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Research Article

Three wheat cultivars' responses to NaCl treatments: changes in lipid peroxidation, cell viability, hydrogen peroxide content and antioxidant defence parameters

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Abstract

The responses of lipid peroxidation, antioxidant defence parameters, the content of hydrogen peroxide (H_2O_2) and cell viability were investigated in callus cultures of *Triticum aestivum* L. (wheat) 'Tekirdağ', 'Pehlivan' and 'Flamura-85' cultivars under various NaCl concentrations (0, 0.05, 0.1 and 0.15 M). According to two-way ANOVA, the interaction between NaCl treatments and cultivars on the antioxidant enzyme activity and malondialdehyde (MDA), proline and H_2O_2 content were statistically significant. Statistically significant differences between cultivars were also detected using two-way ANOVA analyses of antioxidant enzyme activities, MDA and H_2O_2 content. The activity of antioxidant enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) increased under salt treatments, except for catalase activity in Tekirdağ. Proline and oxidized glutathione (GSSG) content increased significantly with NaCl concentrations whereas the reduced glutathione (GSH) increase was not statistically significant. GSH/GSSG ratios decreased under salt treatments in all three wheat cultivars as compared to the control. MDA and H_2O_2 content increased, but the viabilities of callus cultures decreased as salt concentrations increased. Based on the cellular responses of three different wheat calli against salinity, Flamure-85 was the best one because of its superior antioxidant defence system.

Keywords: Antioxidant defence systems, NaCl, plant tissue culture, oxidative stress, wheat.
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Üç buğday çeşidinin NaCl uygulamasına cevapları: Lipit peroksidasyonunda, hücre canlılığında, hidrojen peroksit içeriğinde ve antioksidan savunma parametrelerinde değişiklikler

Özet

Tekirdağ, Pehlivan ve Flamura-85 buğday çeşitlerinin kallus kültürlerinde lipid peroksidasyonunun, antioksidan savunma parametrelerinin, hidrojen peroksit (H_2O_2) ve hücre canlılığı içeriğinin cevapları farklı NaCl (0, 0.05, 0.1 ve 0.15 M) konsantrasyonları altında incelenmiştir. İki yönlü ANOVA analizine göre NaCl uygulamaları ile buğday çeşitleri arasındaki etkileşimin antioksidan enzim aktiviteleri, malondialdehit (MDA), prolin ve H_2O_2 içeriği üzerine etkileri istatistiksel olarak anlamlı bulunmuştur. Ayrıca, antioksidan enzim aktiviteleri, MDA ve H_2O_2 içeriğine iki-yönlü ANOVA analizi uygulanarak çeşitler arasında istatistiksel anlamlı farklar belirlenmiştir. Tekirdağ çeşidinde katalaz aktivitesi hariç, antioksidan enzim aktiviteleri örneğin; süperoksit dismutaz (SOD), guaiakol peroksidaz (POX), katalaz (CAT), askorbat peroksidaz (APX) ve glutatyon redüktaz (GR) tuz uygulamasına bağlı olarak

artmıştır. Prolin ve oksitlenmiş glutatyon (GSSG) içeriği NaCl konsantrasyonları ile önemli ölçüde artış göstermesine rağmen, indirgenmiş glutatyon (GSH) artış istatistiksel olarak anlamlı değildir. Her üç buğday çeşidinde de kontrole göre GSH / GSSG oranları tuz uygulamasına bağlı olarak düşmüştür. MDA ve H_2O_2 içeriği artmış ayrıca tuz konsantrasyonu arttıkça kallus kültürlerinin canlılıkları azalmıştır. Üç farklı buğday kallusunun tuzluluğa karşı gösterdikleri hücresel yanıtına bağlı olarak Flamura-85; diğerlerine göre daha üstün antioksidan savunma sistemi performansına sahip olması yüzünden en iyi genotip olarak tespit edilmiştir.

Anahtar Kelimeler: Antioksidan savunma sistemleri, NaCl, bitki doku kültürü, oksidatif stres, buğday.

