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ORIGINAL ARTICLE

Drought response in winter wheat: protection from oxidative stress and mutagenesis effect

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Abstract

Radiation mutagenesis could provide new drought-tolerant lines for selection purposes in sustainable agriculture. Drought tolerance and yield stability are closely related to coping with oxidative stress, which occurs at severe/prolonged water deprivation. In this study, the response of a newly generated winter wheat mutant line M181/1338K to severe drought stress at seedling stage (3–4th leaf) was compared to that of two established varieties—Guinness (drought tolerant) and Farmer (drought sensitive). Oxidative damage and antioxidant status analyses were performed on second leaves of control, stressed (45–46% leaf water deficit) and recovered plants. Genotypes exhibited similar pattern of stress response, comprising proline accumulation, rise in hydrogen peroxide content and oxidative damage to membrane lipids, increase in total antioxidant and antiradical activities, in phenolic and flavonoid content, in ascorbate and glutathione pools, mobilization of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) enzyme isoforms. Farmer responded to severe water stress with the highest levels of oxidative damage to membranes, proline accumulation, and glutathione content, and slower normalization of the studied parameters upon recovery. Guinness presented a better control of oxidative membrane damage and it the highest accumulation of flavonoids under drought. The new mutant line M181/1338K had similarities with Guinness in its response to severe water stress, such as the same proline and glutathione levels. Unlike Guinness, the mutant genotype had more pronounced oxidative damage to membranes, along with higher POX activities, and tended to accumulate less flavonoids under drought, which could be regarded as secondary effects of the induced mutagenesis.

Keywords *Triticum aestivum* L. · Radiation mutagenesis · Drought · Recovery · Oxidative stress · Antioxidative protection

Abbreviations

AO	Total antioxidant activity
AR	Antiradical activity
ASC	Ascorbic acid
CAT	Catalase
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
FRAP	Ferric reducing antioxidant power assay
FW	Fresh weight
GSSG	Oxidized glutathione
GSH	Reduced glutathione
MDA	Malondialdehyde
POX	Peroxidase
ROS	Reactive oxygen species
RWC	Relative water content
SOD	Superoxide dismutase
TW	Weight at full turgidity

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Introduction

Wheat is a staple food for more than one-third of the world population and crops yields would be significantly influenced by global climatic changes, especially water shortage (Anjum et al. 2011; Hasanuzzaman et al. 2018). Drought is a major growth-limiting factor for wheat plants and a main reason for yield reduction both in quantity and quality

(Daryanto et al. 2016). Economic losses, associated with the agricultural wheat yield reduction, have been estimated to 6–8 billion dollars annually only in the USA (Fontaine et al. 2014). Choice of wheat genotypes with enhanced tolerance to drought stress together with conserved high yield is a major goal in sustainable agriculture. Crop responses to drought depend on the strength and duration of water deficit, on plant species, age and developmental stage; varietal differences are also observed (Chakraborty and Pradhan 2012).

Sustainable agriculture will benefit from the establishment of new lines of wheat plants, resistant to unfavorable environmental conditions, including drought stress. Radiation mutagenesis results in increase of plant variability and has been successfully used as a source of new lines for breeding purposes (Ahloowalia and Maluszynski 2001; Sen et al. 2017). Comparison of the newly created mutant lines with established varieties—tolerant and sensitive to stress, is a prerequisite for their use in sustainable agriculture. The drought-tolerant Guinness was developed from the parental variety Katya, and the sensitive Farmer originates from the parental variety Pobeda at IPGR (Institute of Plant Genetic Resources)—Sadovo, Bulgaria. Both were obtained by gamma irradiation (50 Gy) of the parental seed stocks (Rachovska and Uhr 2010). Variety Katya has been recognized as highly tolerant to drought (Chipilski et al. 2012) while Pobeda has been characterized as cold tolerant. These parental traits were conserved in the newly established varieties. Preliminary screening confirmed the high drought tolerance of Guinness and the relative drought sensitivity of Farmer (Vassileva et al. 2019). The mutant line M181/1338K has also been obtained from Katya variety by treatment of seeds with a higher dosage of gamma irradiation (100 Gy) (Rachovska and Uhr 2010). M181/1338K has shown an improved yield potential and resistance to economically important pathogens but has not been characterized so far for its potential to tolerate drought.

Abiotic stresses in general and drought in particular lead to morphological, physiological, biochemical and molecular alterations which adversely affect plant growth and productivity (Anjum et al. 2011). Plants have developed protective mechanisms for stress tolerance and their diversity depends on the plant species. They are largely orientated towards maintenance of cellular metabolic, redox and ionic homeostasis and protection of plant structures and macromolecules from damage during dehydration and recovery (Shah et al. 2017). Many severe or prolonged stresses, including drought, induce secondary oxidative stress, thus, coping with oxidative stress is an important part of stress-tolerance mechanisms (Singh et al. 2012; Hasanuzzaman et al. 2019). Although molecular oxygen (the terminal oxidant during aerobic respiration) has a relatively low-reactive character and is considered as harmless, its partially reduced intermediate forms, the result of electron transfer to O_2, such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) are highly reactive oxygen species (ROS). Metabolic processes inevitably lead to production of ROS, which are normally synthesized in low-controllable concentrations in every cell compartment and in the apoplast (Foyer and Harbinson 1994). The equilibrium between oxidants and plant antioxidant capacity are strictly maintained and controlled. Different types of environmental stress factors such as water deficiency, low and high temperatures, and osmotic stress may increase the formation of ROS. Disequilibrium in ROS steady-state level has a signaling role, whereas prolonged/severe stress leads to serious shift in redox balance and damage to macromolecules, known as oxidative stress (Mittler et al. 2004). The strongest oxidants are the hydroxyl radicals, formed from $O_2^{\bullet-}$ and H_2O_2 via the Haber–Weiss reaction in the presence of metal ions (Fe^{3+}). They could randomly attack most of the macromolecules and cause significant damage to lipids, proteins and nucleic acids, leading to changes in the structure of cellular components, irreversible disturbances in metabolic functions and ultimately cell death (Noctor and Foyer 1998).

