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ORIGINAL PAPER

Osmotic adjustment and maintenance of the redox balance in root tissue may be key points to overcome a mild water deficit during the early growth of wheat

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Abstract In this investigation we analyzed in detail the consequences of water deficit during the first 4 days of wheat development, focusing on root growth as affected by eventual changes in cell cycle regulation and oxidative processes. Root elongation decreased under water restriction in correlation with the intensity of this limitation, but the total number of cells between the quiescent center and the start of the rapid elongation zone in the root apex did not vary. Neither lipid peroxidation nor protein carbonylation increased in the roots of water-starved seedlings (ψ_w: −0.6 MPa); accordingly, catalase activity increased, and transcript levels of *cat2* gene were enhanced. Superoxide dismutase activity rose at day 2 and 3 and, unlike catalase, displayed quite similar levels on comparing roots and coleoptiles. Proline and total soluble carbohydrates increased in the roots of water-starved seedling. Total conductivity and osmolality were also augmented. No changes in the transcript levels of the markers related to G1-S transition phase of cell cycle could be detected. However, two expansin genes (*TaEXPB8* and *TaEXPA5*) were up-regulated in roots under water deficit. We conclude that wheat root elongation in water-deprived seedlings was simply hampered by lack of water income to cells. The enhanced expression of two root expansin genes is probably related to the eventual need of a quick cell wall expansion to allow the existing root cells to recover normal turgor, in case of sudden rewatering.

Keywords Root growth · *Triticum aestivum* · Water deficit · Root apical meristem · Oxidative stress · Osmolyte accumulation

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Introduction

Sub-optimal availability of water causes important limitations to agricultural production (Delmer 2005). Drought stress may develop along periods ranging from days to weeks, and can be classified as either terminal or intermittent. The former represents a progressive water decrease leading to the plant death; the second is the result of finite periods of inadequate irrigation occurring at one or more intervals during the growing season, and is not necessarily lethal (Neumann 2008). Germination and early seedling growth are critical stages in plant life cycle, and any disturbance on environmental conditions, such as water restriction, can alter survival and/or plant productivity. Therefore, understanding the impact of water shortage at cellular level, especially during these initial stages of plant development, deserves research efforts.

Several abiotic stresses are characterized by altering the cellular redox state, either by modifying the antioxidant defence system or by increasing the levels of reactive oxygen species (ROS), consequently producing oxidative stress (Gallego et al. 2005; Mittler 2006). Cells are equipped with enzymatic and nonenzymatic antioxidant systems to eliminate ROS and maintain redox homeostasis. A major class of enzymatic antioxidants, which catalyze the dismutation of $O_2^{\cdot -}$ to H_2O_2, is known as superoxide dismutase (SOD) (Mittler 2006). Further conversion of

H_2O_2 to $H_2O + O_2$ occurs through the action of catalase, which has been involved in the detoxification of H_2O_2 generated by different stress factors (Guan et al. 2009; Pena et al. 2011), and also under physiological conditions. In wheat (*Triticum aestivum* L.), two sequences encoding catalase, *cat1* and *cat2*, have been described (Luna et al. 2005), and a new sequence designated *cat3* has recently been incorporated to the GeneBank.

Cellular redox signaling hub and osmotic adjustment have been associated to plant growth limitation during abiotic stress (Iglesias et al. 2010; Bartoli et al. 2013). In this sense, the mechanism underlying growth inhibition can be related to their effect on the processes that drive organ growth in plants: cell proliferation and cell expansion.

Plant primary growth (axial) depends on both the presence of actively proliferating cells at root and stem apexes, and the cell expansion in the elongation zone (Shishkova et al. 2008). As in all eukaryotes, plant cell proliferation is regulated by the cell cycle. Cell cycle progression in plants is controlled by cyclin-dependent kinases (CDK) associated with positive regulators called cyclins, or negative regulators known as Kip-related proteins (KRPs) (Inzé 2005). During the G1-S transition stage in the cell cycle, the cyclin-dependent kinase CDKA forms a complex with cyclin D (CYCD). The CYCD holds the cell in a division state related to the Rb-E2F pathway, in which retinoblastoma protein (Rb) is phosphorylated by CDKA-CYCD complex and loses its affinity for the family of E2F-DP transcription factor. The release of E2F-DP heterodimer activates target genes and allows cell cycle progression to S phase (Hirano et al. 2008).

On the other hand, plant expansins comprise a large multigene family, firstly identified as mediators of acid-induced wall extension, involved in cell wall extension in response to a unidirectional extensive force. Four families of genes encoding expansins have been identified and are known as alpha-expansins (*EXPA*), beta-expansins (*EXPB*), expansin-like A (*EXLA*) and expansin-like B (*EXLB*) (Choi et al. 2008). These proteins act selectively on the cross-linking polymers between parallel microfibrils (Marga et al. 2005), resulting in physical effects, such as polymer creep and stress relaxation of stretched cell walls (Cosgrove 2000).

Wheat (*Triticum aestivum* L.) is a C3 grass species widely sown in many temperate climate regions, including drought prone-environments. In water-deficit conditions, the maintenance of root growth is essential for plant survival because root elongation allows plants to get access to deeper layers of the soil profile, where water contents are usually higher. The purpose of this work was to evaluate the consequences of a moderate water restriction during the early phase of wheat root development, focusing on growth components at cellular level and on membrane integrity, in relation to eventual oxidative processes.

