TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2014) 38: 89-98

doi:10.3906/bot-1305-1

Expression of SOD gene and evaluating its role in stress tolerance in NaCl and PEG stressed *Lycopersicum esculentum*

Semra AYDIN[1,*], İlker BÜYÜK[2], Emine Sümer ARAS[2]

[1]Medical Biological Products Laboratories, Department of Medicine, Biological and Medical Products Laboratory, Turkish Medicines and Medical Device Agency, Ministry of Health of Turkey, Ankara, Turkey

[2]Department of Biology, Faculty of Science, Ankara University, Ankara, Turkey

Received: 04.05.2013 • **Accepted:** 14.10.2013 • **Published Online:** 02.01.2014 Printed: 15.01.2014

Abstract: Antioxidant enzyme mechanisms are important for protecting crop productivity against stressful environmental conditions. Therefore, the current study was designed to evaluate lipid peroxidation (via malondialdehyde (MDA)) levels, superoxide dismutase (SOD) gene expression profiles, and SOD enzyme activities in tomato (*Lycopersicum esculentum* L.) plants subjected to different concentrations (100 and 150 mM) and time periods of NaCl and polyethylene glycol (PEG) stress (3 h, 6 h, 9 h, 12 h, and 48 h). Enhancement of lipid peroxidation in plants may be attributed to the increased accumulation of reactive oxygen species (ROS). Results indicated the antioxidant responses of tomato plants could be reflected as changes in gene transcripts and enzyme activities of SOD, but SOD gene expression patterns and changes in SOD enzyme activity revealed no positive correlation. As a result, stressful conditions led to stress in tomato plants (indicated by increased MDA levels) and triggered the expression levels of the SOD gene. The antioxidative response of tomato plants to PEG stress was found to be more vigorous than the response to NaCl stress. All these results pointed to the importance of SOD for stress defense in tomato. The overexpression and silencing of the SOD gene in tomato plants under NaCl and PEG stresses remain to be identified in further studies.

Key words: Abiotic stress, SOD, real-time PCR

1. Introduction

The rapidly growing world population has made it necessary to minimize stressful environmental conditions that result in negative plant responses and loss of crop productivity (Maathuis et al., 2003; Tester and Davenport, 2003; Mazzucotelli et al., 2008; Tardideu, 2013). Agricultural productivity is severely affected by high soil salinity and is responsible for the decline in soil fertility throughout the world (Mahesh and Satish, 2008). Improper irrigation management and usage of salt-rich irrigation water are the major sources of salinity in arid and semiarid regions (Plaut et al., 2013). Abiotic stress conditions, including drought and salinity, are projected to be the most important reasons for arable land loss in the future, as 19% of the world's soil is hyperarid and 56% is at risk of desertification (FAO, 2011).

Drought and salinity stresses in plants lead to water potential imbalance, turgor decrease, hyperosmotic stress, and oxidative stress. Oxidative stress is caused by the generation of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) (Smirnoff, 1993; Zhu, 2001). ROS have 2 different kinds of effects. They not only act as oxidative damaging factors, leading to lipid peroxidation, DNA mutation, protein denaturation, and many physiologic disorders at the cellular level; they also function as protective or signaling factors (Bowler et al., 1992; Scandalios, 1993; Mittler, 2002; Apel and Hirt, 2004; Behnamnia et al., 2009). In addition, plants have efficient detoxification systems including both nonenzymatic and enzymatic mechanisms for scavenging ROS. Nonenzymatic antioxidants include glutathione (GSH), proline, carotenoids, tocopherol, etc. The enzymatic antioxidants are monodehydroascorbate reductase (MDHAR), dehydro ascorbate reductase (DHAR), glutathione reductase (GR), ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) (Verma et al., 2003; Verma and Dubey, 2003; Halliwell, 2006; DalCorso et al., 2008).

SOD plays a determinant role in the protection against the toxic effects of oxidative stress by scavenging superoxide radicals and providing their conversion into O_2 and H_2O_2 (Verma et al., 2003). Four different classes of

* Correspondence: semrasoydam@gmail.com

SOD have been distinguished, depending on the metal at the active center: manganese (Mn), iron (Fe), copper (Cu), and zinc (Zn) (Miller and Sorkin, 1997). Previous studies established that most SODs are intracellular enzymes; these are Cu/Zn SOD (which is also extracellular), MnSOD, and FeSOD. Cu/Zn SODs are generally found in the cytosol of eukaryotic cells and chloroplasts; membrane-associated MnSODs are found in mitochondria and reported in chloroplasts and peroxisomes in some plants; the dimeric FeSODs, which are not found in animals, have been reported in chloroplasts of some plants (Salin and Bridges, 1980; del Rio et al., 1983; Droillard and Paulin, 1990; Van Camp et al., 1994; Fridovich, 1995; Gomez et al., 2004).

