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Drought and watering-dependent oxidative stress: effect on antioxidant content in *Triticum aestivum* L. leaves

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Abstract

The purpose of the present work was to evaluate both oxidative stress and the antioxidant response system in leaves from wheat (*Triticum aestivum* cv. Buck Poncho) subjected sequentially to drought and watering. Drought was imposed by withholding water until soil water potential reached −2.0 MPa and maintained under those conditions for 24 h. DCFDA oxidation by wheat leaves was not significantly affected by drought, but watering led to an approximately 2-fold increase in DCFDA oxidation rate. However, no significant effect either on lipid radical content or on hydroperoxide content was measured after drought and drought followed by watering. Microsomes isolated from leaves exposed to drought, and from leaves exposed to drought followed by watering, generated a significantly higher amount of hydroxyl radical as compared to microsomes isolated from control leaves, suggesting a higher production of hydroxyl radical in the cellular water-soluble phase, after drought and watering as compared to control values. The content of α-tocopherol in wheat leaves was increased 2.4-fold after drought and β-carotene content was increased by 2.6-fold after drought. Hydration lowered lipid-soluble antioxidant content to control values. Total thiol content was increased by 70% after drought, and watering did not significantly alter the enhanced values. Drought decreased by 28.5% the content of reduced ascorbic acid. Taken as a whole, active species formed at wheat membranes after exposure to moderate water stress, are efficiently removed upon rehydration by reaction with an increased content of α-tocopherol and β-carotene. Moreover, a co-ordinated response involving glutathione reductase activity, thiols and ascorbic acid is triggered to limit free radical dependent effects.

Key words: Antioxidants, lipid radicals, oxygen radicals, water stress, wheat.

Introduction

Oxygen toxicity is an inherent feature of aerobic life since it has been estimated that 1% of the oxygen consumed by plants is diverted to produce activated oxygen (Asada and Takahashi, 1987) in various subcellular loci (Elstner, 1987; Del Rio *et al*., 1992). It has been proposed that water-stress conditions, in particular, may trigger an increased formation of the superoxide radical and hydrogen peroxide, which can directly attack membrane lipids and inactivate SH-containing enzymes (Navarri-Izzo *et al*., 1994; Menconi *et al*., 1995). Moreover, Moran *et al*. (1994) reported in pea plants that augmented levels and decompartmentation of catalytic metals occurring during water stress could be responsible for the oxidative damage observed *in vivo*. On the other hand, plants are endowed with an array of enzymes and small molecules to cope with free radicals. During water-deficit stress, this defence mechanism may be induced. Sgherri *et al*. (1994*a*) found that during rehydration of *Boea hygroscopica*, antioxidants such as glutathione and ascorbate were accumulated. Moreover, it was found that in *Sporobolus stapfianus*, the activities of related defence enzymes, glutathione reductase and dehydroascorbate reductase, were increased (Sgherri *et al*., 1994*b*). Nevertheless, it is important to point out that desiccation is

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a physically and physiologically different situation to water stress. In sunflower seedlings it was reported that there was an induction of defence enzyme activities and an increase in glutathione content when plants reached a moderate level of water-deficit stress (Sgherri and Navarri-Izzo, 1995). In wheat plants subjected to water stress, Menconi *et al.* (1995) showed that after moderate water deficit, the good functionality of the ascorbate/glutathione cycle allowed the plants to maintain hydrogen peroxide at the control level despite a greater capacity of the thylakoid membranes to leak electrons towards oxygen. Regarding the effect of drought on the content of lipid soluble antioxidants, Price and Hendry (1987, 1989) have reported that drought enhanced the synthesis of α-tocopherol in leaves of a number of grasses. However, this response has not been observed in mosses (Seel *et al.*, 1992) or in other higher plants.

The importance of water stress in plant physiology has been recognized for decades, particularly in studies of the growth and development of mature tissue (Hanson and Hitz, 1982). Similarly, the impact of water stress immediately following radicle emergence has been well documented (Crevecoeur *et al.*, 1976; Leprince *et al.*, 1993). But the impact of water stress in the days following germination, that challenges seedling survival, is almost certainly one of the major limitations for the establishment of species in many habitats. Substantially different models have been used to study water stress in plants, since some authors stressed plants gradually, others used a shock treatment while there were others who rewatered the plants. These discrepancies severely restricted comparisons among studies (Beltrano *et al.*, 1997).