Materials and methods

Plant material and growth conditions

Twenty seeds of bread wheat (*Triticum aestivum* L. cv 75 Aniversario, supplied by Buck, Argentina) were placed in Petri dishes (10 cm diameter) containing 10 mL aqueous solution. Water deficit was simulated with solutions obtained by mixing deionized water with different amounts (3.4, 11.8, 17.6, 20.0, 22.2, 26.9, and 32.4 % w/v) of polyethylene glycol (PEG) 6000, resulting in water potentials (ψ_w) of −0.03, −0.2, −0.4, −0.5, −0.6, −0.8 and −1.2 MPa, respectively (Michel and Kaufmann 1973). The ψ_w of −0.03 MPa, representing the ψ_w at field capacity in loamy soils (Schulze et al. 2005), was considered control (C) in this work. The osmotic potential of the PEG solutions (equivalent to water potentials, under our experimental conditions) was measured with a vapour pressure osmometer (Wescor Vapro 5520). Petri dishes were kept at 24 ± 2 °C in the dark and PEG solution was daily replaced. After 1–4 days of imbibition, seedlings were gently washed with distilled water and the length of the root and coleoptile was determined.

Germination percentage was assessed at day 4 of imbibition by counting seeds with protruding radicles of 1 mm or longer, measuring from the seed coat. Root length was assessed by measuring the radicle root of each seedling. Coleoptile length was measured too. Dry weight (DW) was determined after drying the roots at 80 °C for 7 days or up to constant weight. RGR (relative growth rate) was calculated using the following formula: ln F2-ln F1/T2–T1, where F1 and F2 are initial and final biomass, and T1 and T2 are initial and final time, respectively (Poorter and Garnier 2007).

Root analyses

Microscopic analysis of root apical meristem

Roots of 2 and 3 days-old seedlings were fixed overnight in FAA [paraformaldehyde:ethanol:glacial acetic acid (1:4:1)] and processed by the inclusion of paraffin technique (Johansen 1940). Root sections were stained with safranin-fast green and mounted in DPX. Cell lengths and numbers between the quiescent center (QC) and the start of the rapid elongation zone were measured using the software analysis package ImageJ.

Oxidative damage to lipids and proteins

The amount of thiobarbituric acid reactive substances (TBARS) was determined as an index of lipid peroxidation, according to Heath and Packer (1968). Roots (0.3 g) were

homogenized in 3 mL of 20 % (w/v) trichloroacetic acid (TCA). The homogenates were centrifuged at 3,000×*g* for 20 min. To 1 mL aliquots of the supernatants, 1 mL of 20 % (w/v) TCA containing 0.5 % (w/v) TBA and 100 µL 4 % (w/v) butylated hydroxytoluene (BHT) in ethanol was added. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. The homogenates were centrifuged at 10,000×*g* for 15 min and the absorbance measured at 532 nm. The concentration of TBARS was calculated using an ε of 155 mM^{-1} cm^{-1}.

For protein oxidation assessment, root samples (1 g) were homogenized in 3 mL of extraction buffer containing 100 mM phosphate buffer (pH 7.4), 120 mM KCl, and 1 mM EDTA. The homogenates were centrifuged at 10,000×*g* for 20 min and the supernatant fraction was used for the assay. Proteins were derivatized with 2,4-dinitrophenylhydrazine (2,4-DNPH) (Levine et al. 1990) and then 50 µg-protein samples were separated by 10 % (w/v) SDS–PAGE (Laemmli 1970). Two gels were runned simultaneously, one for immunodetection and the other for protein staining with Coomassie Brilliant Blue R-250. Derivatized proteins were transferred onto nitrocellulose membranes and detected with rabbit anti-DNP primary antibody 1:20,000 (Sigma–Aldrich, St Louis, USA). Bands corresponding to oxidized proteins were visualized by secondary goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase 1:2,000 (DakoCytomation) and using 3,3′-diaminobenzidine (DAB) as substrate (information available on http://www.encorbio.com/protocols/blotting.htm). Membranes were photographed with Fotodyn and analyzed with GelPro software based on absolute integrated optical density of each line.

Proline and soluble carbohydrate content

Proline content was determined in root extracts prepared in 3 % (w/v) of 5-sulfosalicylic acid, according to the method described by Bates et al. (1973). After centrifuging the homogenates at 3,000×*g* for 15 min, the supernatant fractions were used for the assay. The estimation of carbohydrate content was performed in root aqueous extracts using the anthrone reagent (Yemm and Willis 1954). Proline and glucose were used to obtain the corresponding standard curves, to allow quantification.

Conductivity and osmolality

To estimate root membrane integrity, roots (0.5 g) were separated and placed in vials containing 20 mL of deionized water for 4 h at room temperature. Then the electrical conductivity (EC1) of the solution was measured. After that, vials were kept in an autoclave (110 °C) for 20 min and total conductivity (EC2) was determined. The electrolyte leakage was calculated as: EC1-ECw/EC2-ECw, where ECw is the deionized water conductance (Flint et al. 1967). In a similar experiment, root total solute contents were estimated based on osmolality of the solution after heating. EC and osmolality were determined using a HI 9813 portable conductivity-meter (Hanna Instruments, Inc., Woonsocket, RI) and a vapour pressure osmometer (Wescor Vapro 5520), respectively.

Root and coleoptile analyses

Antioxidant enzymes

Extracts for determination of catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidise (APOX) and guaiacol peroxidase (GPX) activity were prepared from root and coleoptile samples as previously described (Davenport et al. 2003). CAT activity was determined in the homogenates by measuring the decrease in absorbance at 240 nm in a reaction medium containing 50 mM potassium phosphate buffer (pH 7.2) and 2 mM H_2O_2. The pseudo-first order reaction constant ($k' = k \times [CAT]$) of the decrease in H_2O_2 absorption was determined, and CAT content was calculated using ($k = 4.7 \times 10^7$ M^{-1} s^{-1} [CAT]) (Chance et al. 1979). Total SOD activity was determined by assessing the inhibition of the photochemical reduction of NBT, as described by Becana et al. (1986). One unit of SOD was defined as the amount of enzyme which produced a 50 % inhibition of NBT reduction under the assay conditions. GPX activity was determined by measuring the increase in absorption at 470 nm due to tetraguaiacol formation (extinction coefficient: 26.6 mM^{-1} cm^{-1}) in a reaction mixture containing 10 mM guaiacol. APOX activity was determined by the decrease in absorption due to ascorbate oxidation at 290 nm (extinction coefficient: 2.8 mM^{-1} cm^{-1}) (Nakano and Asada 1981).