Introduction

Excessive soluble salts in the soil are harmful to most cultivated plants. They affect the physiological, biochemical and molecular pathways in plants and consequently, lead to the reduction in agricultural productivity and quality worldwide (Gandonou et al. 2006). More than 45 million hectares of irrigated land which account for 20% of total land have been damaged by salt worldwide, and 1.5 M ha are taken out of production each year due to high salinity levels in the soil (Hasanuzzaman et al. 2013). As for in Turkey, a total of over 2.7 million ha land is affected by salinity and sodicity (Sönmez and Beyazgül 2015). Salt stress occurs naturally, but a significant proportion of recently cultivated agricultural land has become saline due to human-induced processes. Irrigation causes dissolved salts to accumulate in soil water and inhibit plant growth (Hasanuzzaman et al. 2013). Besides these developmental and agronomic problems, salt stress also induces oxidative stress in plants (Lokhande et al. 2011). Oxidative stress is considered to be unbalanced between pro-oxidant and anti-oxidant molecules in the cell (Mittler 2002; Gill and Tuteja 2010). It results in the overproduction of reactive oxygen and/or nitrogen species (ROS and/or RNS). These molecules damage macromolecules, including cell membranes, photosynthetic pigments, protein, nucleic acids and lipids. Therefore, keeping control over them is important to protect plants from the damage they do. Plant cells have developed complex mechanisms to prevent overproduction of ROS or limit their damaging effects with enzymatic and non-enzymatic antioxidant defence systems, which detoxify ROS at the proper site and time. Superoxide dismutase (SOD, EC 1.15.1.1) is a key scavenger of superoxide, whose enzymatic action forms H_2O_2, which is then scavenged by catalase (CAT, EC 1. 11.1.6) and guaiacol peroxidase (POX, EC 1.11.1.7). Glutathione reductase (GR, EC 1.8.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11) are the keys to the detoxification process of H_2O_2. Plant cells also accumulate non-enzymatic antioxidants including reduced glutathione (GSH) and proline to scavenge ROS (Mittler 2002; Gill and Tuteja 2010). Malondialdehyde (MDA) is a decomposition product of polyunsaturated fatty acids. Thus, it is widely used as an indicator of the rate of oxidative processes (Davey et al. 2005).

Tissue culture techniques have great potential for studying the physiological, biochemical and molecular mechanisms that operate at the cellular level in response to stressors. Therefore, these techniques are frequently used to investigate plants' cellular response to salinity (Lokhande et al. 2011).

Eliminating salinization worldwide is not realistic. Although temporary solutions have reduced the negative impact of salinity on plants (Khan et al. 2014), implementing them is not always possible. Thus, the most effective solution is to determine salt-tolerant genotypes among the population. Biochemical parameters are frequently used for screening plant genotypes for salinity response, such as antioxidant enzyme activity or osmoprotectant molecule levels, (Patada et al. 2008; Nikam et al. 2014). In this study, this was done by investigating the responses of three wheat cultivars to NaCl treatments on antioxidant defence parameters (activities of SOD, POX,

CAT, APX and GR and contents of GSH, GSSG and Proline), cell viability, H_2O_2 and MDA content in wheat callus.

Materials and methods

Plant materials and culture conditions

Three widely-cultivated wheat cvs, Tekirdağ, Pehlivan and Flamura-859, were obtained from the Thrace Region of Turkey, provided by the Trakya Agricultural Research Institute in Edirne, Turkey.

For the induction of callus from mature wheat embryos, seed surfaces were sterilized according to Ozgen et al. (1998) and imbibed in sterile water for 2h at 35°C. Afterwards, mature embryos were removed with the help of sterile lancets and forceps under aseptic conditions. Then, these explants were inoculated in petri dishes containing callus induction medium, whose pH was pre-adjusted to 5.8 with mineral salts of MS (Murashige and Skoog, 1962), 2 mgl^{-1} 2,4-dichlorophenoxyacetic acid, 20 gl^{-1} sucrose, 0.8% (w/v) agar and different concentrations of NaCl (0, 0.05, 0.1 and 0.15 M). Then, these cultures were incubated in a growth chamber for a 16h light / 8h dark photoperiod with an irradiance of 500 µmol $m^{-2}s^{-1}$ photon flux density, at 26°C for 28 days.

