. The concentrations of H_2O_2 were estimated following the method of Ferguson (Ferguson *et al*., 1983): 100 mg fresh leaf was ground with 3 ml acetone for 30 min at 4°C, and the sample was then filtered through eight layers of gauze cloth. After the addition of 0.15 g active carbon, the sample was centrifuged twice at 3000 *g* for 20 min at 4°C. Next, 0.2 ml 20% $TiCl_4$ in HCl and 0.2 ml ammonia were added to 1 ml of the supernatant. The post-reacted compound was centrifuged at 3000 *g* for 10 min; the supernatant was discarded and the pellet was dissolved in 3 ml 1 mol L^{-1} H_2SO_4 and the absorbance value was determined at 410 nm. The standard curve was made using H_2O_2, and the H_2O_2 content in leaf was calculated from the absorbance at 410 nm compared with the standard curve.

Lipid peroxidation. Membrane lipid peroxidation was assessed by measuring the content of malonyldialdehyde in tissue. Fresh leaf samples were homogenized in 0.1% (w/v) TCA (Trichloroacetic acid) solution. The homogenate was centrifuged at 15000 *g* for 10 min. An aliquot of the supernatant was added to

0.5% TBA (Thiobarbituric acid) in 20% TCA (Trichloroacetic acid). The mixture was heated at 90°C for 30 min in a shaking water bath, and then cooled in an ice bath. The samples were centrifuged at 10 000 g for 5 min, and the absorbance of the supernatant was read at 532 and 600 nm (Hernandez *et al*., 2000). The MDA concentration was calculated as the difference of absorbance at 600 nm and 532 nm.

Protein extraction. Aliquots of frozen fresh leaves were ground to a fine powder with liquid nitrogen and extracted (100 mg FW) at 4°C in 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM EDTA, 50 mM KCl, 20 mM $MgCl_2$, 0.5 mM PMSF, 1 mM DTT, 0.1% (v/v) Triton X-100, and 10% (w/w) PVP. The homogenate was centrifuged at 14000 *g* for 30 min at 4°C, and the supernatant was utilized for protein content and the determination of antioxidative enzyme activities. Three replicates per treatment were used. Protein concentration was determined according to Bradford (1976), using bovine serum albumin as standard.

Native gel electrophoresis and enzyme activity staining. Samples of crude *Paspalum vagintum* leaf extracts were separated by gel electrophoresis in 10% (POD) polyacrylamide slab gel, at pH 8.9 under native conditions according to Davis (1964). POD isoforms were visualized on gels according to Vallejos (1983).

The gel was first incubated in a 0.1 M sodium acetate buffer (pH 4.0) containing 1% (v/v) guaiacol for 30 min. Then, in a solution containing a final concentration of 4.7 mM 3-amino-9-ethylcarbazole, 38 mM N,N-dimethyl formamide, 0.1 M sodium acetate buffer (pH 5.0), 0.1 M $CaCl_2$, 3 mM and H_2O_2 the revelation was achieved.

Statistical analysis

Statistical differences were assessed using ANOVA of SAS (9.0) by t-test. The experimental design was a randomized complete block design with three replications. All data were statistically analyzed using least significant difference (LSD) to separate entry means. When the interaction was significant, a subroutine (PDMix800, SAS) was used to compare means at (p<0.05%).

3. Results

Local weather conditions in field induced changes in turfgrass coverage, in photochemical efficiency of PSII, and growth parameters

Data on minimum green coverage collected in winter (December) indicated that turfgrass coverage was significantly (p<0.05) altered by local weather conditions (Fig. 4, Table 1). With spring green up (March) turfgrass coverage of *Paspalum vaginatum* 'Seaspray' and 'Salam' exhibited an increase of 44.12% and 75%, respectively (Fig. 2, 4). Photochemical efficiency of phtosystem II (*Fv/Fm)* followed a significant (p<0.05) similar pattern of decrease then increase through the winter and spring green up. It decreased significantly (p<0.05) and was most pronounced in the second measurement time (Table 2). The percentages of decrease were, respectively, 15 and 6.5%. By the end of the trial, DW and WC were slightly different in both Paspalum cultivars and were not significantly affected (Table 2).