Semiquantitative RT-PCR

The expression level of several target genes was assessed. Total RNA was extracted from coleoptiles and/or from roots (apical and subapical region, 0–10 mm) using a modified TRIzol (Invitrogen; Carlsbad, CA, USA) procedure, treated with DNase I (Promega) and then converted to cDNAs with oligo (dT)15 using the RevertAid™ MMuLV Reverse Transcriptase (Fermentas). PCR primers and conditions for amplifications are described in Table 1. PCR reactions were performed using a programmable Termocycler T 18 (Ivema). The PCR products were electrophoresed through 1.5 % (w/v) agarose and visualized with ethidium bromide. Fragments of wheat actin gene were amplified as internal standards to normalize for differences of total RNA amounts. Gels were photographed with Fotodyn and analyzed with

Table 1 PCR primers and conditions for amplification of target genes

Gene	Sense and antisense primers	PCR conditions	Amplicon size (pb)	Accession number (GenBank ID)
cat1	5′-ACTACGACGGGCTCATG 5′-GCCCTGAAGCAGATTTCT	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 52 °C, 30 s 72 °C; 10 min 72 °C	373	E16461
cat2	5′-CCTTAATCAGCAGGGATG 5′-AGATAGAACACGCGGAG	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 53 °C, 45 s 72 °C; 10 min 72 °C	611	X94352
cat3	5′-AGCTTCGACACCAAGACGAC 5′-GACGAGGGTGGCCTCGTC	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 50 °C, 45 s 72 °C; 10 min 72 °C	699	HQ860268
pcna	5′-CACCAAGGAGGGTGTCAAGT 5′-GATCTTGGGGTGCCAGATAA	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 58 °C, 30 s 72 °C; 10 min 72 °C	367	AK335595
rdr	5′-TTCCCCATCCGGTTCCCGCA 5′-TGAGCCCGCGCTTCTTGAGC	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 53 °C, 45 s 72 °C; 10 min 72 °C	508	TC287177
mcm2	5′-ACGACGGCGCCACCGTTATC 5′-TTGCGATGAAGCGCCGGACT	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 58 °C, 45 s 72 °C; 10 min 72 °C	552	AY532594
EXPB8	5′-GGTTGTTCATAGTCGACCAATATGG 5′-AGAGCAGGAAGGGTTGTTGG	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 50 °C, 30 s 72 °C; 10 min 72 °C	351	AY543542
EXPB10	5′-GAAGACCTGTAGTGCCAATATGGCT GG 5′-GTCGGTGATGACGATCCTCC	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 50 °C, 30 s 72 °C; 10 min 72 °C	373	AY543544
EXPA5	5′-CGACGACATGGCGGTCATCAAGATG 5′-CCCGTTGATGGTGAACCTGA	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 53 °C, 30 s 72 °C; 10 min 72 °C	499	AY543531
EXPA6	5′-ATGGCAGCTGGGATGCGCTTCCT 5′-CGGTAGATGGCGAGGTTCTC	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 53 °C, 30 s 72 °C; 10 min 72 °C	422	AY543532
EXPA8	5′-CGAGCATCTCCGCACTTGCAA ATTCAAG 5′-GCCTGGGCGATCTTAGTGAA	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 53 °C, 30 s 72 °C; 10 min 72 °C	482	AY543534
Actin	5′-GGATCGGTGGCTCTATTTTG 5′-TGTACCCCTTATTCCTCTGAGG	3 min 95 °C; 30 cycles: 30 s 95 °C, 30 s 53 °C, 30 s 72 °C; 10 min 72 °C	224	TC441720

cat catalase, *pcna* proliferating cell nuclear antigen, *rdr* ribonucleotide reductase small subunit, *mcm2* minichromosomal maintenance factor, *EXPB*, beta-expansins, *EXPA* alpha-expansins

GelPro software, data expressed as arbitrary units (assuming control value equal to 1), based on absolute integrated optical density of each band. Each expression profile shown was representative of at least three experiments.

Protein determination

Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as standard.

Statistics

Experiments were repeated three times. Data reported are the mean values of three to five replicates (±SEM). Differences among treatments were analyzed by 1-way ANOVA, taking $P < 0.05$ as significant according to Tukey's multiple range test.

Results

Early growth declined under water restriction, but root tip organization remained unaltered

Germination percentage under control conditions was above 98 %, and this germination rate remained unaltered at water potentials up to −0.6 MPa. However, as it is shown in Fig. 1, seed germination decreased by about 25 % when ψ_w was below −0.6 MPa.

Although seeds were able to germinate even at ψ_w below −0.8 MPa, the length of both root and coleoptile significantly decreased under water restriction. As shown on Fig. 2, decreases in growth were dependent on PEG concentration.

Since under a ψ_w of −0.6 MPa germination rate was still unaffected; this water potential was considered to generate a "moderate water deficit" and thus used for further comparative analyses.