Plant cells cope well with oxidative stress through the joint functioning of various antioxidant enzymes and non-enzymatic components, which modulate ROS levels in different subcellular compartments. Non-enzymatic antioxidants are predominantly low-molecular mass molecules such as glutathione, cysteine, hydroquinone and ascorbate, and lipophilic antioxidants such as α-tocopherol, flavonoids, carotenoid pigments, alkaloids and a variety of other plant components with antioxidant activity (Larson 1988). The ascorbate–glutathione cycle involves both enzymatic (ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase) and non-enzymatic components (ascorbate, glutathione, H_2O_2 and NADPH). The tripeptide glutathione (γ-glutamylcysteinylglycine) has multiple functions in plants in different cell organelles, the greatest concentration being in chloroplasts and cytosol (Noctor and Foyer 1998). Glutathione can reduce H_2O_2, $O_2^{\bullet-}$ and $OH^{\bullet}$ both directly and in a catalytic reaction with glutathione peroxidase. Its major role in plants is its participation in the ascorbate–glutathione cycle (Katerova and Miteva 2010). Normally, plants contain higher quantities of reduced glutathione (GSH) many times exceeding its oxidized form—GSSG. Ascorbate (vitamin C) is one of the major plant antioxidants and can be found in cytosol, different organelles and in the apoplast, the reduced form being up to 90% of the ascorbate pool. Ascorbate can directly reduce superoxide and hydroxyl radicals, singlet oxygen and H_2O_2 or it can act as a substrate of the enzymes from the antioxidant defense system. Ascorbate also participates in α-tocopherol regeneration and in the synthesis of zeaxanthine in the xanthophyll cycle (Smirnoff 2000).

Besides the enzymes of the ascorbate–glutathione cycle which scavenge ROS in cytosol, chloroplasts and mitochondria, the enzymatic antioxidant defense system consists of superoxide dismutases which degrades $O_2^{\bullet-}$ in cytosol, chloroplasts and mitochondria, catalases which effectively remove H_2O_2 in peroxisomes, and various peroxidases, which detoxify H_2O_2 in the cytosol, cell wall and apoplast (Scandalios 1993, 1994). Superoxide dismutases (SOD, E.C.1.15.1.1) are a group of metal-containing enzymes which catalyze the dismutation of superoxide radicals to less harmful O_2 and H_2O_2 in different cell compartments. Depending on the metal in the prosthetic group, SODs are classified as Cu/Zn-, Mn- or Fe-containing enzymes (Tyagi et al. 2017). Catalase (CAT, EC 1.11.1.6; H_2O_2: H_2O_2 oxidoreductase) is a tetramer heme-containing enzyme found in all aerobic organisms, that participates in the fast degradation of H_2O_2. CAT is one of the most active catalysts found in living organisms. At high-substrate concentrations, catalase breaks down H_2O_2 at a very high rate in a reaction where peroxide acts both as an acceptor and a donor of hydrogen (Mhamdi et al. 2012). The joint function of catalase and SOD, which are most effective in removing the $O_2^{\bullet-}$ and in degrading H_2O_2, leads to lower formation of the highly reactive $OH^{\bullet}$. Peroxidases (POX, EC 1.11.1.7) participate in H_2O_2 degradation in plants by converting H_2O_2 to H_2O, coupled with reduction of a substrate. Under lower H_2O_2 concentration ($< 10^{-6}$ M), POXs oxidize various hydrogen donors.

Several studies report upregulation of the major antioxidant enzymes to counteract drought-induced oxidative stress in wheat (Chakraborty and Pradhan 2012; Singh et al. 2012; Mohammadkhani and Sharifi 2016; Tyagi et al. 2017). Monitoring the antioxidative protection at seedling stage is considered a suitable parameter for distinguishing varieties with different drought tolerance with respect to yield (Lascano et al. 2001), while poor capacity of the antioxidant defense system is related to reduced yield potential (Singh et al. 2012).

Having in view the central role of the antioxidative defense in alleviating the negative effects of severe or prolonged drought stress, the present study aimed at studying oxidative stress protective mechanisms in three wheat genotypes with different agronomic characteristics. A drought-sensitive (Farmer) and a drought-tolerant variety (Guinness) have been compared with a new line M181/1338K which shares a common parental genetic background (the highly drought-tolerant variety Katya) with Guinness but it has been obtained by mutagenesis with a higher dosage of gamma irradiation. We hypothesized that (i) the new line will conserve some drought resilience traits from Katya similarly to variety Guinness, (ii) will have oxidative stress response close to that observed in Guinness, and (iii) Guinness and Farmer will differ substantially in the antioxidative stress protection mechanisms as they differ in drought tolerance. Stress severity and extent of recovery were monitored by changes in water status, proline accumulation, oxidative damage to membranes, and content of H_2O_2. Total antioxidant and antiradical activity was registered along with estimation of several low-molecular antioxidants such as phenolics, flavonoids, glutathione and ascorbate pools, and the activity pattern of key antioxidant enzymes such as SOD, CAT and POX.