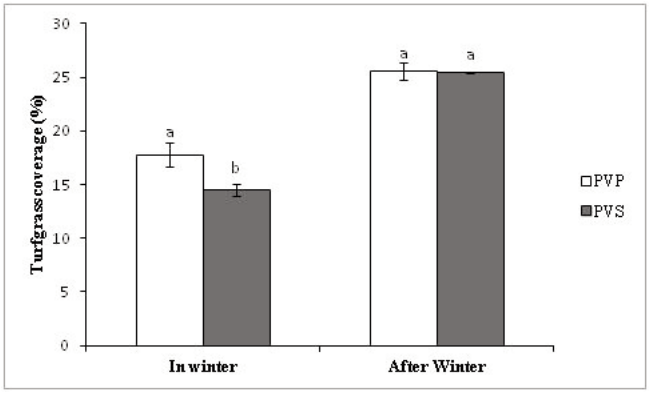

Fig. 4 - Turfgrass coverage on minimum green coverage (in winter) and on maximum green coverage (spring green up) in Paspalum vaginatum Salam (PVS) and Seaspray (PVP). Data are the mean ± SE of three plants.

Table 2 - Changes in photochemical efficiency of PSII (Fv/Fm ratio), dry weight (DW), and water content (WC) in *Paspalum vaginatum* 'Salam' and *Paspalum vaginatum* 'Seaspray' under local weather conditions

Date	'Salam'	'Seaspray'
	Fv/Fm	
D1	0.71 b	0.72 b
D2	0.70 b	0.71 b
D3	0.79 a	0.80 a
	DW (g)	
	21.30 a	20.28 b
	WC (%)	
	247.73 a	238.61 b

Data are the mean ± SE of twelve replications (n=12). Different letters indicate significant differences at a p<0.05 probability level. Measurements were taken three times during the trial D1= December; D2= January; D3= March).

Effect of pretreatment with H_2O_2 and long-term cold stress on growth and photosytem II efficiency of Paspalum vaginatum *'Salam' and 'Seaspray'*

The effect of foliar pretrement using low concentrations of H_2O_2 on biomass production (DW) and water content (WC) of both seashore cultivars is

shown in figure 5. Cold stress exposure reduced the DW of pretreated cultivar Salam compared to the control with no significant difference observed between pretreated and untreated 'Seaspray' ($p>0.05$). Biomass production (DW) in pretreated cultivars and their controls were above 300% and leaves did not wilt during the cold stress process however not significant levels were noted ($p>0.05$). These results seem to be independent of stress exposure and chemical pretreatment of these cultivars. The photochemical efficiency of PSII *(F*v/*F*m) was monitored three times throughout the cold exposure (Table 3) and the *Fv/Fm* ratio remained above 0.80 in both control and pretreated seashore cultivars at 10 mM ($p<0.05$).

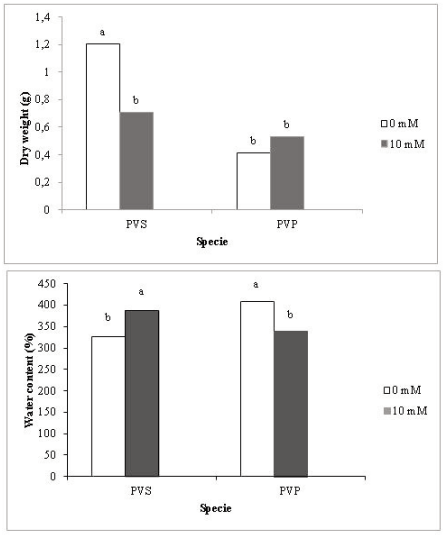

Fig. 5 - Dry weight and water content of *Paspalum vaginatum* 'Salam' (PVS) and *Paspalum vaginatum* 'Seaspray' (PVP). Values expressed as mean ± SE (n=3). Different letters indicate significant differences at probability level $p<0.05$.

Table 3 - Changes in Fv/Fm ratio in pretreated plants with 10 mM H_2O_2 and in control leaves of *Paspalum vaginatum* 'Salam' and *Paspalum vaginatum* 'Seaspray' during cold stress exposure

Genotype	Date	0 mM	10 mM
Paspalum vaginatum 'Salam'	D1	0.78 b	0.80 ab
D2	D2	0.81 a	0.80 a
D3	D3	0.81 a	0.80 ab
Paspalum vaginatum 'Seaspray	D1	0.80 ab	0.80 ab
	D2	0.81 a	0.80 a

Data are the mean ± SE of six replications (n=6). Different letters indicate significant differences at a probability level $p<0.05$. Measurements were taken three times during stress time (Dates 1, 2, and 3) under controlled conditions (See Materials and Methods).