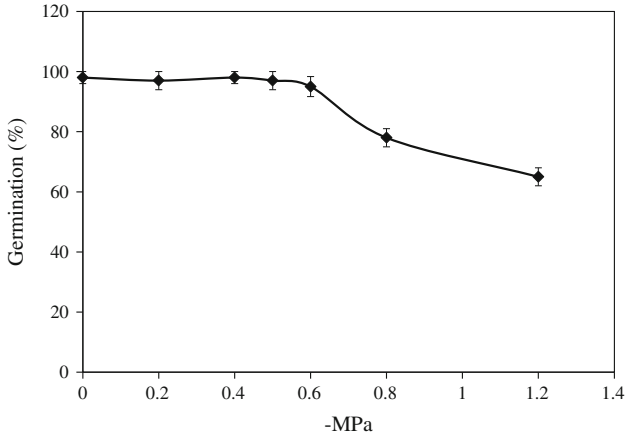

Fig. 1 Germination of wheat seeds subjected to water deficit. Germination percentage was assessed after 4 days of imbibition in solutions of different water potentials obtained by increasing PEG 6000 concentrations, as described in "Materials and methods" section. Mean values ± SEM of three replicated experiments with five replicates per treatment are shown

Between days 2 and 3 post-imbibition, the growth rate of roots and coleoptiles decreased in water-starved seedlings (−0.6 MPa) as compared to controls (−0.03 MPa) (14 vs. 23 mm day^{-1} and 5 vs. 13 mm day^{-1}, respectively) (Fig. 2a, b).

Fresh weight (FW) of roots and coleoptiles and dry weight (DW) of coleoptiles were also significantly diminished in water-starved seedlings at days 2 and 3 of treatment. However, water restriction did not affect root dry weight (Table 2). An increase in root/coleoptile ratio was observed in seedlings growing under water shortage, especially by day 3 post-imbibition (Table 2). RGR between day 2 and 3 was calculated from data given on Table 2. Only the RGR of coleoptiles was significantly affected by water shortage (0.468 vs. 0.748 mg/day in controls), as dry biomass accumulation in roots was preserved.

Microscopic examination of root apical sections at day 2 and 3 revealed that control and water-stressed seedlings had a similar number of cells between the quiescent center (QC) and the start of the rapid elongation zone. The average length of these cells was also similar (Fig. 3).

A moderate water restriction did not generate oxidative damage in roots, but significantly enhanced osmolyte content

Lipid peroxidation and protein oxidation were determined in order to verify if water restriction induced oxidative stress in roots during early seedling growth stage (Fig. 4). Under our experimental conditions neither protein carbonyl

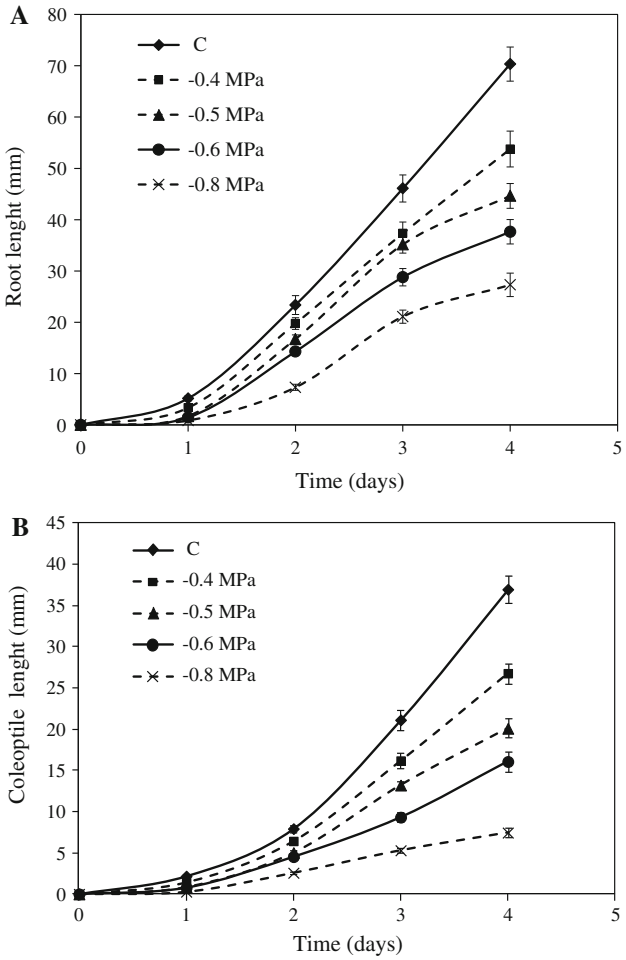

Fig. 2 Time-course of wheat seedlings growth under water deficit. **a** root; **b** coleoptile. Wheat root and coleoptiles growth was recorded after 4 days. The water potential of control (C) was −0.03 MPa. Mean values ± SEM of three independent experiments with five replicates per treatment

group content (Fig. 4b) nor TBARS (Fig. 4d) increased in the roots of water-stressed seedlings. Validating carbonyl-group analysis, a similar pattern of carbonylated (Fig. 4a) and soluble (Fig. 4c) proteins was obtained from control and water-stressed seedlings.

In line with these results, integrity of root cells (estimated from electrolyte leakage calculations based on conductivity measurements) was unaffected in seedlings grown at a water potential of −0.6 MPa (data not shown). Nevertheless, total conductivity and osmolality at day 2 and 3 was increased in the roots of seedlings subjected to this moderate water restriction compared to control ones (Fig. 5a), indicating solute accumulation in water-starved tissues.