Enzyme extraction and assay

To measure antioxidant enzymes, homogenates were obtained from 100 mg of frozen callus tissue using a mortar and pestle with a 1 ml extraction buffer containing 100 mM phosphate buffer (pH 7.0), 1% PVP40 (w/v) and 0.1 mM Na-EDTA. They were centrifuged at 15,000 rpm for 25 min, and the supernatant fraction was used to prepare the enzyme assays. All of the procedures in the preparation of enzyme extracts were performed at 4°C. Protein content was determined using Bradford's (1976) method. Superoxide dismutase was assayed by monitoring the superoxide radical-induced NBT reduction at 560 nm (Dhinsa et al. 1981). One unit of SOD activity was defined as the amount of enzyme, which causes a 50% inhibition of the photochemical reduction of nitro blue tetrazolium chloride. Guaiacol peroxidase activity was measured at 470 nm by using H_2O_2 and guaiacol as substrates (Panda et al. 2003). Catalase activity was determined by monitoring the disappearance of H_2O_2 at 240 nm according to Aebi (1984) method. Ascorbate peroxidase activity was determined by measuring the consumption of ascorbate by tracking absorbance at 290 nm (Nakano and Asada 1981). Glutathione reductase activity was determined by measuring the enzyme-dependent oxidation of NADPH by the following absorbance at 340 nm (Foyer and Haliwell 1976).

MDA content

Lipid peroxidation was measured as the amount of MDA as determined by the thiobarbituric acid (TBA) reaction (Heath and Packer 1968). Briefly, 100 mg callus were homogenized in 1 ml 0.1 % (w/v) TCA, and then, the homogenate was centrifuged at 10000 g for 5 min. Collected supernatant was mixed with 1 ml for 0.5 % TBA diluted in 20% TCA. The mixture was heated in 100ºC for 1 h, and then cooled down to room temperature and centrifuged at 10000 g for 5 min. The optical density value of the supernatant was measured at 532 and 600 nm. The MDA value was calculated according to molar extinction coefficient of the MDA (156 mM^{-1} cm^{-1}).

Proline content

Proline was determined by the ninhydrin method (Bates et al. 1973). Approximately 100 mg of fresh or frozen plant material was homogenized in 2 ml of 3% aqueous sulfosalicyclic acid and filtered through Whatman's No. 2 filter paper. One ml of filtrate was mixed with 1 ml acid-ninhydrin and 1 ml of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100°C. The reaction mixture was extracted with 2 ml toluene and the chromophore containing toluene was aspirated, cooled to room temperature, and the absorbance was measured at 520 nm. The amount of proline was determined from a standard curve in the range of 20 - 100 µg.

Cell Viability Assay

To determine the reduction of viability in callus cultures under salt stress, we used the 2,3,5-Triphenyltetrazolium Chloride (TTC) method (Steponkus and Lanphear

1967). 100 mg callus were placed in glass vials containing 8 ml of 0.6% TTC solution in 0.05 M potassium phosphate buffer, pH 7.4 at overnight in the dark. Then, the callus was macerated in 95% ethanol in a boiling water bath for 30 min, and the formazan concentration of the extract was determined by measuring the optical density at 530 nm.

H_2O_2 content

The spectrophotometric assay of H_2O_2 was done as described by Holland and Story (1981). A total of 0.5 mL of homogenate was added to the tubes containing 1.5 mM ferricytochrome. The formation of H_2O_2 in the mixture was measured at 550 nm by estimating the oxidation product of ferrocytochrome.