Materials and methods

Plant material and growth conditions

Two well-established winter wheat (*Triticum aestivum* L.) varieties (Guinness and Farmer) from the selection of Institute of Plant Genetic Resources "Konstatnin Malkov"—Sadovo, South Bulgaria, were compared to one new line (M181/1338K) from the same selection. Plants were grown in pots (9.5-cm diameter, 12-cm deep, 18 plants per pot) on a mixture of leached meadow cinnamonic soil (400 g, pH 6.2, optimally fertilized with N, P and K) and sand in a ratio 3:1, at relative soil humidity 70% of the maximal field capacity (for this mixture, maximal field capacity corresponded to 26.32% w/w soil water content). Optimal soil humidity (70% of maximal soil water capacity) was monitored gravimetrically and was maintained by daily watering. Growth chamber conditions were: day/night temperatures of 25/21 °C, 250 µmol. m^{-2} s^{-1} photosynthetically active radiation at the leaf level, and 16-h photoperiod.

Stress treatment and recovery

Drought stress was imposed on 20-day-old plants (with developed second leaf and emerging third one) by withholding irrigation for 6 days, followed by recovery for 4 days, restoring the optimal water supply. Control plants were watered daily. By the end of drought treatment, soil water content has dropped to 20% of the maximal field capacity, which corresponded to severe stress conditions. Biochemical analyses were performed on samples derived from the second leaf of stressed/recovered and control plants, as fresh material or quick-frozen in liquid nitrogen and stored at − 70 °C until analyses.

Water status

Relative water content (RWC) of 2–3 s leaves was estimated using the formula $RWC\% = \left[(FW - DW)/(TW - DW)\right] \times 100$, where FW is the fresh weight, TW is the weight of the same leaf material at full turgidity (after floating one night at cold in 20-ml

distilled water) and DW is the measure of the same leaf material after drying 8 h at 105 °C to constant weight.

Proline content

Fresh leaf material (approximately 300 mg) was homogenized with 0.1% (w/v) trichloroacetic acid for determination of proline, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content. Free proline was derivatized with acid ninhydrin and absorbance was read at 520 nm according to Bates et al. (1973).

Malondialdehyde (MDA) content was determined as thiobarbituric acid reagent product according to Kramer et al. (1991) using the extinction coefficient 155 mM^{-1} cm^{-1}.

Hydrogen peroxide content was estimated spectrophotometrically according to Alexieva et al. (2001). The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H_2O_2.

Total antioxidant activity

The FRAP (Ferric reducing antioxidant power assay) procedure described by Benzie and Strain (1999) was followed for measuring the total antioxidant activity in leaf extract prepared by grinding plant material (1 g) in ice bath with 10 ml 80% ethanol (v/v) and immediately centrifuging the homogenate at 10,000 g for 20 min at 4 °C. The principle of this method is based on the reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants. Aliquots of 100-µL sample from the decanted clear supernatant were mixed with 3-mL FRAP reagent and, after incubation at 37 °C for 10 min, the absorbance of the reaction mixture was measured spectrophotometrically at 593 nm. The values were expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mmol/L $FeSO_4$.

Antiradical activity

Free radical-scavenging activity was estimated spectrophotometrically according to Brand-Williams et al. (1995) using DPPH• (1,1-diphenyl-2-picrylhydrazyl) radical. The percent inhibition of the DPPH• (I%) was calculated by the following equation: $I\% = \left[\left(A_{blank} - A_{sample}\right)/A_{blank}\right] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the extract), and A_{sample} is the absorbance of the extract with reagents.

Total phenolics and flavonoids

Total phenolics content was determined in leaf extract in 0.1% (w/v) trichloroacetic acid, as described for proline determination, with Folin–Ciocalteu reagent supplemented with sodium carbonate and absorbance was read at 725 nm according to the method of Swain and Goldstein (1964). Gallic acid was used as a reference standard. *Total flavonoid* content was measured by the aluminum chloride assay (Zhishen et al. 1999) at 510 nm, using a standard curve of catechin, and expressed as mg catechin equivalents (CE). g^{-1} fresh mass.

GSH and ASC quantification

The state of redox buffers ascorbate and glutathione was analysed as described by Zaharieva and Abadía (2003) starting from the same extract of 0.5 g FW sample in 3 ml 2% w/v metaphosphoric acid. The ascorbate content (total and reduced) was assayed by reduction of Fe^{3+} to Fe^{2+} by ascorbate in acidic solution and complexation of Fe^{2+} with α,α'-dipyridyl, giving a pink-colored product with absorbance at 525 nm. Ascorbate content was quantified using a standard curve. Oxidized ascorbate was estimated from the difference between total and reduced ascorbate. Glutathione content was assayed with dithiobisdinitrobensoic acid (DTNB) and glutathione reductase. Absorbance at 412 nm was read and the total glutathione content was estimated using a standard curve. Oxidized glutathione was estimated by derivatization with divinylpiridine.

ROS-scavenging enzymes

All steps in the extraction were performed at 4 °C. The leaf material was homogenized with 100 mM Tris–HCl buffer pH 7.6 supplemented with 2 mM EDTA, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$, 0.005% v/v Triton X 100, 1 mM phenylmethylsulphonylfluoride, and 2% w/v Polyclar AT (1:5 w/v). After centrifugation at 12,000 g for 30 min, protein content in the supernatant was determined according to Bradford (1976) with bovine serum albumin as a standard. The enzymes in the crude extract were separated by non-denaturing electrophoresis in polyacrylamide gel at 4 °C.

Isoenzymes of SOD were resolved in 10% gel, and SOD activity was developed with nitroblue tetrazolium. Specific isoforms were located by inhibition with H_2O_2 and KCN as previously described (Simova-Stoilova et al. 2009).

CAT and POX isoenzymes were separated on 7.5% polyacrylamide gel. CAT activity was visualized with ferricyanide–ferrichloride according to Woodbury et al. (1971). POX activity was visualized with benzidine (Ornstein 1964). Equal protein load per lane was applied in the gels.