In agreement with the increase in osmolality, a rise in proline (90 and 125 % over the control for days 2 and 3, respectively) and in total soluble carbohydrates (115 and 79 % over the control, for days 2 and 3, respectively) was also

Table 2 Effect of a moderate water restriction on biomass accumulation

mg per plant	Root		Coleoptile		Root/coleoptile ratio	
	C	T	C	T	C	T
Day 2						
FW	12.0 ± 0.2a	6.5 ± 0.1b	10.2 ± 1.17a	3.4 ± 0.1b	1.2	1.9
DW	0.69 ± 0.02a	0.64 ± 0.01a	1.02 ± 0.04a	0.58 ± 0.01b	0.7	1.1
Day 3						
FW	23.6 ± 0.4a	13.1 ± 0.3b	27.1 ± 0.5a	6.9 ± 0.2b	0.9	1.9
DW	1.73 ± 0.03a	1.65 ± 0.02a	2.12 ± 0.08a	0.93 ± 0.02b	0.8	1.8

Wheat seedlings were germinated and grown at ψ_w of −0.03 MPa (3.4 % w/v PEG; C) or −0.6 MPa (22.2 % w/v PEG; T). Fresh weight (FW) and dry weight (DW) is expressed by seedling

Different letters between columns (for the same tissue) indicate a significant difference ($P < 0.05$) according Tukey's multiple range test (n = 15)

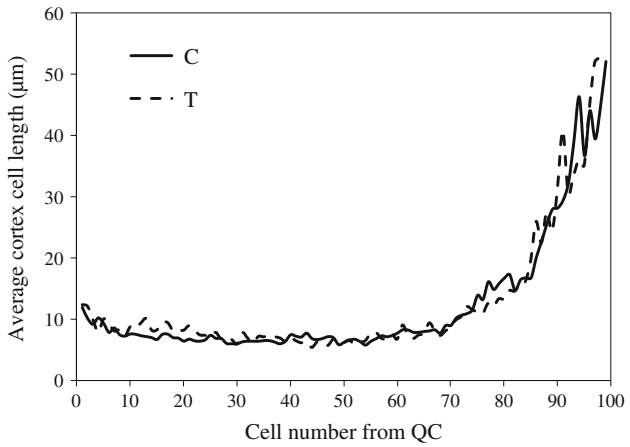

Fig. 3 Cell lengths in different zones of the root meristem. Wheat seedling were germinated and grown at −0.03 MPa (C) or −0.6 MPa (T) for 3 days and cell lengths and numbers between the quiescent center (QC) and the start of the rapid elongation zone were measured

observed in roots of seedling subjected to water restriction (Fig. 5b).

SOD and CAT activities in roots increased under a moderate water restriction, along with up-regulation of *cat2* gene

Catalase activity in roots markedly increased when wheat seedlings were subjected to a moderate water restriction. In coleoptiles, however, no variation could be detected at day 2, while this activity was significantly reduced respect to that of control by day 3 post-imbibition (Fig. 6a). SOD activity was enhanced in water-starved seedlings at day 2 and 3, and, unlike catalase, displayed quite similar levels on comparing roots and coleoptiles (Fig. 6b). By contrast, APOX activity diminished in water-starved plants, while GPX did not vary in response to water shortage, being about 5-fold higher in roots than in coleoptiles (data not shown).

In roots, *cat1* transcript abundance remained constant for control and treated seedlings along time and was not affected by the moderate water restriction imposed (Fig. 7a). By opposite, *cat2* transcript amount clearly increased as a consequence of water restriction, in coincidence with the rise observed for specific catalase activity (Fig. 7b). Expression of these catalase genes showed quite a different pattern in coleoptiles, where *cat1* transcript levels significantly increased over time in control seedlings, as it happened with specific catalase activity in this tissue (Figs. 6a, 7a). This increment was not observed in water-stressed seedlings. Oppositely, *cat2* transcript amounts showed negligible changes in this tissue (Fig. 7b). *cat3* was not detected in our samples.

Cell cycle genes were not affected by a moderate water restriction, but two expansin genes were upregulated

In order to link our observations with eventual changes in the transcript levels of certain genes related to cell cycle and/or cell expansion, we also analyzed some target genes of cyclin D-Rb-E2f system in the root apical meristem (RAM) and five root expansin genes in the apical plus subapical root region.

No significant differences between control and water-starved seedlings regarding transcript levels of the markers related to G1-S transition phase, like minichromosome maintenance (*mcm2*), ribonucleotide reductase small subunit (*rdr*) and proliferating cell nuclear antigen (*pcna*), could be detected in the root apical region (5 mm) (Fig. 8a). On the other hand, from the five expansin genes (*TaEXPA5*, *TaEXPA6*, *TaEXPA8*, *TaEXPB8* and *TaEXPB10*) analysed by semiquantitative RT-PCR in the apical plus subapical root region (10 mm), two of them, *TaEXPB8* (at days 2 and 3) and *TaEXPA5* (only at day 2) were found to be remarkably up-regulated under water restriction (Fig. 8b).