The purpose of the present work was to evaluate both oxidative stress and the antioxidant response system in early seedlings of wheat subjected sequentially to drought and watering. Oxidative stress was assessed by oxidation of the probe 2',7'-dichlorofluorescein, and by EPR determination of lipid and hydroxyl radical content in wheat leaves. Enzymatic antioxidants (catalase, superoxide dismutase, ascorbate peroxidase, glutathione reductase), lipid-soluble antioxidants (α-tocopherol, β-carotene) and water-soluble antioxidants (total thiols, ascorbic acid) were measured.

Materials and methods

Plant material

Wheat leaves were isolated from plants (*Triticum aestivum* L. cv. Book Poncho) grown during 1 month in a culture room at $26 \pm 1\,^{\circ}C$ and a photosynthetic photon flux of 350 μmol photon $m^{-2}\,s^{-1}$. A group of plants were kept at a soil water potential of −0.7 MPa (control group). Drought was imposed by withholding water until soil water potential reached −2.0 MPa and maintained under those conditions for 24 h (drought group). A group of plants was watered and samples were taken after 90 min (watered group). Soil and leaf water potentials (ψ) were measured with a Wescor HR33T dew point psychrometer.

Net photosynthetic rate and stomatal conductance

Gas exchange measurements were performed using a Li-Cor LI-6200 Portable Photosynthesis System at an irradiance of 800 μmol photon $m^{-2}\,s^{-1}$ and 330–370 μmol CO_2 mol^{-1}. Under the same conditions the stomatal conductance was measured on the adaxial leaf surface employing a Li-Cor LI-1600 steady-state porometer.

Reactive oxygen species generation

An *in vivo* assay was performed by placing a leaf segment (approximately 30 mg) in 8 ml of 40 mM TRIS-HCl buffer (pH 7.0), in the presence of 100 μM 2′,7′-dichlorofluorescein diacetate (DCFDA, Molecular Probes Inc, Eugene, OR) at 30 °C. Supernatants were removed after 60 min and fluorescence monitored in an Hitachi spectrofluorometer with excitation at 488 nm and emission at 525 nm (Simontacchi *et al.*, 1993). To differentiate reactive oxygen species from other long-lived substances able to react with DCFDA additional controls were performed. Leaf segments were incubated without DCFDA for 60 min and after removing the leaf segments DCFDA was added to the medium and incubated for 60 min before fluorescence was determined. This fluorescence value was considered as the contribution of long-lived substances to the total fluorescence and subtracted from all readings to assess the fluorescence that depends on reactive oxygen species. Corrections for autofluorescence were made by the inclusion in each experiment of parallel blanks (assay mixture without plant material).

Hydroperoxide content

Hydroperoxides were extracted with ethyl acetate and assayed as described by Simon (1994). The reaction mixture contained 250 mM ammonium ferrous sulphate, 100 mM xylenol orange, 25 mM H_2SO_4, 4 mM BHT prepared in 90% (v/v) methanol, and an aliquot of the sample. After 30 min of incubation at room temperature the reaction was added with triphenylphosphine to specifically reduced hydroperoxides to distinguish from H_2O_2 (Nourooz-Zadeh *et al.*, 1994).

Lipid radical generation by EPR-spin trapping

Wheat leaves were homogenized with a Potter-Elvejhem homogenizer in 60 mM phosphate buffer–100 mM KCl (pH 7.0) filtered through four layers of cheesecloth and then centrifuged at 750 *g* for 10 min. Membrane fractions were isolated by centrifugation at 3000 *g* followed by a centrifugation at 105 000 *g*. The pellet, containing an enriched fraction of membranes, was supplemented with α-(4-pyridyl 1-oxide)-*N*-t-butyl nitrone (POBN). EPR spectra were obtained at room temperature using a Bruker Spectrometer ECS 106, operating at 9.75 GHz with 50 kHz modulation frequency. EPR instrument settings for the spin trapping experiments were: microwave power 20 mW; modulation amplitude 1.232 G; time constant 81.92 ms; receiver gain 2×10^4 (Jurkiewicz and Buettner, 1994). Quantitation of the spin adduct was performed using an aqueous solution of 2,2,5,5-tetramethyl piperidine 1-oxyl (TEMPO) introduced into the same sample cell used for spin trapping. EPR spectra for both sample and TEMPO solutions were recorded at exactly the same spectrometer settings and the first derivative EPR spectra were double-integrated to obtain the area intensity, then the concentration of spin adduct was calculated according to Kotake *et al.* (1996).