Statistics and figures

All experiments were repeated three times with three replicates each. Data are expressed as mean values ± SD. The

significance of differences was analyzed using XLSTAT Version 2014.5.03, applying Duncan's multiple range test at level of significance of 0.05. SigmaPlot version 10 was used for graphics. Areas of enzyme activity bands were calculated using ImageJ and composite image of gels was built applying IrfanView 64 program.

Results

The response of one new mutant line of winter wheat—M181/1338K—to strong but reversible drought stress was compared to the one observed in a drought-tolerant variety (Guinness) and a sensitive variety (Farmer). The applied stress induced substantial changes in the water status of second leaves—diminution in RWC by about 50%, accompanied with considerable proline accumulation which is indicative for stress severity in all tested genotypes (Table 1). After resuming water supply, water status of the recovered plants reached the one of the age controls. Proline declined approximately to the control levels in Guinness and in the mutant line M181/1338K. In the sensitive variety Farmer, which manifested the highest proline content under drought, the levels of this compatible solute remained distinctly increased upon recovery (Table 1). Changes in the water status were similar in the three genotypes. However, basal proline levels in Guinness and in the mutant M181/1338K controls were higher than in the one detected in the sensitive variety Farmer.

Table 1 Leaf water status and proline accumulation in age controls, drought-stressed and recovered winter wheat seedling varieties Guinness and Farmer and line M181/1338K

Variant/treatment	Leaf RWC [%]	Proline [μmol.g^{-1} FW]
Guinness		
CD	97.1 ± 0.7^{a}	0.584 ± 0.300^{f}
D	44.9 ± 6.7^{c}	39.764 ± 1.095^{b}
CR	97.0 ± 1.2^{a}	0.520 ± 0.084^{g}
R	86.3 ± 0.7^{b}	0.924 ± 0.084^{d}
M181/1338K		
CD	97.8 ± 3.1^{a}	0.462 ± 0.060^{f}
D	45.5 ± 4.1^{c}	37.492 ± 0.500^{b}
CR	98.5 ± 1.2^{a}	0.526 ± 0.230^{ef}
R	84.1 ± 6.9^{b}	1.272 ± 0.309^{d}
Farmer		
CD	94.9 ± 0.8^{a}	0.253 ± 0.025^{g}
D	46.5 ± 3.8^{c}	53.653 ± 2.100^{a}
CR	96.6 ± 0.9^{a}	0.748 ± 0.080^{e}
R	85.2 ± 0.7^{b}	6.582 ± 0.980^{c}

Values are given as means ± standard deviation from three replicates

CD age control of drought, *D* drought, *CR* age control of recovery, *R* recovery, *FW* fresh weight, *DW* dry weight, *RWC* relative water content

Different letters after values indicate statistically significant differences at $p < 0.05$

The analyses of oxidative damage and antioxidant status were performed in samples derived from the second fully developed leaf of control, stressed and recovering plants. Hydrogen peroxide accumulation is indicative for shifted redox balance and increased oxidative strain, whereas MDA reflects the degree of membrane lipid peroxidation as a result of oxidative damage (Fig. 1). Water deprivation provoked a strong increase in H_2O_2 content in all variants (3, 2.5, and 3.2 times in Guinness, M181/1338K and Farmer, respectively). The measured hydrogen peroxide content during recovery stage was close to the control ones in all tested varieties. The observed pattern of H_2O_2 changes is indicative for similar shift in ROS equilibrium levels toward oxidative stress in all studied genotypes. MDA basal level in control Guinness and M181/1338K plants was higher than the one measured in Farmer. Under drought stress, MDA

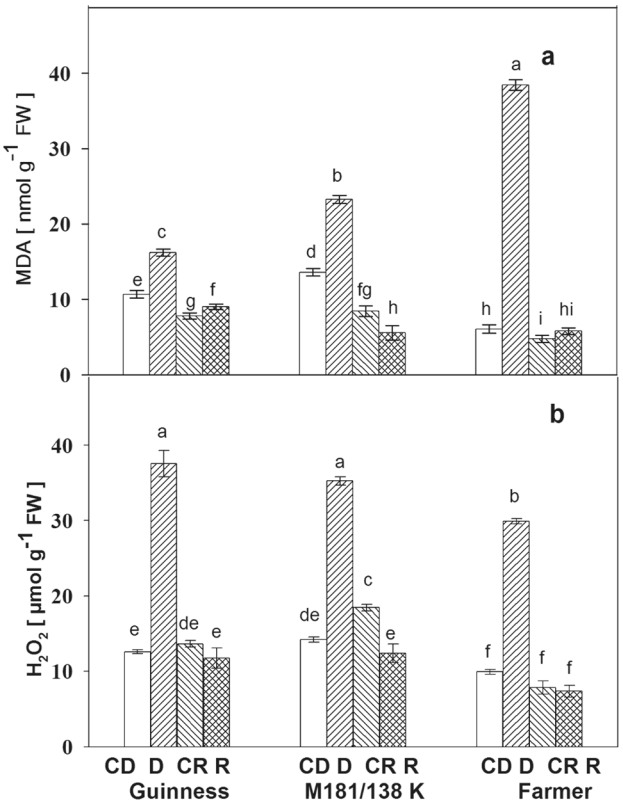

Fig. 1 Malondialdehyde (**a**) and hydrogen peroxide (**b**) content in the second fully developed leaves of age controls, drought-treated and recovered wheat plants from varieties Guinness and Farmer, and line M181/1338K. *CD* age control of drought-stressed plants, *D* drought-stressed plants, *CR* age control of recovered plants, *R* recovered plants. Values are given as means ± standard error from three replicates. Different letters above the columns represent statistically significant differences between mean values at $p < 0.05$ level

accumulation generally increased by 63% in Guinness, 76% in M181/1338K and up to 533% in Farmer. This indicates that the sensitive variety Farmer experienced the most severe oxidative damage while the tolerant variety Guinness managed to sustain relatively lower MDA content. Membrane damage documented in M181/1338K samples, although a bit higher resembled the one detected in the drought-tolerant variety Guinness. After re-watering, MDA levels diminished and were close to control values in Guinness and in Farmer, whereas in M181/1338K, they were even below than the one measured in the respective recovery control.