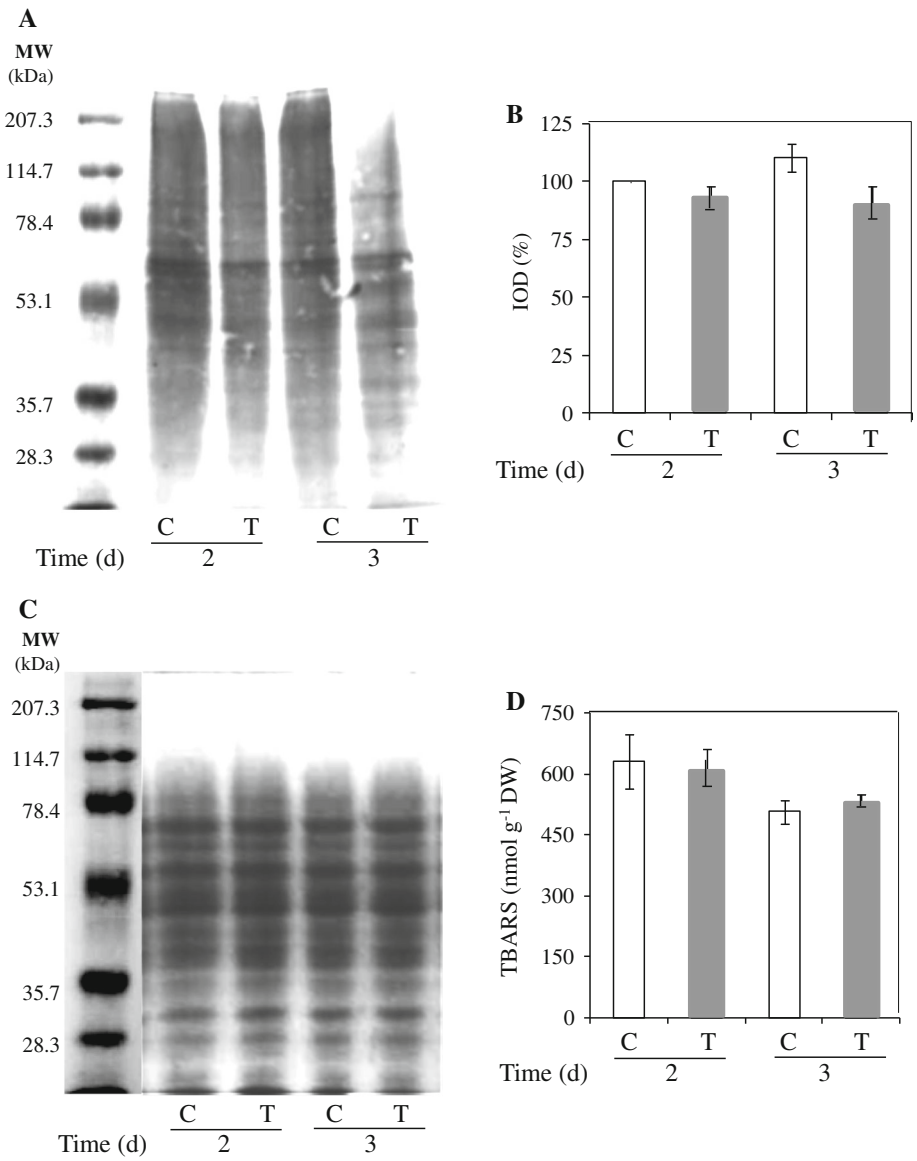

Fig. 4 Protein oxidation and lipid peroxidation in roots of wheat subjected to a moderate water restriction. Wheat seedlings were germinated and grown at −0.03 MPa (C) or −0.6 MPa (T) for 2 and 3 days. **a** Western blot of carbonylated proteins; **b** integrated optical density of bands shown in (**a**); **c** Coomassie Brilliant Blue R-250 staining of soluble proteins. Photographs are representative of four protein gel blotting and electrophoreses. **d** TBARS content. Values are mean ± SEM. Significant differences with respect to control at $*P < 0.05$ and $***P < 0.001$ according to Tukey's multiple range test (n = 5)

Discussion

Reduced germination and decreased seedling growth are common signs in crops subjected to water deficits (Soltani et al. 2006 and references therein; Seki et al. 2007). Our results corroborated that, as it was already documented for other plant species, water restriction negatively affected seed germination and early seedling growth in wheat (Fig. 1 and 2; Table 2). Increased root/shoot ratios have been associated to reduction in water consumption and increase in water absorption (Erice et al. 2010), thus improving plant resistance to drought (Bajji et al. 2000; Wu et al. 2001), as was observed in wheat seedlings, where only the RGR of coleoptiles was significantly affected by water deficit, due to the maintenance of dry biomass accumulation in roots.

Several investigations on wheat plants have related changes in the antioxidant system under water starvation to prevent oxidative damage (Khanna-Chopra and Selote 2007; Jubany-Marí et al. 2010; Hameed et al. 2011). In an attempt to know if the root growth restriction observed was associated to oxidative stress, oxidative damage of lipids and proteins was investigated. TBARS and carbonylated proteins levels in the roots of water-starved seedlings were comparable to those of controls; therefore, we assume that cells were able to maintain the redox balance and still growing at a water potential of −0.6 MPa. In support of this finding, H_2O_2 was not detected neither by the staining technique with DAB nor by the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (data not shown).

SOD and catalase specific activities increased in wheat roots under the mild water restriction here imposed

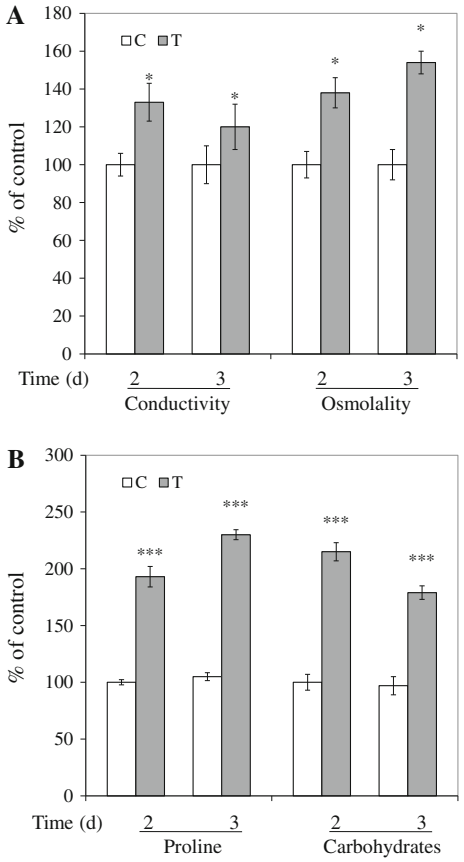

Fig. 5 Compatible solutes in roots of wheat exposed to a moderate water restriction. Wheat seedlings were germinated and grown at −0.03 MPa (C) or −0.6 MPa (T) for 2 and 3 days. **a** Total electrical conductivity and osmolality; **b** proline and soluble carbohydrates content. Values are mean ± SEM. Significant differences with respect to control at $*P < 0.05$ and $***P < 0.001$ according to Tukey's multiple range test. Control values at day 2 correspond to 45.8 μg proline g^{-1} FW and 45.7 mg hexose g^{-1} FW