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Lipid soluble antioxidants content

The content of α-tocopherol (αT) and β-carotene in the homogenates was quantified by reverse-phase HPLC with electrochemical detection using a Bioanalytical Systems LC-4C amperometric detector with a glassy carbon working electrode at an applied oxidation potential of 0.6 V (Desai, 1984). Extraction from the samples was performed with 1 ml of methanol and 4 ml of hexane. After the samples were centrifuged at 1500 *g* for 10 min, the hexane phase was removed and evaporated to dryness under N_2. Samples were dissolved in methanol:ethanol (1:1, v/v) and injected for HPLC analysis. *d*,*l*-α T from synthetic phytol (Sigma) and β-carotene (Sigma) were used as standards.

Hydroxyl radical production by EPR-spin trapping

Prior to use, 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was purified, following the method of Green and Hill (1984), to remove contaminants that contribute to the EPR background signal. In this procedure, 10 ml of 1 mM DMPO in doubly distilled water are mixed with 1.25 g activated charcoal for 1 min, allowed to stand for 1 h, and then filtered. This purification procedure is repeated twice. The basic incubation system consisted of 100 mM DMPO, leaf membranes (0.5 mg protein ml^{-1}), 0.5 mM sodium azide, 0.6 mM DTPA, and 50 mM potassium phosphate (pH 7.4). Reactions were started by addition of 0.5 mM NADPH and incubated for 15 min at 37 °C. The reaction system was transferred to a Pasteur pipette for direct observation of the reaction in a Bruker ECS 106 EPR spectrometer at room temperature. The EPR spectrometer settings were as follows: field scan 100 G, scan time 167.772 s, time constant 655.36 ms, modulation amplitude 0.490 G, modulation frequency 50 kHz, and microwave power 20 mW (McCay *et al*., 1992).

Total thiol content

Total thiol content was assayed in acid-soluble extracts (0.2 g FW ml^{-1}) as described by Tietze (1969). The homogenate was prepared in TCA 3% (w/v) and after a brief centrifugation, the supernatant was diluted 10-fold in 100 mM phosphate buffer (pH 7.5). Thiols content was determined measuring absorbance at 412 nm, in the presence of 0.5 mM 5,5′-dithiobis 2-nitrobenzoic acid (DTNB), 0.5 U ml^{-1} glutathione reductase and 0.2 mM NADPH.

Reduced ascorbic acid content

Wheat leaves (0.5 g) were homogenized in 1 ml 5% (v/v) metaphosphoric acid and centrifuged at 22 000 *g* for 10 min. Supernatants were added with 5 mM EDTA, 1.7% (w/v) TCA, 7.6% (w/v) *o*-phosphoric acid, 44 mM α-α′-dipyridyl, and 16 mM $FeCl_3$, in potassium phosphate buffer (pH 7.4). After 40 min of incubation at 40 °C the absorbance at 525 nm was determined (Law *et al*., 1983).

Enzyme assays

Wheat leaves were homogenized with a Potter-Elvejhem homogenizer in 60 mM phosphate buffer–100 mM KCl (pH 7.0) filter through four layers of cheesecloth and then centrifuged at 750 *g* for 10 min. The homogenates were added with 1% (w/v) polyvinylpolypyrrolidone (PVP). Superoxide dismutase (SOD) activity was determined spectrophotometrically as the inhibition of xanthine oxidase-dependent reduction of nitroblue tetrazolium (Beauchamp and Fridovich, 1971). The reaction mixture contained 0.1 mM nitroblue tetrazolium, 0.1 mM EDTA, 50 μM xanthine, and xanthine oxidase in 50 mM potassium phosphate buffer (pH 7.8). One unit of SOD is defined as the amount of enzyme that inhibits by 50% the control rate (0.025 units of absorbance at 550 nm min^{-1}) (McCord and Fridovich, 1969). Catalase activity was measured according to Aebi (1984). The reaction mixture contained 15 mM H_2O_2, up to 100 μl of homogenate (7 mg protein ml^{-1}) with 0.2% (w/v) Triton X-100 in 50 mM potassium phosphate buffer (pH 7.0). Ascorbate peroxidase (AP) activity was determined according to Nakano and Asada (1981) using homogenates previously supplemented with 0.5 mM ascorbic acid and 0.1 mM EDTA. Parallel experiments in the presence of *p*-chloromercuribenzoate 50 μM were performed to ruled out interference from guaiacol peroxidases (Amako *et al*., 1994). Glutathione reductase (GR) activity was measured according to Schaedle and Bassham (1977). The reaction mixture contained 50 mM TRIS-HCl (pH 7.6), 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG), 3 mM $MgCl_2$ and up to 100 μl of homogenate (7 mg protein ml^{-1}). NADPH oxidation was followed at 340 nm.