The shift towards oxidative stress was counteracted by mobilization of the antioxidant system, which was monitored by changes in total antiradical activity (AR) and antioxidant activity (AO). These parameters reflect mainly the participation of different low-molecular antioxidants such as ascorbic acid, phenolics, flavonoids and others, in total. Drought provoked increased antiradical activity in Guinness by 172% above that of the control plants (Fig. 2). Recovered Guinness plants had nearly 50% lower AR activity compared to respective age controls, so that this variety manifested the maximal amplitude of changes in this parameter. In Farmer, water stress resulted in 3.7 times higher AR activity which upon recover remained two times higher than the controls. Drought caused only 51% increase in AR activity in M181/1338K, which was the variety with the highest basal levels of this parameter. The recovered M181/1338K plants had lower AR activity than the respective age controls. The documented AO increase in drought-stressed Guinness and Farmer plants was nearly three times higher than the corresponding controls, while in M181/1338K line it was only 72% higher compared to the control (Fig. 2b). Recovering plants had AO activity close to the control levels. AR and AO changes in the three tested genotypes reflect general mobilization in ROS protective mechanisms. However, some genotype-linked differences were also observed. M181/1338K had higher basal level of antioxidant and antiradical activities but moderate increase in these parameters under stress. Contrary to Guinness and M181/1338K, the variety Farmer preserved relatively higher AR activity at recovery stage.

Secondary metabolites such as plant phenolic compounds have multiple functions in plants, including ROS scavenging. A three- to fourfold increase in total phenol content was observed under drought in M181/1338K, Farmer and Guinness (Fig. 3). Diminution of the phenolics content was observed upon recovery from water stress reaching the control levels. Flavonoids are one of the major classes of plat phenols that participate in plant protection. Drought induced nearly sevenfold increase of flavonoid content in Guinness and about fourfold increase in Farmer, while the increase in line M181/1338K was only about 62% (Fig. 3a). Flavonoids content in recovering plants was comparable to the control

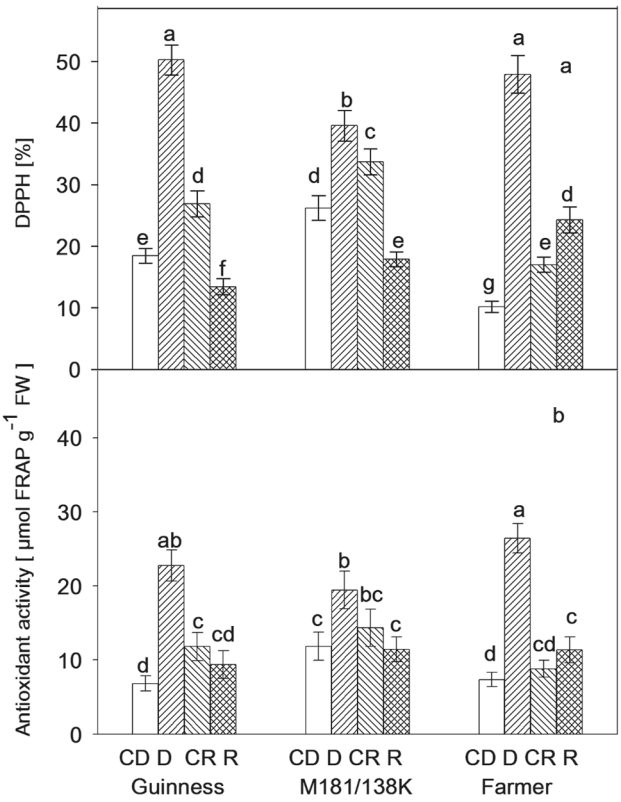

Fig. 2 Total antiradical (**a**) and antioxidant (**b**) activities in the second fully developed leaves of age controls, drought-treated and recovered wheat plants from varieties Guinness and Farmer, and line M181/1338K. *CD* age control of drought stressed, *D* drought-stressed plants, *CR* age control of recovered plants, *R* recovered plants. Values are given as means ± standard error from three replicates. Different letters above the columns represent statistically significant differences between mean values at $p < 0.05$ level

levels in all studied varieties. Guinness accumulated more flavonoids under stress than the other genotypes.

Substantial rise in ascorbate and glutathione content, both in the reduced and in the oxidized form, was observed in all drought-stressed plants from the tested wheat genotypes (Fig. 4). Upon recovery, the amount of ascorbate measured in the recovered-from-stress Farmer samples remained higher than the controls with a detected increase in the proportion of oxidized ASC. Significant accumulation of glutathione under drought was observed in all studied variants—threefold in Guinness, 3.5-fold in M181/1338K and fivefold in Farmer. During recovery from stress glutathione content dropped to control levels in M181/1338K line, while in the other two genotypes, it remained somewhat higher.