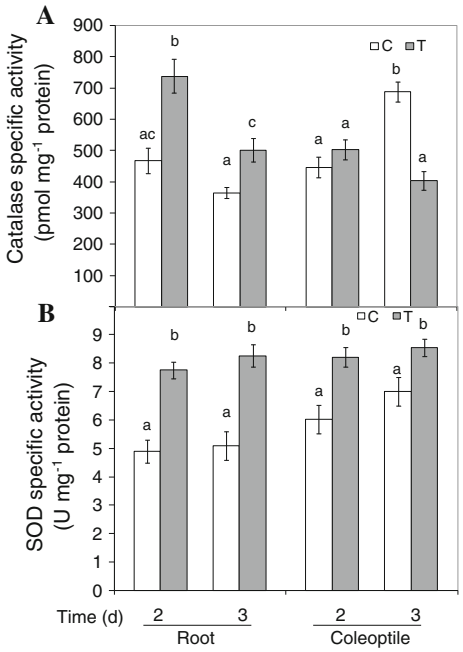

Fig. 6 Specific CAT and SOD activity in root and coleoptile of wheat seedlings subjected to a moderate water restriction. CAT (**a**) and SOD (**b**) activity was determined in protein extracts obtained from roots and coleoptiles of wheat seedlings growing at a water potential of −0.03 MPa (C) or −0.6 MPa (T). Mean values ± SEM of three independent experiments with five replicated measurements are shown. *Bars* showing different letters (for the same tissue) indicate a significant difference ($P < 0.05$) according Tukey's multiple range test

(Fig. 6a, b). In coincidence with the rise of CAT activity—which was the sole peroxidase activity significantly altered in response to water starvation under our experimental setting, we found that *cat2* transcript amount, but not *cat1*, increased in root tissue (Fig. 7). An enhanced catalase activity and biosynthesis should have contributed to prevent oxidative damage through reduction of H_2O_2 levels, as it was widely demonstrated during abiotic stress in plants (Contento and Bassham 2010).

Accumulation of compatible solutes is regarded as an important indicator of physiological tolerance to different stresses, such as drought or salinity (Liu et al. 2011). In this sense, we clearly verified enhancement of total conductivity and of proline and soluble carbohydrates in roots in response to water restriction, representing an increase in compatible osmolytes (Fig. 5). Accumulation of ions and low molecular mass compounds enhances osmotic potential and facilitates water uptake (Parida and Das 2005; Ashraf and Foolad 2007), while compatible organic solutes also possess the ability to exert a protective antioxidant role against membrane damage, allowing the plant to sustain growth even under adverse environmental conditions (Ashraf and Foolad 2007; Contento and Bassham 2010). Moreover, compatible solutes might contribute to the protection of cells against the increased levels of ROS (Bartels and Sunkar 2005; Miller et al. 2010), cooperating with the antioxidant defense system in the maintenance of cellular redox buffering capacity at low water potential (Sharma et al. 2011).

Concerning the role of proline during water deficit in wheat, Vendruscolo et al. (2007) reported that the tolerance exhibited by transgenic plants that accumulated high levels of proline was not a consequence of an osmotic adjustment but of protection mechanisms against oxidative stress. Additionally, an increase in proline content has been associated to the induction of an antioxidant gene expression during salt stress (Banu et al. 2009), and its role in the protection of cell membranes against ROS damage and of protein and DNA structure has been documented (Matysik et al. 2002).

Electrolyte leakage from control and water-starved wheat roots was similar; suggesting that plasma membrane integrity was unaffected by this moderate water restriction.

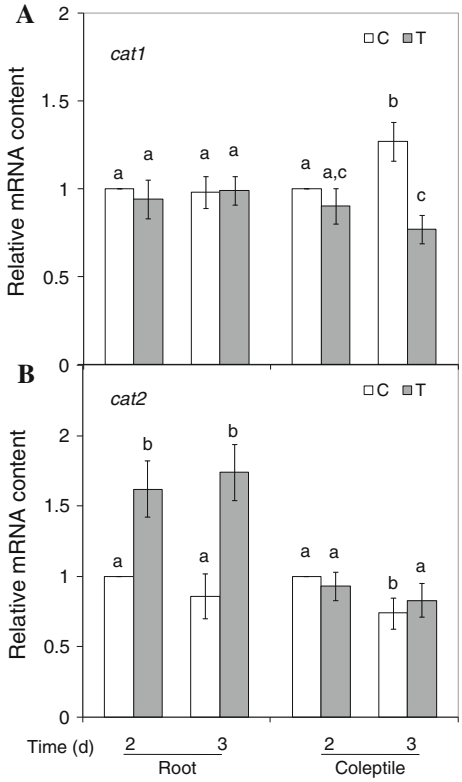

Fig. 7 *cat* transcripts accumulation in root and coleoptile of wheat seedlings subjected to a moderate water restriction. Wheat seedling were germinated and grown in −0.03 MPa (C) or in −0.6 MPa (T) for 2 and 3 days. Semiquantitative RT was performed using specific primers. Actin transcripts were used as housekeeping genes and to calculate the mRNA values (assuming control value equal to 1). *Bars* showing different letters (for the same tissue) indicate a significant difference ($P < 0.05$) according Tukey's multiple range test