GSH and GSSG contents

Total and oxidized glutathione contents were measured using the methods of Tietze (1969) and Griffith (1980) with minor modifications. Briefly, 100 mg callus were ground with a mortar and pestle in the presence of 1 ml of 5% (w/v) meta-phosphoric acid. Homogenates were centrifuged at 10,000 × *g* for 20 min at 4°C. Glutathione (as GSH + GSSG) was measured using 1 ml of assay mixture containing 100 mM sodium phosphate buffer (pH 7.5) plus 6.3 mM EDTA, 300 µM NADPH, 6 mM DTNB, 25 µl of homogenate and 0.5 U GR. The change in absorbance at 412 nm was recorded for 2 min. Glutathione concentrations were calculated from a standard curve constructed using commercial GSH over the range 0–0.1 mM. To determine the content of GSSG, 100 µl of the samples were incubated with 2 µl of pure 2-vinylpyridine and 6 µl of 1.5 M triethanolamine for 1 h at 25°C. GSSG concentrations were calculated from a standard curve constructed using commercial GSSG over the range 0–0.01 mM.

Statistical Analysis

Analysis of variance (two-way ANOVA) and multiple comparison tests (LSD test) was run in the SPSS computer program (IBM Inc, Chicago, IL, USA). The data are reported as ean ± standard deviation (SD).

Results and Discussion

Clarifying the physiological, biochemical and molecular mechanisms that lead to salt tolerance in plants has agronomic significance for its potential to increase resistance against salt stress. Thus, callus cultures as plant tissue culture techniques have been frequently used as *in vitro* model for stress treatments in large populations, in a limited space, in a short period, homogeneity of stressor applications and monitoring responses to salinity at morphological, physiological and biochemical levels (Niknam et al. 2011).

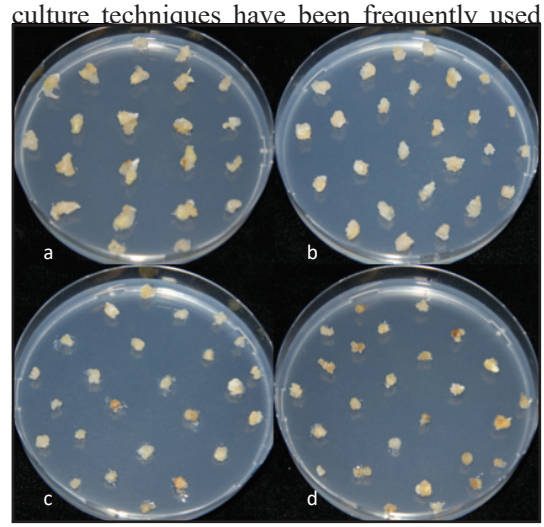

Figure 1. *28-day-old wheat callus from Flamura-85 cultivar; a) Control, b) 0.05 M NaCl; c) 0.1M NaCl and b) 0.15M NaCl.*

In this study, wheat calli from Tekirdağ, Pehlivan and Flamura-85 cultivars were exposed to different concentrations of NaCl for four weeks (Fig.1). The behaviour of antioxidant defence systems was investigated, namely the activities of SOD, POX, CAT, APX and GR and content of GSH, GSSG and Proline, cell viability, H_2O_2 and MDA content, to test NaCl treatments on wheat cultivars.

The general assumption is that increasing levels of MDA and H_2O_2 are an inevitable result of oxidative stress and that they decrease cell viability (Table 1).

Table 1: The contents of MDA (nmolmg^{-1}protein) and H_2O_2 (µmolg^{-1}FW) and viability (A_{530} mg^{-1}FW) assay in Tekirdag, Pehlivan and Flamura-85 wheat callus cultures under different concentrations of NaCl treatments.