Four isoforms of SOD were visualized in the leaves of wheat plants from the three examined genotypes (Fig. 5). The highest activity (90–97% of the total) was observed for Cu/Zn SOD isoenzymes. Control plants from M181/1338K had higher leaf total SOD activity in comparison with Guinness and Farmer. Under drought, an increase in total SOD

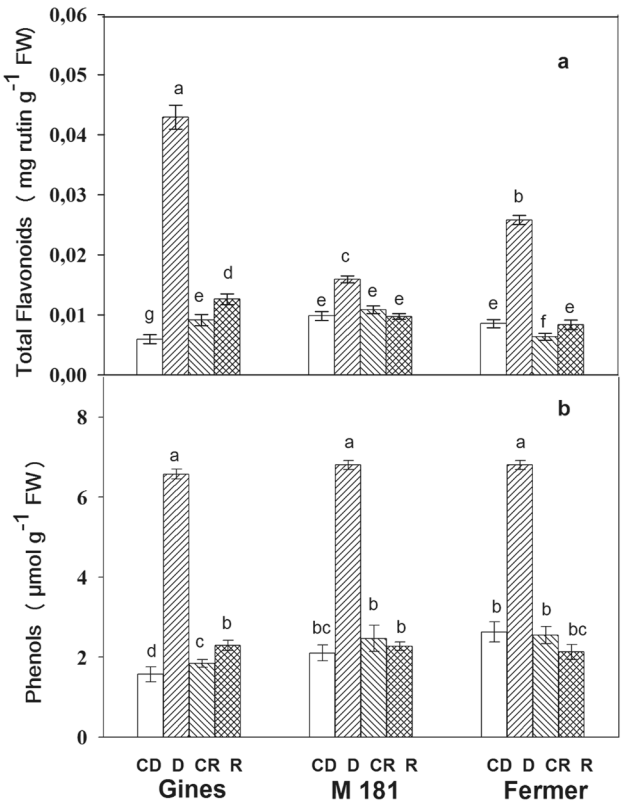

Fig. 3 Total flavonoid (**a**) and phenolic (**b**) content in second leaves of the age controls, drought-treated and recovered wheat plants from varieties Guinness and Farmer, and line M181/1338K. *CD* age control of drought-stressed plants, *D* drought-stressed plants, *CR* age control of recovered plants, *R* recovered plants. Values are given as means ± standard error from three replicates. Different letters above the columns represent statistically significant differences between mean values at $p < 0.05$ level

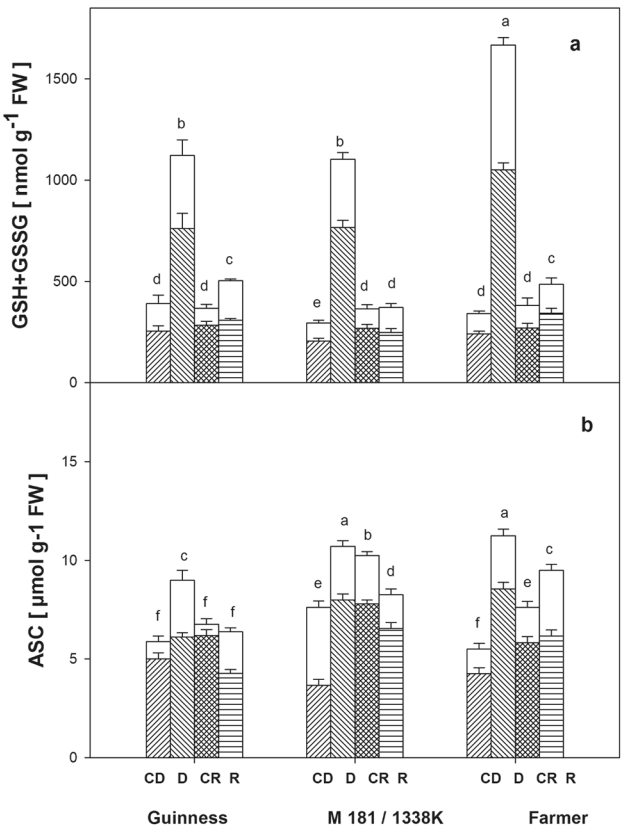

Fig. 4 Glutathione content in its reduced (GSH) and oxidized (GSSG) form (**a**), and ascorbate in its reduced and oxidase form (**b**) in the second leaves of age controls, drought-treated and recovered wheat plants from varieties Guinness and Farmer, and line M181/1338K. Hollow bars depict oxidized form of glutathione and ascorbate, hatched bar represents reduced form of glutathione and ascorbate. *CD* age control of drought-stressed plants, *D* drought-stressed plant, *CR* age control of recovered plants, *R* recovered plants. Values are given as means ± standard error from three replicates. Different letters above the columns represent statistically significant differences between mean values at $p < 0.05$ level

isoenzyme activity was observed in the three variants—nearly 200% in Guinness, with 44% in M181/1338K and 59% in Farmer. Two activity staining areas appeared below each of Cu/Zn SOD bands in samples derived from water-deprived Farmer plants, which could be due to some modification of the main forms. All SOD isoforms (Mn, Fe and Cu/Zn containing ones) were with increased activities in drought-stressed samples. The rise in Fe SOD activity was the most pronounced and persisted as such at recovery stage as well (Table S1 presents the relative volumes of individual peaks and ratios "drought/control of drought D/CD" and "recovery/ control of recovery R/CR"). Relatively higher SOD activity was observed in the leaves of Guinness and Farmer plants recovering from stress, while the measured total SOD activity in M181/1338K dropped close to the control levels.

One CAT activity band was revealed by the in-gel staining assay (Fig. 5). In M181/1338K control, a significantly higher catalase activity was detected compared to Guinness and Farmer samples. Drought provoked increase in catalase activity in the leaves of all variants, by 57%, 53%, and 59% for Guinness, M181/1338K and Farmer, respectively. In plants recovering from stress, CAT activity remained high in Guinness. The detected CAT activity in samples derived from recovered plants was close to the control levels in M181/1338K, and lower with respect to the control in Farmer.