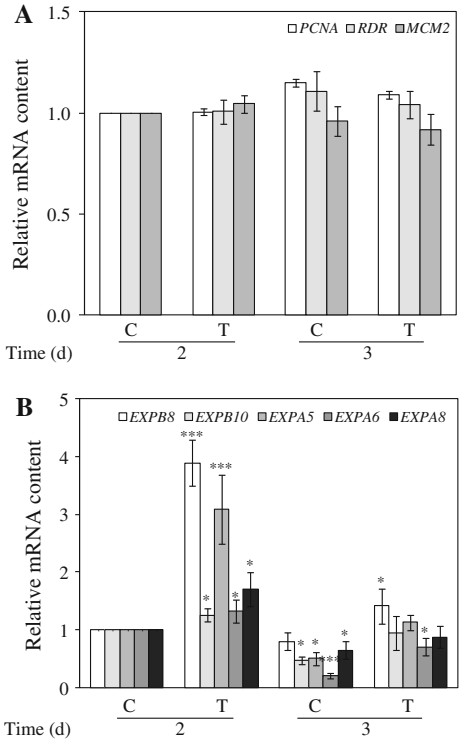

Fig. 8 Cell cycle related genes (**a**) and expansin genes (**b**) transcript accumulation in root tips of wheat exposed to a moderate water restriction. Wheat seedling were germinated and grown in −0.03 MPa (C) or in −0.6 MPa (T) for 2 and 3 days. Semiquantitative RT-PCR was performed using specific primers. Actin transcripts were used as housekeeping genes and to calculate the mRNA values (assuming control value equal to 1). Asterisks indicate significant differences with respect to control at $*P < 0.05$ and $***P < 0.001$, according to Tukey's multiple range test

These results are in line with those reported by Banu et al. (2009) for tobacco cells, where proline significantly suppressed the increase in membrane permeability induced by 200 mM NaCl.

Soluble sugars have also been implicated in drought stress tolerance in plants. As proline, they not only contribute to the regulation of osmotic adjustments but are also involved in the metabolism and protection of both ROS-producing and ROS-scavenging pathways (Couée et al. 2006; Seki et al. 2007). Several studies have associated the presence of particular soluble sugars with the acquisition of stress tolerance (Farrant 2010; Pinheiro and Chaves 2011). Zhang et al. (2012) showed that transgenic *Arabidopsis TaMYB30-B* plants accumulated higher amounts of free proline and soluble sugars than the WT plants under drought stress, suggesting that proline and soluble sugars were key factors that contributed to *TaMYB3-B* plants tolerance to drought stress.

Taking into account that root shortening under water starvation may be the result of the inhibition of cell proliferation or of cell expansion (or of both), we analyzed here the mRNA relative content of target genes including several genes activated by the CYD-Rb-E2f pathway, selected as cell cycle marker. Under our experimental conditions, the expression of *mcm2*, *rdr* and *pcna* remained unaltered after water restriction. Hence, it could be inferred that the proliferative zone in the RAM kept on being functional. In fact, as shown on Fig. 3, the number and the size of the cells in the RAM remained unaltered.

It was recently reported that osmotic stress caused cell cycle arrest in the early leaf development in *Arabidopsis* (Skirycz et al. 2011). Therefore, the effect of water deficit on cell proliferation seems to be different in roots and leaves, reinforcing the importance of plant capacity to redirect growth and to redistribute resources in order to increase its survival chances. The maintenance of the redox balance could be necessary for root tissue proliferation. In this sense, we have recently found that the redox imbalance generated by cadmium during wheat early seedling growth was associated to blockage of cell cycle progression (Pena et al. 2012).

To analyze factors potentially affecting cell wall plasticity, we examined mRNA accumulation of five expansin genes, characterized by having higher expression levels in roots than in leaves or sheaths (Lin et al. 2005). Of the five expansin genes expressed in roots, two expansin genes were strongly up-regulated at low water potential in the apical and the subapical (0–10 mm) zones (Fig. 8b). Thus, in wheat, as it was observed in other plant species, changes in cell wall elasticity and cell wall proteins might be important to sustain growth at low water potential (Wu et al. 2001). It has been reported that in maize, at least two expansin genes were up-regulated in apical regions of the root elongation zone in plants exposed to low water potential (Wu et al. 2001). Recently, Li et al. (2011) found that the increase of cell wall flexibility by over-expression of the *TaEXPB23* gene enhanced drought resistance in tobacco plants. In this sense, the understanding of the molecular and physiological mechanisms by which plants adapt to water deficit is essential for the improvement of agricultural practices.

As far as we know, this is the first report focusing on wheat growth under water deficit at the very beginning of plant development, and demonstrates that both fresh root biomass and root elongation are compromised under a moderate water-restricted regime, but this did not involve an impairment of root apical meristem functionality, as cell cycle genes remained at similar transcript levels. Osmotic adjustment and maintenance of the redox balance allowed root tissues to continue growing, though at lower rate.

Root cells of water-starved seedlings could have adjusted their cellular redox balance, at least in part, by induction and/or activation of SOD and CAT. Increases in osmolytes could also have been implicated in redox adjustments in roots, in addition to their well-known osmotic role. These responses resulted in the absence of oxidative damage and cell membrane disruption in roots, but were not enough to prevent a significant drop in root elongation rate. Under field conditions, this drop would imply a serious obstacle for young wheat seedlings to properly expand and access to soil water, in a phenological stage where water supply is crucial. Since two expansin genes were found to be up-regulated in roots under low water potential, we hypothesized that changes in cell wall plasticity may be of particular relevance at this stage of wheat development, probably to facilitate cell expansion and to allow a prompt recovery of normal turgor of existing cells in case of sudden rewatering. Further studies directed to gain a deeper knowledge of plant cell homeostasis under different degrees of water restriction are certainly needed.

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