The protein content was assayed according to Bradford (1976) using bovine serum albumin as standard.

Statistical analyses

Data are expressed as mean ± SEM from 4–6 independent experiments. Effect of treatments were tested for significance using a single-factor analysis of variance (ANOVA). Statistical tests were carried out using Statview SE+, v 1.03 (Abacus Concepts Inc, Berkeley, CA).

Results

Physiological parameters

Drought was imposed on *Triticum aestivum* L. cv. Book Poncho by withholding water until soil water potential reached −2.0 MPa and those conditions were maintained for 24 h. Under those conditions the seedlings showed clear symptoms of water stress, manifested by winding and flaccidity of their leaves. A group of plants was watered and the water potential values were recovered to those measured in irrigated plants (Table 1). Together with the decrease in leaf water potential, photosynthetic rate and stomatal conductance were significantly reduced in droughted plants and recovered after rehydration (Table 1). Chlorophyll content was not affected by drought or drought followed by watering (11.2 ± 0.7; 10 ± 1 and 10.3 ± 0.9 μg mg^{-1} DW for control, drought and watering group, respectively).

Oxidative stress and lipid oxidation

The oxidation of DCFDA to a fluorescent compound was used as an *in vivo* assay to assess oxygen radical generation by wheat leaves. DCFDA oxidation by wheat leaves was not significantly affected by water stress, however, watering led to an approximately 2-fold increase in DCFDA oxidation rate (Fig. 1). The addition of catalase (0.1 μM) to the reaction medium quenched wheat leaf-dependent fluorescence, reaching values not significantly different from the blanks (reagents without leaves), suggesting that hydrogen peroxide diffusing from the tissue is the main oxidant leading to DCFDA oxidation.

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Table 1. *Effect of drought and watering on physiological parameters in wheat leaves*

Treatment	Ψ (MPa)	Photosynthetic rate (μmol m^{-2} s^{-1})	Stomatal conductance (mmol m^{-2} s^{-1})
Control	−0.66±0.09	11±2	147±10
Drought	−2.00±0.25*	nd	15±3*
Watering	−0.70±0.08	4.9±0.9*	74±14*

*Significantly different from controls without treatment (ANOVA, $P \leq 0.05$). Data are expressed as mean±SEM of 4–6 independent experiments. Watering represents samples taken after 90 min of watering the plants previously exposed to drought conditions; nd stands for non-detectable values.

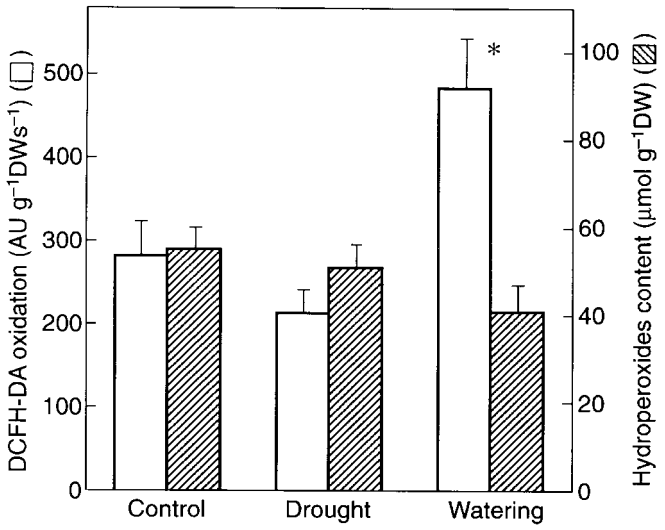

Fig. 1. DCFH-DA oxidation rate and hydroperoxide content exposed to drought and drought followed by watering. Watering represents samples taken after 90 min of watering the plants previously exposed to drought conditions. *Significantly different from controls without treatment (ANOVA, $P \leq 0.05$).