Seven specific bands of POX were detected by in-gel activity staining with co-substrate benzidine (Fig. 5). The isoforms with middle mobility (POX4, POX5) manifested higher intensity. The two fast moving bands (POX6, POX7) exhibited lower signal. Overall, line M181/1338K presented the highest total intensity of POX bands. Increased POX4 and POX 5 activity in drought-stressed Guinness plants was documented. The stress treatment resulted in higher POX1

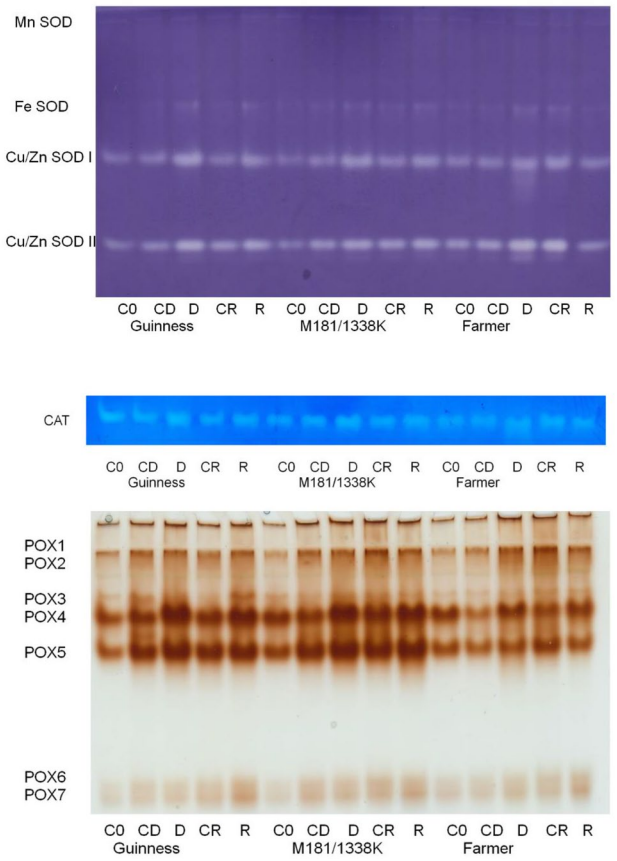

Fig. 5 Isoenzyme activity patterns of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) in extracts from second leaves of age controls, drought-treated and recovered wheat plants from varieties Guinness and Farmer, and line M181/1338K. *C0* control at the beginning of the treatment, *CD* age control of drought-stressed plants, *D* drought-stressed plants, *CR* age control of recovered plants, and *R* recovered plants. Equal protein load was applied – 30 µg per lane for SOD, 40 µg—for CAT and 20 µg—for POX, respectively

and POX4 signals in M181/1338K, and POX1 and POX2 in Farmer. In recovering plants, an enhanced activity of POX7 was registered in all tested genotypes, with the highest increase documented in Guinness. For some of the bands in Farmer POX profile, a decrease in activity was observed at recovery, compared to the respective age control.

Discussion

Abiotic stresses, including drought, are usually accompanied by secondary oxidative stress. Direct relation has been found between efficient protection against oxidative stress and wheat tolerance to dehydration stress (Chakraborty and Pradhan 2012; Singh et al. 2012). The poor antioxidative defense system capacity of drought-sensitive wheat genotypes contributes to the reduced yield under water deficit (Lascano et al. 2001; Simova-Stoilova et al. 2009; Singh et al. 2012). The ability to maintain physiological functions under drought conditions and to rapidly recover after rewatering during vegetative period is considered an important prerequisite for wheat final productivity (Abid et al. 2018). A synergism has been observed between modulation of primary metabolism and mobilization of the antioxidant system in protection against dehydration (Nemati et al. 2019). Significant positive correlation has been found between enzymatic and non-enzymatic ROS protection (Singh et al. 2012; Mohammadkhani and Sharifi 2016). The mobilization of the antioxidant system depends on drought stress severity and prolongation. It has been established that mild water stress rarely induced increase in activities of ROS-scavenging enzymes, contrary to severe drought; in the latter case, a difference in antioxidant enzyme response between tolerant and sensitive wheat varieties was manifested (Hameed et al. 2011). Therefore, the three genotypes in our study were subjected to severe but recoverable drought stress to reveal differences in ROS protective mechanisms in relation to drought tolerance/sensitivity.

The primary response of wheat plants to water deficiency is osmotic regulation, which is achieved by accumulation of compatible solutes such as proline. In addition to its role as osmotic regulator, proline plays the role of a chaperone, redox buffer and ROS-scavenger protecting membranes and proteins during drought stress (Verbrugge and Hermans 2008). In our study, the lowest basal level and the highest increase in proline content was detected in the drought-sensitive genotype, while proline values in Guinness and the mutant line changed in the same manner without statistically significant differences between them.

The increase in the steady-state level of H_2O_2 is indicative for a shift in the cell redox balance towards oxidative stress (Mittler et al. 2004). According to the obtained results on H_2O_2 accumulation in the present study, all three genotypes developed secondary oxidative stress to a comparable extent under severe drought stress, with complete restauration of H_2O_2 levels at recovery (Fig. 1).