Cultivars	NaCl Concentrations (M)	H_2O_2	MDA	Viability
Tekirdağ	**Control**	12.94±0.509^{a}	2.55±0.221^{a*}	7.29±0.505^{a}
	0.05	15.92±0.359^{b}	3.11±0.474^{b}	5.05±0.563^{b}
	0.10	17.94±0.517^{c}	3.39±0.307^{c}	3.91±0.225^{c}
	0.15	18.54±0.281^{d}	5.58±0.544^{d}	2.86±0.192^{d}
Pehlivan	**Control**	11.93±0.477^{a}	2.91±0.270^{a}	7.49±0.403^{a}
	0.05	15.95±0.304^{b}	4.32±0.432^{b}	4.91±0.410^{b}
	0.10	18.16±0.391^{c}	4.57±0.381^{c}	3.99±0.477^{c}
	0.15	18.62±0.253^{d}	6.56 ±0.402^{d}	2.79 ±0.451^{d}
Flamura-85	**Control**	11.13±0.661^{a}	2.43±0.442^{a}	7.57±0.307^{a}
	0.05	14.96±0.294^{b}	2.71±0.239^{b}	5.07±0.463^{b}
	0.10	16.96 ±0.776^{c}	5.56±0.265^{c}	4.20±0.402^{c}
	0.15	18.71±0.155^{d}	5.74±0.200^{d}	3.06±0.108^{d}

*Letters indicate significant differences ($P<0.05$) between experimental groups according to one-way ANOVA and post hoc LSD tests (±SD, n=5)

In accordance with these expectations, H_2O_2 content increased significantly (one-way ANOVA, LSD test at $P< 0.05$) in all cultivars but most in Flamura-85, followed by Pehlivan and Tekirdağ, which increased 1.68, 1.56 and 1.43 times higher than the control, respectively. Salt stress induces lipid peroxidation through ROS production, thus making the membranes injury. Like H_2O_2, in MDA content increased most in Flamura-85, followed by Pehlivan and Tekirdağ (3.23, 2.25 and 2.19 times higher, respectively). On the other hand, the rate of cell viability decreased linearly in all cultivars by 62.75% in Pehlivan, 60.77% in Tekirdağ and 59.58% in Flamura-85, depending on the increasing concentrations of NaCl. Two-way ANOVA analyses confirmed significant changes in both H_2O_2 and MDA content and viability depending on the salt treatments. Additionally, a two-way ANOVA showed significant differences between cultivar pairs Tekirdağ and Pehlivan and Tekirdağ and Flamura-85 in the MDA content and between Tekirdağ and Flamura-85 and Pehlivan and Flamura-85 in the H_2O_2 content (Table 2 and 3).

Table 2: Statistical significance of F values (two-way ANOVA) for single and combined effects of cultivars and NaCl treatments as indicated by biochemical parameters in the 28-day-old wheat callus samples.

Treatments	SOD	POX	APX	CAT	GR	MDA	Proline	Viability	H_2O_2	GSH	GSSG
NaCl-treatments	430.798*	1564.685*	200.248*	45.132*	374.885*	384.568*	287.642*	264.963*	647.608*	195.380*	354.614*
Cultivars	2381.843*	846.636*	167.139*	22.859*	26.248*	45.852*	1.805^{ns}	1.119^{ns}	22.609*	3.486^{ns}	4.658^{ns}
NaCl treatments x Cultivars	134.514*	46.036*	51.406*	34.435*	17.340*	25.343*	3.902*	0.175^{ns}	3.474*	2.762^{ns}	0.089^{ns}

*P<0.005; ns=Not Significant.

Table 3: Statistically significant relationship (two-way ANOVA) between wheat cultivars based on F values of biochemical parameters in the 28-day-old callus samples under NaCl stress treatments.

Varieties	SOD	POX	APX	CAT	GR	MDA	Proline	Viability	H_2O_2	GSH	GSSG
Tekirdag x Pehlivan	2.791^{ns}	136.265*	1.266*	0.471*	0.880*	0.934*	0.248^{ns}	0.021^{ns}	0.171^{ns}	0.069^{ns}	0.609^{ns}
Tekirdag x Flamura-85	92.488*	98.295*	2.641*	0.634*	0.898*	0.976*	0.148^{ns}	0.198^{ns}	0.895*	0.254^{ns}	0.353^{ns}
Pehlivan x Flamura-85	89.697*	234.560*	1.376*	0.164*	0.017^{ns}	0.042^{ns}	0.100^{ns}	0.178^{ns}	0.725*	0.328^{ns}	0.292^{ns}

* P<0.005; ns=Not Significant

Increased H_2O_2 and MDA productions were reported to occur in response to salt stress in chickpea plants (Sheokand et al. 2010). Niknam et al. (2011) reported that under NaCl stress, MDA and H_2O_2 levels increased more in *Acanthophyllum sordidum* than *A. glandulosum.* Cucumber callus viability was significantly reduced for each increase in NaCl concentration (Abu-Romman and Suwwan 2009).