The increased DCFDA oxidation rate persisted in leaves excised after 3 h of hydration, and started to decline after 4–5 h of hydration (data not shown).

Oxidative stress has been extensively associated with lipid oxidation. Figure 1 shows that under these experimental conditions hydroperoxide content in the membranes was not affected either by drought or by drought–watering. To characterize further the effect of drought and drought–hydration to the membrane the content of lipid radicals was measured. Lipid radicals combined with the spin trap POBN resulted in adducts that gave a characteristic EPR spectrum with hyperfine coupling constants of $a^N = 15.8$ G and $a^H = 2.6$ G, in agreement with computer spectral simulated signals obtained using those parameters (Fig. 2). Even though these constants could be assigned to lipid radicals (Buettner, 1987), spin trapping studies cannot readily distinguish between $ROO^{\cdot}$, $RO^{\cdot}$ and $R^{\cdot}$ adducts, owing to the similarity of the corresponding coupling constants (Buettner, 1987). No significant effect on lipid radical content was measured after drought and drought followed by watering, as compared to controls (3.3×10^{-3}, 3.2×10^{-3}, and 3.1×10^{-3} AU, respectively) (Fig. 2).

Since DCFDA oxidation measurements suggested that hydrogen peroxide generation could be increased after watering, and according to the Haber–Weiss reaction hydrogen peroxide could result in the generation of an hydroxyl radical, the generation of an hydroxyl radical by microsomal membranes isolated from wheat leaves was assessed by EPR. Figure 3 shows that microsomes isolated from leaves exposed to drought, and from leaves exposed to drought conditions followed by hydration generated a significantly higher amount of hydroxyl radical as compared to microsomes isolated from control leaves. The integrated areas were 1.8×10^{-3}, 3.3×10^{-3}, and 5.2×10^{-3} AU for control microsomes, microsomes obtained from leaves exposed to drought, and microsomes from leaves exposed to drought and hydration, respectively.

Water stress effect on antioxidant content in wheat leaves

The content of α-tocopherol in wheat leaves was increased by 2.4-fold after drought. Accordingly, β-carotene content was increased by 2.6-fold after drought. Hydration lowered lipid-soluble antioxidant content to control values (Table 2). Water-soluble antioxidant content showed a distinctive profile. Total thiol content in wheat seedlings was increased by 35% and 83.6%; whereas ascorbic acid content decreased by 28.5% and 35% as compared to control values, after exposure to drought and drought followed by hydration, respectively (Table 3). Regarding the enzymatic antioxidants, neither superoxide dismutase activity nor catalase activity was affected by water stress under these experimental conditions. However, ascorbate peroxidase activity was increased by 40% after drought followed by hydration, as compared to control values. GR activity was increased by 92.3% and 61.5% after exposure to drought and drought followed by hydration, respectively, as compared to control values (Table 4).

Discussion

According to the definition of Hsiao (1973), wheat plants reached a moderate level of water stress following the drought period performed here. It is important to point out that the time frame of the treatment (minutes) used in this work, did not add the problem of different ageing conditions between control and stressed plants.