The negative effect of ROS on membranes is manifested as an increased MDA content, resulting in lower membrane stability, as MDA could react with free amino groups of proteins and phospholipid components (Kocheva et al. 2014). Higher MDA content has been reported for drought-sensitive wheat varieties (Singh et al. 2012; Mohammadkhani and Sharifi 2016; Nemati et al. 2019). In our study, the highest increase in lipid peroxidation was observed in the sensitive variety Farmer and the lowest in the tolerant variety Guinness, and lower values of oxidative damage to membranes were also documented in the mutant line M181/1338K (Fig. 1). The negative effect of ROS was counteracted by increased total AO and AR activities (Fig. 2). These parameters reflect, besides

classical antioxidants such as ascorbate, also secondary metabolites with potent antioxidant activity. Phenolics are aromatic compounds with benzene rings and one or more hydroxyl groups, which production by plants is increased under abiotic stress, including drought (Gregorová et al. 2015; Varela et al. 2016). These compounds play an important role in plant development, in biosynthesis of pigments and lignin, in plant protection (pathogen defense, ultraviolet screening, anti-herbivory, and cell wall strengthening), as well as in ROS scavenging (Varela et al. 2016). A close connection has been observed between the antioxidant activity and the presence of phenolic compounds in vegetables and fruits (Fu et al. 2011). The positive linear correlation between antioxidant properties and total phenol and flavonoid content suggests that phenolic compounds are the main components contributing to the antioxidant activities in a number of medicinal plants (Spiridon et al. 2011). A previous work reported that the total phenolic and flavonoid content in wheat leaves were enhanced during drought, with higher accumulation found in the tolerant wheat variety (Ma et al. 2014). In our study, the tolerant variety Guinness presented the highest accumulation of flavonoids under drought stress, but line M181/1338 K had the lowest flavonoid content, which could be due to mutagenesis effect (Fig. 3). The role of plant phenolic compounds in drought stress deserves further elucidation.

Ascorbic acid is a key component in total AO and AR activity; its capacity to act as a donor of electrons and hydrogen ions in numerous enzymatic and non-enzymatic reactions make it an important component of the antioxidant defense system (Smirnoff 2000). In plant metabolism, glutathione has two main functions: in sulfur exchange and as endogenous defense constituent against abiotic and biotic stresses (Noctor and Foyer 1998). The ratio between reduced and oxidized forms of these redox buffers contributes to the maintenance of cell redox potential. Ascorbate and glutathione are constituents of the ascorbate–glutathione cycle—a major ROS detoxification system in cytosol, chloroplasts and other organelles (Hasanuzzaman et al. 2019). In our study, we observed prominent response of both redox buffers in wheat leaves under drought stress, with rise in total pools and a transient increase in the oxidized form (Fig. 4). Similar mobilization of ascorbate and glutathione under drought was reported in other studies on wheat varieties (Lascano et al. 2001; Mohammadkhani and Sharifi 2016). Dalmia and Sawhney (2004) reported a decrease in the level of glutathione and ascorbate with increasing magnitude of water stress. Diminished ASC and GSH pools were also observed by Simova-Stoilova et al. (2008) in other wheat varieties under severe drought at an earlier developmental stage, which was not detected in the present study. Probably different antioxidants could compensate and partly substitute for each other, such as the secondary metabolites and ROS enzymes, depending on plant habitus and ambient conditions.

Studies in dynamics revealed that peroxidase activity increased gradually with water stress prolongation, whereas SOD and CAT activities initially increased, then diminished in sensitive varieties (Chakraborty and Pradhan 2012). According to Nemati et al. (2019), high level of Cu/Zn SOD isoforms might improve stress resistance. Higher levels of SOD, CAT and POX were observed in the leaves of tolerant wheat varieties under field drought (Singh et al. 2012; Mohammadkhani and Sharifi 2016). It is considered that the role of H_2O_2-scavenging enzymes (such as CAT, POX) is particularly important under drought (Hameed et al. 2011). In our study, a strong response of all studied ROS enzymes to severe drought stress was documented, particularly the ones of Fe SOD, Cu/Zn SOD, CAT and some isoforms of POX. Increased activity POX7 isoform was evident at recovery stage of the experiment (Fig. 5). The mutant line M181/1338K had the highest intensity of POX bands, even stronger compared to Guinness. We suggest that this interesting characteristic could be due to a mutagenesis effect since both varieties share the same parental genetic background.

Comparing Guinness and M181/1338K, with the sensitive variety Farmer differences were observed mainly at recovery stage. Farmer maintained relatively higher MDA and proline content, higher AO/AR activities and ASC content, whereas the measured ROS enzymes' activities tended to be diminished. The comparatively slower recovery of this variety is one of the factors that contribute to its lower drought tolerance. The tolerant variety Guinness was characterized with high flavonoid content under severe drought, whereas line M181/1338K had higher POX activities.

Conclusion

This study confirmed the utility of using oxidative stress markers as a tool for estimating the effects of induced mutagenesis in winter wheat. The comparative analyses show that all three genotypes responded readily to severe drought stress by efficiently mobilizing the enzymatic and low-molecular antioxidative protection, with some (although not substantial) differences between the sensitive and the tolerant variety. The drought-sensitive variety Farmer responded to severe water stress with the highest increase in oxidative damage to membranes, proline accumulation, and glutathione content; it was characterized with a delayed normalization of the studied parameters back to the control levels upon recovery. The drought-tolerant variety Guinness responded to severe drought by better control over oxidative membrane damage, maintaining the highest accumulation of flavonoids under drought. As expected, the new mutant

line M181/1338K presented similarities with Guinness in its response to severe water stress, such as the same proline and glutathione level. Differently to Guinness, M181/1338K experienced higher oxidative damage to membranes and accumulated lower flavonoid content under drought, along with higher POX activities. We presume that these could be secondary effects of the induced mutagenesis which require further detailed studies.

Author contribution statement E.K. conducted the experiments, performed analyses on proline, H_2O_2, MDA, AR, AO, phenol and flavonoid content, discussed the results and wrote the paper draft. D.P. performed SOD, CAT and POX analyses and discussed the results. L.S. performed water status, ASC and GSH analyses, discussed the results and worked on the paper draft. All the authors approved the final manuscript.

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