Mechanism of ROS detoxification exists in all plants and can be categorized as enzymatic (SOD, CAT, POX, APX, GR etc) and non-enzymatic (Glutathione, Proline etc) antioxidant defence mechanism. The level of stress-response depends on the species, development and metabolic state of plant, as well duration and intensity of stress. Plant resists the stress-induced production of ROS by increasing component of antioxidant defence systems (Gill and Tuteja 2010).

The first component of this system is SOD. SOD is the major $O_2^{\bullet-}$ scavenger and its enzymatic action results in H_2O_2 and O_2 formation. The H_2O_2 is then scavenged by CAT, POX and Ascorbate–Glutathione Cycle's components. CAT catalyses H_2O_2 into H_2O and O_2 in several cellular compartments including peroxisomes, cytosol and mitochondria. POX decomposes H_2O_2 via co-substrates such as phenolic compounds. APX uses ascorbate as the electron donor in the first step of the Ascorbate–Glutathione Cycle. GR, GSH and GSSG are other important components in the Ascorbate–Glutathione Cycle (Gill and Tuteja 2010).

The increase of SOD activity in Flamura-85 was the highest, followed by Pehlivan and then Tekirdağ, of 2.97, 2.14 and 1.66 times higher than the control, respectively. POX

activity increased significantly in all the three cultivars (P<0.05). The increase in Pehlivan was observed to be much higher than increases in both Flamura-85 and Tekirdağ, of 3.10, 2.19 and 2.11 times greater under 0.15 M NaCl treatment than the control, respectively. On the other hand, under NaCl-treatment, CAT activity decreased in Tekirdağ while it increased in Pehlivan and Flamura-85, by 2.35 times and 1.85 times over the control, respectively. The increase in APX activity in all cultivars reached a maximum in Flamura-85, followed by Pehlivan and Tekirdağ, of 2.65, 2.25 and 1.47 times greater under 0.05 M NaCl treatment than the control, respectively. GR activity increased significantly in all the three cultivars (P<0.05). The increase in Pehlivan was the highest, followed by Tekirdağ and then Flamura-85, of 2.23, 2.04 and 1.72 times greater than the control, respectively (Table 4).

Table 4: The effects of different concentrations of NaCl treatment on activities of SOD (Umg^{-1} protein), CAT (Umg^{-1} protein), POX (ΔA_{470} mg^{-1} protein), APX (ΔA_{290} mg^{-1} protein) and GR (Umg^{-1} protein) in Tekirdag, Pehlivan and Flamura-85 callus cultures.

Cultivars	NaCl Concentrations (M)	SOD	CAT	POX	APX	GR
Tekirdag	**Control**	32.06±2.924^{a*}	2.88±0.424^{a*}	353.82±28.713^{a*}	3.51±0.307^{a*}	5.25±0.537^{a*}
	0.05	53.15±2.633^{b}	1.53±0.301^{b}	654.95±19.299^{b}	5.16± 0.424^{b}	8.79±0.416^{b}
	0.10	52.34±4.018^{b}	2.00±0.311^{c}	657.38±15.466^{b}	4.10±0.381^{ab}	10.69±0.486^{c}
	0.15	51.30±3.327^{b}	1.76±0.312^{bc}	746.09±13.642^{c}	4.13±0.407^{ab}	7.91±0.497^{b}
Pehlivan	**Control**	30.82±2.653^{a}	1.76±0.078^{a}	185.74±7.958^{a}	3.06±0.348^{a}	5.31±0.386^{a}
	0.05	56.45±3.532^{b}	1.95±0.073^{a}	564.87±12.164^{b}	6.58±0.436^{b}	9.63±0.390^{b}
	0.10	65.93±5.150^{c}	4.13±0.279^{b}	576.42±15.623^{c}	6.88±0.433^{b}	11.82±0.471^{c}
	0.15	46.81±3.090^{ab}	2.22±0.029^{a}	540.14±15.452^{b}	5.44±0.462^{b}	9.42±0.437^{b}
Flamura-85	**Control**	60.16±5.380^{a}	1.88±0.033^{a}	401.51±24.037^{a}	4.24±0.057^{a}	6.19±0.440^{a}
	0.05	152.5±7.777^{b}	2.14±0.016^{a}	881.09±13.276^{b}	11.24±0.673^{b}	9.31±0.439^{b}
	0.1	178.6±6.772^{c}	3.23±0.024^{b}	794.31±16.249^{b}	5.92±0.405^{ab}	10.07±0.409^{c}
	0.15	167.5±6.094^{c}	3.47±0.047^{b}	728.51±24.468^{b}	6.07±0.417^{ab}	10.67±0.441^{d}