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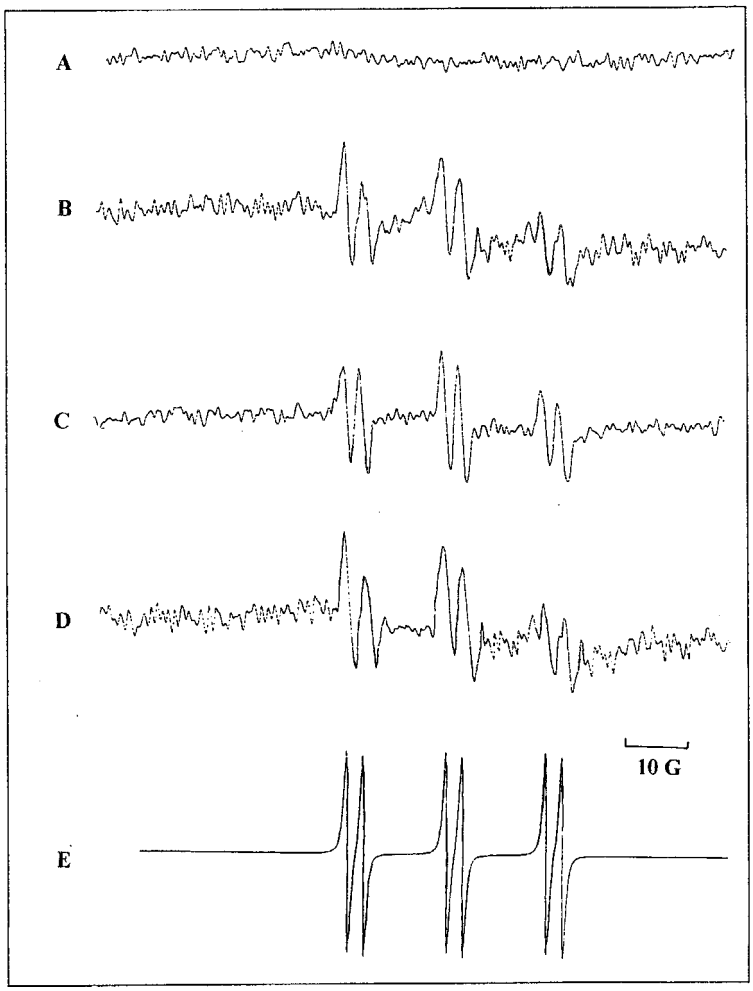

Fig. 2. Typical lipid radical EPR spectra of wheat leaves in the presence of POBN. (A) Spectrum without the addition of microsomes. (B) Spectrum after the addition of homogenate from control samples. (C) Spectrum after the addition of homogenate from samples after drought. (D) Spectrum after the addition of homogenate from samples after drought followed by watering. (E) Computer simulated spectrum.

Some authors have proposed that water stress should be considered as an oxidative stress (Dhindsa *et al.*, 1981; Burke *et al.*, 1985) since it was reported that lipid peroxidation caused alterations in the membrane similar to those noticed under certain conditions of dehydration. Several studies have shown lipid peroxidation in water-deficit conditions (Dhindsa and Matowe, 1981; Dhindsa *et al.*, 1981; Paulin *et al.*, 1986). However, Quartacci and Navarri-Izzo (1992) have reported a decreased level in polar lipids in water-stressed sunflower seedlings, with no accumulation of MDA, which constitutes the final product of unsaturated fatty acid degradation. The results presented here indicate that in wheat exposed to moderate drought conditions there is neither a significant oxidative stress, assessed as DCFH-DA oxidation, nor an increase in hydroperoxide content. After rehydration, DCFH-DA oxidation significantly increased, suggesting that radicals formed as soon as water uptake started and were able to lead to chain reactions. Surprisingly, neither hydroperoxide content nor lipid radical content were affected by drought or drought followed by watering. This lack of effect could be ascribed either to lipid peroxidation mainly related to de-esterification reactions by free radical action on phospholipids (Navarri-Izzo *et al.*, 1990, 1991) or to oxidative stress mainly related to phenomena taking place in water-environments instead of an effect centred on lipids. In this regard, the results included in Fig. 3 showing a significant increase in hydroxyl radical production after drought and even higher after hydration, suggest that water stress could mainly act at the cytosolic level. Nevertheless, it should be pointed out that distribution in cellular compartments could be a critical factor under *in vivo* conditions, since the effect of drought and watering on specific isozymes could be different to what is detected in the homogenate (Mittler and Zilinskas, 1994).