Changes in H_2O_2, MDA and protein content

H_2O_2 pretreatment, exposure duration to cold stress, and the types of paspalum cultivars significantly affected the production of H_2O_2 and MDA content ($p<0.05$). In cultivar Salam, H_2O_2 content in control and pretreated plants did not differ significantly during cold stress, whereas MDA content decreased by 54% in pretreated plants when compared to control ones. In 'Seaspray' prior to cold stress (0 day), H_2O_2 content was higher in pretreated plants than in controls (Fig. 6). During the third day of cold stress, H_2O_2 content decreased by 83.77% compared to their controls, before it increased again after 6 days of cold stress. H_2O_2 pretreated plants lowered (77%) significantly ($p<0.05$) the MDA content in 'Seaspray', reaching the minimum levels during the third day of cold exposure as compared to the controls (Fig. 6).

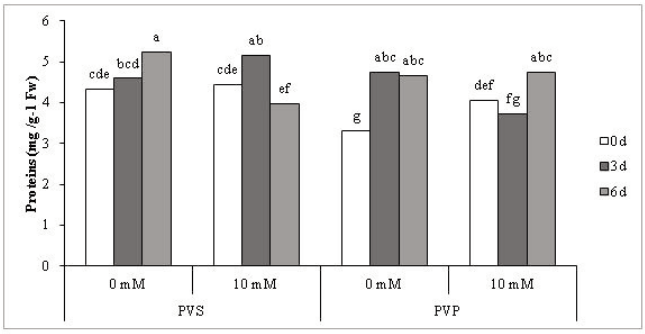

Fig. 6 - Protein content of *Paspalum vaginatum* 'Salam' and *Paspalum vaginatum* 'Seaspray' leaves in control plants (0 mM) and plants pretreated with 10 mM H_2O_2 after 0, 3, and 6 days of cold stress exposure. Values expressed as mean ± SE (n=3). Treatment ($p<0.001$), Species ($p<0.001$), Time ($p<0.001$) and interaction ($p<0.001$). Different letters indicate significant differences at probability level $p<0.05$.

After six days of stress exposure, MDA content increased slightly but was lower than MDA content recorded on the first day (0 d). Protein content in the leaves of 'Salam' changed slightly and remained at constant concentrations during the stress period. On the other hand, protein content in 'Seaspray' pretreated with 10 mM H_2O_2 varied significantly ($p<0.05$) compared to their controls (Fig. 7).

Chemical pretreatment and cold stress exposure altered relative density of Peroxydase isoforms

Peroxidase activity was assessed on polyacrylamide gel to distinguish the different POD isoforms, and to quantify their activities using image j software. Paspalum cultivars show different POD activity reactions under cold stress conditions. POD relative density in all pretreated *Paspalum vaginatum* 'Salam' (Fig. 8) plants increased with increased levels of cold exposure, compared to controls. However, POD relative density in pretreated *Paspalum vaginatum* sea-

spray was much higher compared to control plants and then decreased during the third day of stress (Fig. 8).

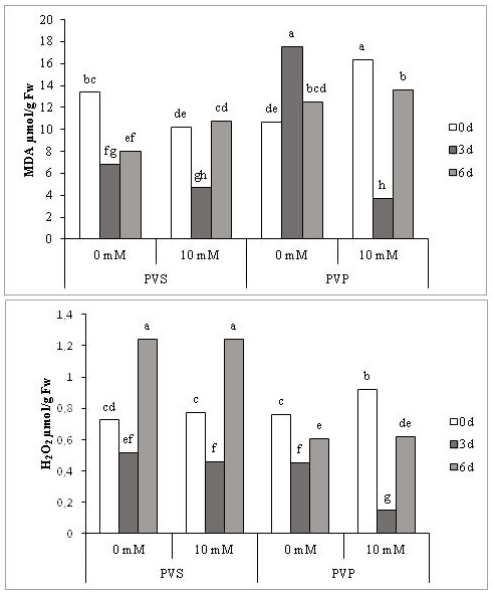

Fig. 7 - H_2O_2 and MDA contents of *Paspalum vaginatum* 'Salam' (a) and *Paspalum vaginatum* 'Seaspray' (b) leaves in control plants (0 mM) and plants pretreated with 10 mM H_2O_2 after 0, 3, and 6 days of cold stress exposure. Values expressed as mean ± SE (n=3). Treatment ($p<0.001$), Species ($p<0.001$), Time ($p<0.001$) and interaction ($p<0.001$). Different letters indicate significant differences at probability level $p<0.05$.