*Letters indicate significant differences (P<0.05) between experimental groups according to one-way ANOVA and post hoc LSD tests (±SD, n=5)

Antioxidant enzyme capacity can be different in species depending on their responses to NaCl treatments, and various researchers recorded variable enzyme responses under

stress treatments. Yang et al. (2010) reported that SOD, CAT and APX activity increased significantly in *Nitraria tangutorum* Bobr. calli treated with NaCl, while POX activity decreased. While, the activities of SOD, POX and APX enhanced, CAT decreased, and GR is not changed in NaCl-treated chickpea (Sheokand et al. 2010). Patade et al. (2012) observed that the activity of SOD and CAT increased, but APX decreased in sugarcane calli treated with 150 mM NaCl. Sharma and Ramawat (2014) reported that low concentration NaCl treatments enhanced the activities of SOD and CAT, but higher application reduced their activities in three halophytes. These results indicated that antioxidant enzymes behave in a way to compensate for the responsibility in each under stress conditions.

In the presented study, two-way ANOVA analysis of variance confirmed the significant differences in antioxidant enzyme activities in wheat callus dependent, for interaction effects between cultivars and NaCl treatments. Additionally, a two-way ANOVA showed the statistically significant relationship between cultivars under salinity stress and the activity of antioxidant enzymes, except for SOD activity and H_2O_2 content between Tekirdağ and Pehlivan and GR activity and MDA content between Pehlivan and Flamura-85 (Table 2 and 3). Besides increasing levels of antioxidant enzyme activities, the accumulation of compatible solutes such as proline tends to enhance response to salinity stress. Torabi and Niknam (2011) noted that proline is involved in the protection of enzymes, cellular structures and acts as a free radical scavenger. Our study confirmed the significant differences in proline content. The proline increase in all cultivars was statistically significant, according to a one-way ANOVA and post-hoc LSD tests ($P<0.05$). The greatest increase under 0.15 M NaCl treatment was in Tekirdağ, followed by Pehlivan and Flamura-85, of 2.57, 2.04 and 2.02 times greater than the control, respectively (Table 4).

The enhancing level of proline accumulation was detected in cucumber callus (Abu-Romman and Suwwan, 2009), sugarcane calli (Patade et al. 2012) and three halophytes callus culture under NaCl stress treatments (Sharma and Ramawat, 2014). These results indicated that the increasing levels of proline accumulation could be important adaptive mechanisms of salinity tolerance in plants.

GSH has a central role in plant cell against oxidative stress via the Ascorbate-Glutathione Cycle. Besides this, GSH and GSSG redox plays an important role in the maintenance of cellular redox homeostasis and the signalling system, including several oxidants, antioxidants and stress hormones, and is also used in plants as an indicator of oxidative stress. Numerous researchers have reported that GSH/GSSG ratios decrease under abiotic stress due to decreasing GSH content (reviewed by Szalai et al. 2009).