Under the experimental conditions used in this study, the content of lipid-soluble antioxidants was drastically

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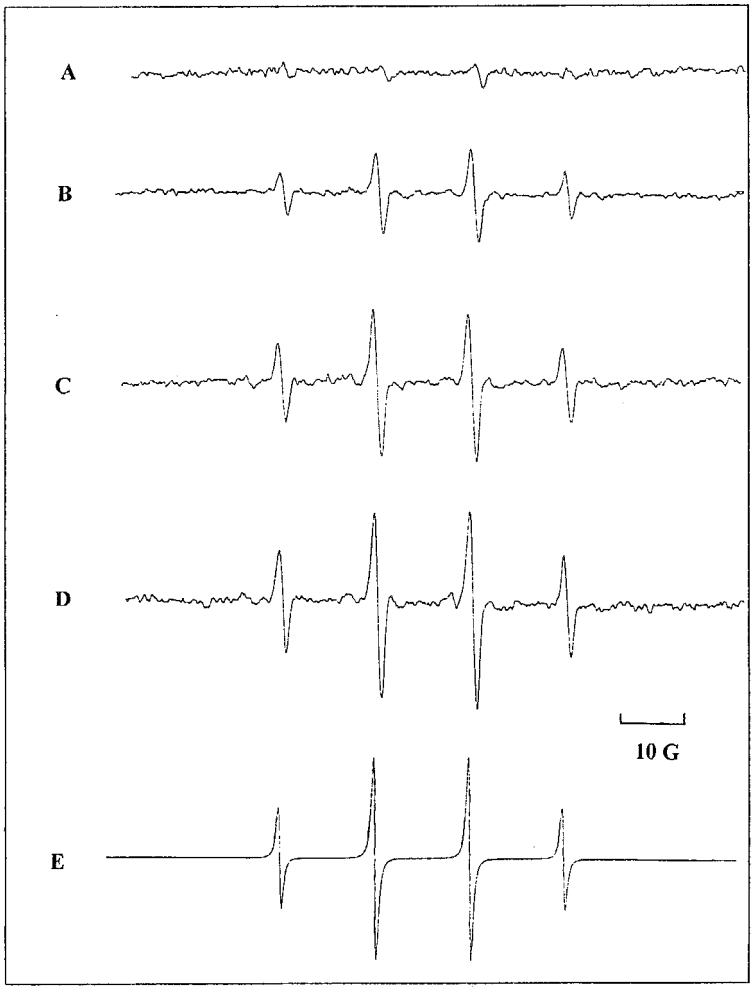

Fig. 3. Typical hydroxyl radical EPR spectra of wheat leaves microsomes in the presence of DMPO. (A) Spectrum without the addition of microsomes. (B) Spectrum after the addition of microsomes from control samples. (C) Spectrum after the addition of microsomes from samples after drought. (D) Spectrum after the addition of microsomes from samples after drought followed by watering. (E) Computer simulated spectrum.

Table 2. *Effect of drought and watering on lipid soluble antioxidants*

Treatment	α-tocopherol (nmol g^{-1} DW)	β-carotene (nmol g^{-1} DW)
Control	133 ± 13	12 ± 3
Drought	322 ± 54*	32 ± 2*
Watering	151 ± 22	13 ± 2

*Significantly different from controls without treatment (ANOVA, $P \leq 0.05$). Data are expressed as mean ± SEM of 4–6 independent experiments. Watering represents samples taken after 90 min of watering the plants previously exposed to drought conditions.

Table 3. *Effect of drought and watering on water-soluble antioxidants content in wheat leaves*

Treatment	Total thiols (μmol g^{-1} DW)	Reduced ascorbic acid (μmol g^{-1} DW)
Control	1.47 ± 0.07	15.4 ± 0.6
Drought	1.99 ± 0.03*	11 ± 2*
Watering	2.7 ± 0.4*	10 ± 1*

*Significantly different from controls without treatment (ANOVA, $P \leq 0.05$). Data are expressed as mean ± SEM of 4–6 independent experiments. Watering represents samples taken after 90 min of watering the plants previously exposed to drought conditions.

increased after drought, since α-tocopherol and β-carotene content was increased by 2.4-fold and 2.6-fold, respectively. The reported increase in antioxidant content could be postulated as a key factor to control the oxidation at the membrane level, limiting the increase in hydroperoxide and lipid radical content. However, to assess the real role of these antioxidants, experiments should be performed with plants, e.g. mutants or transgenics, in which drought could not change the antioxidant content and membrane damage could be measured. In our study, no significant changes in the content of antioxidant enzymes, such as catalase and superoxide dismutase were measured in wheat exposed to drought and drought followed by hydration, suggesting that enzymatic activities would not be responsible for controlling free radical-dependent damage.