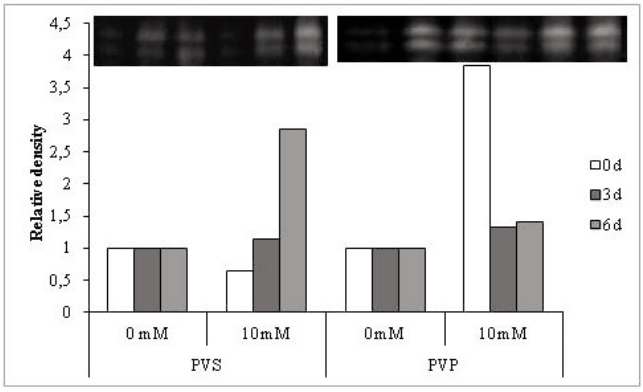

Fig. 8 - POD isoforms in gel, relative density in control plants (0 mM) and plants pretreated with 10 mM H_2O_2 under cold stress condition.

4. Discussion and Conclusions

Results showed that both cultivars were affected by environmental conditions and could have an impact on turfgrass coverage and on *Fv/Fm*. Lower *Fv/Fm* ratio values were close to 0.7 (date 1), suggesting an altered performance of PSII. Remarkably, both paspalum cultivars required longer to recover, reaching 0.8 (*Fv/Fm)* (date 3). A close relationship has been reported between low temperatures and photosynthetic rates. The recovery of photosynthetic activity under cold stress was faster in other species such as sorghum, maize and pennisetum, while it was slightly slower in soybean, and ryegrass (Taylor and Rowley, 1971).

Some of the excessive energy is quenched into chlorophyll fluorescence to minimize damage to photosynthetic systems, particularly in photosystem II (PSII) and subsequent electron carriers (Krause and Weis, 1991; Araboui *et al*., 2010). Changes in membrane structure were considered as the primary lesion of stress injury and may lead to a loss of membrane permeability and metabolic disfunction (Lyons, 1973; Montillet *et al*., 2005). The peroxidation of lipids is also a damaging process that can be taken as a single parameter to determine the level of lipid destruction under various stresses. During lipid peroxidation, products are formed from polyunsaturated precursors that include hydrocarbon fragments; such as ketones, MDA, and compounds related to them (Grag and Machanda, 2009). The increase of MDA indicated the deterioration of peroxidation and membrane injury induced by ROS under cold stress. Our findings suggest that within three days of cold stress exposure, 10 mM of H_2O_2 remarkably decreased MDA in cultivar Seaspray. He *et al*. (2009) shown that H_2O_2 pretreatment enhanced the membrane stability of wheat seedlings, as revealed by a greatly reduced membrane damage rate (MDA) and malondialdehyde (MDA) content. This was associated with a slight increase in protein content. Improving tolerance of both Paspalum cultivars to chilling stress also leads to the over production of ROS such as O_2.- and H_2O_2 in plant tissues (Desikan *et al*., 2003). ROS are extremely active molecules that may damage membranes and other cellular components to avoid cold stress or other stress-induced injuries. However, ROS could be considered as a signal for molecules that mediate responses to various stimuli. Compared to the other ROS, H_2O_2 can be the most suited to act as a signaling molecule due to its higher stability and longer half-life. The fluctuation of H_2O_2 level in plants should spatially and temporally reflect changes in the environment (Desikan *et al*., 2003, 2004).

Our study provides evidence that exogenous H_2O_2 resulted in a significant decrease ($p<0.05$) of the endogenous content of H_2O_2 and MDA in the first three days of exposure to cold stress in pretreated 'Seaspray' plants. The higher H_2O_2 content (0 d) in pretreated 'Seaspray' was followed by a systematic

high relative density of POD isoforms. With further cold stress, relative densities of POD decreased but remained higher than control plants. This type of response would permit pretreated Seaspary Cv. plants to still upright to overcome the wilting and mechanical weakness imparted by cold stress.

In conclusion, our results have demonstrated that pretreatment with 10 Mm of H_2O_2 could improve the tolerance of seashore paspalum to cold stress, especially cultivar Seaspray which showed a better response to cold stress than 'Salam'. Exogenous H_2O_2 could constitute a signaling molecule that significantly increases H_2O_2 detoxifying and POD activity, and decreases MDA and H_2O_2 content.

The meaning of presented results can be important for fundamental and practical sciences. These results would help to understand what are plant reactions to cold stress. I will be also possible to apply low concentrations of hydrogen peroxide in golf course and sport fields, making these fields more viable and sustainable.

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