In our study, GSH increases were not statistically significant in all cultivars while GSSG increases were statistically significant. Under NaCl treatments, GSSG ratios in Tekirdag were 1.9 times higher than the control, and in Pehlivan and Flamura-85, 1.8 times higher. On the other hand, GSH/GSSG decreased in all wheat calli under NaCl treatment (Table 5).

A two-way ANOVA showed significant differences in GSH and GSSG contents under NaCl treatment. There was no statistically significant relationship between salinity stress and cultivars in terms of proline, GSH and GSSG content (Table 2 and 3). Similarly, Sheokand et al. (2010) reported that glutathione content increased under salt stress, however, sat stress conditions resulted in a decrease in the GSH/GSSG ratio. The reason for this decrease was the increase in GSSG contents under stress treatments. An increase in GSH and GSSG ratios may indicate a compensatory mechanism in which there is increased recycling of glutathione to keep it in its active reduced form under salt stress because of increased activities of APX and GR. These results suggested that increased capacity of the antioxidant system to scavenge H_2O_2.

Table 5: The contents of Proline (μmolmg^{-1}protein) GSH (nmolmg^{-1}protein), GSSG (nmol mg^{-1} protein) and the ratios of GSH/GSSG and in Tekirdag, Pehlivan and Flamura-85 wheat callus cultures under different concentrations of NaCl treatments.

Cultivars	NaCl Concentrations (M)	Proline	GSH	GSSG	GSH/GSSG
Tekirdag	**Control**	3.24±0.663^{a*}	22.89±0.406^{a*}	5.06±0.381^{a*}	4.52
	0.05	5.84±0.609^{b}	25.15±0.403^{a}	7.59±0.401^{b}	3.31
	0.10	7.27±0.503^{c}	25.34±0.394^{a}	8.28±0.439^{c}	3.06
	0.15	8.34±0.438^{d}	25.59±0.418^{a}	9.61±0.372^{d}	2.66
Pehlivan	**Control**	3.70±0.361^{a}	22.14±0.518^{a}	5.07±0.387^{a}	4.37
	0.05	5.93±0.313^{b}	25.55±0.244^{a}	7.65±0.379^{b}	3.34
	0.10	6.53±0.344^{c}	25.32±0.339^{a}	8.38±0.349^{c}	3.04
	0.15	7.54 ±0.321^{d}	25.67 ±0.324^{a}	9.69±0.334^{d}	2.65
Flamura-85	**Control**	3.74±0.336^{a}	22.78±0.452^{a}	5.50±0.315^{a}	4.14
	0.05	6.14±0.309^{b}	26.09±0.619^{a}	7.96±0.528^{b}	3.28
	0.10	6.67 ±0.257^{c}	25.74±0.370^{a}	8.51 ±0.359^{c}	3.02
	0.15	7.56±0.291^{d}	25.17±0.209^{a}	9.91±0.404^{d}	2.54

*Letters indicate significant differences ($P<0.05$) between experimental groups according to one-way ANOVA and post hoc LSD tests (±SD, n=5).

Conclusion

The present study investigated the responses of lipid peroxidation, antioxidant defence systems, H_2O_2 content and viability in callus cultures of Tekirdağ, Pehlivan and Flamura-85 wheat cultivars under different NaCl concentrations. Interaction effects between cultivars and NaCl treatments were significant in terms of antioxidant enzyme activities and MDA, proline and H_2O_2 content. Statistically significant differences between cultivars were detected for antioxidant enzyme activities, MDA and H_2O_2 contents, except for SOD activity and H_2O_2 content between Tekirdağ and Pehlivan and GR activity and MDA content between Pehlivan and Flamura-85. Additionally, with increasing levels of NaCl stress, lower values of cell viability and higher values of H_2O_2 and MDA content were detected in all wheat calli, indicating that greater activity of antioxidant defence mechanisms was not efficiently scavenging free radicals at the cellular level. Decreasing GSH/GSSG ratios indicated that oxidative stress disturbed the redox balance at the cellular level in all wheat

calli under NaCl stress. Based on the cellular responses of three different wheat calli against salinity, Flamure-85 was the best one based on its superior antioxidant defence system.

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