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Table 4. *Effect of drought and watering on antioxidant enzymes activity in wheat leaves*

Treatment	SOD ($U\ mg^{-1}$ protein)	Catalase ($\mu mol\ substrate\ min^{-1}\ mg^{-1}$ protein)	AP ($\mu mol\ substrate\ min^{-1}\ mg^{-1}$ protein)	GR ($\mu mol\ substrate\ min^{-1}\ mg^{-1}$ protein)
Control	20 ± 1	119 ± 6	0.95 ± 0.07	0.13 ± 0.01
Drought	17 ± 1	120 ± 20	1.0 ± 0.1	0.25 ± 0.02*
Watering	18 ± 1	129 ± 16	1.4 ± 0.1*	0.21 ± 0.05*

*Significantly different from controls without treatment (ANOVA, $P \leq 0.05$). Data are expressed as mean $\pm$ SEM of 4–6 independent experiments. Watering represents samples taken after 90 min of watering the plants previously exposed to drought conditions.

The cellular thiol-disulphide redox status can be enormously altered under conditions of oxidative stress. Navarri-Izzo *et al.* (1995) proposed that during drying of *Boea hydroscopica* the accumulation of reduced glutathione, in combination with tocopherol, may protect membrane components by suppressing lipid peroxidation. The accumulation of glutathione may also protect enzymes that possess exposed thiol groups and may enable the cell to maintain the percentage of -SH in the thylakoid proteins during rehydration. The same effect was observed in wheat leaves exposed to drought that significantly increased total thiol content, even more, it was further increased after watering. A major function of reduced glutathione in the protection of cells against the toxic effects of free radicals is to keep the free radical-scavenging ascorbate in its reduced and hence, active form by involvement in the ascorbate-glutathione cycle (Zhang and Kirkham, 1996). Reduction in ascorbate concentrations in response to drought were reported in *Vigna catjang* (Mukherjee and Choudhuri, 1983), *Cohlearia atlantica* and *Armenia maritima* (Buckland *et al.*, 1991) and sorghum (Zhang and Kirkham, 1996). Results reported here showed a decrease in ascorbic acid content by 28.5% in wheat exposed to drought conditions. Ascorbate participates in the removal of H_2O_2 as a substrate of ascorbate peroxidase, directly reduces O_2^-, quench $^1O_2$ and regenerate reduced α-tocopherol (Foyer, 1993). Any of the routes for ascorbate oxidation listed above, as well as a slow synthesis rate of ascorbate or a decreased reduction rate of both oxidation products (monodehydroascorbate and dehydroascorbate), could lead to the decrease in ascorbate content in stressed wheat. An increased consumption of ascorbic acid in wheat by an increased ascorbate peroxidase activity did not seem to be responsible for the drought-dependent decrease in the content of ascorbic acid since ascorbate peroxidase activity only increased, as compared to controls, after watering.

Another factor that would contribute to maintaining reduced glutathione content at the cellular level is the activity of the enzyme GR. Increases in GR activity under stresses of cold, high light, ozone, and water were reported (Creissen *et al.*, 1994; Gamble and Burke, 1984; Burke *et al.*, 1985). An increase in GR activity was also observed in a drought-resistant wheat cultivar subjected to 100% oxygen and water stress and ascribed to *de novo* synthesis (Pastori and Trippi, 1993). Under these experimental conditions, GR in wheat increased by 92% after drought and the increase over control values was maintained after rehydration. The elevated levels of GR might be able to increase the ratio of $NADP^+$/NADPH, thereby ensuing the availability of $NADP^+$ to accept electrons from the photosynthetic electron transport chain (Baisak *et al.*, 1994).

Taken as a whole, active oxygen species formed at wheat membranes after exposure to moderate water stress, are efficiently removed upon rehydration by reaction with an increased content of α-tocopherol and β-carotene. Moreover, a co-ordinated response involving GR, thiols and ascorbic acid is triggered to limit free radical dependent effects. However, adaptive changes in enzymatic and non-enzymatic antioxidants were not sufficient to prevent oxidative stress since hydroxyl radical production and DCFH-DA oxidation were significantly increased. Further studies will be performed to determine if an experimental increase in water-soluble antioxidants would improve wheat resistance to drought and rehydration.

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