Numerous studies have reported that ROS induce oxidative stress, which has been correlated with SOD enzyme activity under drought and salt stress in plants (Bowler et al., 1992; Scandalios, 1993; Foyer et al., 1994; Donahue et al., 1997; Fadzilla et al., 1997; El-Saht, 1998; Dionisio-Sese and Tobita, 1998; Baloğlu et al., 2012). In tomato and maize plants, enhanced transcript abundances of Mn-SOD and Cu/Zn-SOD were shown by northern blot analysis in previous reports (Hernández et al., 2000; Alscher et al., 2002; Munir and Aftab, 2009).

The classical methods used for determining gene expression levels, such as northern blot and RNA protection assays, suffer from disadvantages such as large RNA requirements and being resource-consuming (Chelly and Kahn, 1994). With the development of new technology, the more rapid, sensitive, and specific real-time PCR (RT-PCR) method began to be used in the quantification of gene expression. RT-PCR uses cDNA as a template of PCR reaction, which reduces the RNA requirement. It uses reliable internal control genes (housekeeping genes), which encode proteins essential to cell viability and whose expression levels show minimal changes due to changes in environmental condition of plants (Stürzenbaum and Kille, 2001).

In this regard, the current study was conducted in 3 phases after NaCl and PEG stress treatments. In the first part, in order to obtain evidence that the plants were in stress, malondialdehyde (MDA) levels were determined after the NaCl and PEG treatments. In the second part of the study, the abundance of the steady state level of SOD mRNA level was determined by quantitative real-time PCR. Finally in the third part, the SOD enzyme activities were determined in order to obtain an idea about the final stage of the SOD gene expression in tomato samples.

2. Materials and methods

2.1. Plant material, growth conditions, and stress treatment

Tomato (*Lycopersicum esculentum* L. 'Falcon') seeds were germinated and grown hydroponically in pots containing 0.2 L of modified 1/10 Hoagland solution. Hoagland solution includes macronutrients (K_2SO_4, KH_2PO_4, $MgSO_4$, $7H_2O$, Ca $(NO_3)_2$, $4H_2O$, and KCl) and micronutrients (H_3BO_3, $MnSO_4$, $CuSO_4$, $5H_2O$, NH_4Mo, $ZnSO_4$, and $7H_2O$) with a final concentration of ions as 2 mM Ca, 10^{-6} M Mn, 4 mM NO_3, 2.10^{-7} M Cu, 1 mM Mg, 10^{-8} M NH_4, 2 mM K, 10^{-6} M Zn, 0.2 mM P, 10^{-4} M Fe, and 10^{-6} M B. Six plants were grown in each pot in a controlled environmental growth chamber with light of 250 mmol m^{-2} s^{-1} photosynthetic photon flux at 25 °C and 70% relative humidity. Twenty-five–day–old plants grown in controlled media were used for stress treatments. The plants were treated with salt through the addition of NaCl to the nutrient solution, to a final concentration of 100 mM and 150 mM. Osmotic pressures of the NaCl solutions were determined by Vapor Pressure Osmometer 5520. The average osmotic pressure of the 100 mM NaCl solution was estimated as 190 mmol/kg, while the 150 mM NaCl solution was found to be approximately 290 mmol/kg. For the drought treatment (Verslues et al., 2006), Polyethylene glycol 6000 (PEG 6000) was added to the nutrient solution until the osmolality of the solution, measured by a vapor pressure osmometer, was the same as that of the NaCl solutions. In this way, an isoosmotic level of stress was applied to both the NaCl and PEG-treated plants, with a goal of matching the stem water potentials of the plants exposed to these treatments. Six plants in a single pot were harvested 0, 3, 6, 12, 24, and 48 h after the plants were exposed to these treatments. Harvested whole tomato plants (total leaves, stems, and roots) were ground in liquid nitrogen and used for estimation of lipid peroxidation, SOD enzyme activity assay, RNA extraction, and gene expression analysis. All 3 replicates were tested twice with the purpose to verify results.

2.2 Estimation of lipid peroxidation

MDA is a marker of oxidative lipid injury, and it changes in response to environmental factors that lead to stress in plants. TBA-MDA content was determined as described by Hodges et al. (1999). Whole plant samples (total leaves, stems, and roots) were homogenized with liquid nitrogen and used for each MDA assay. All samples were homogenized with 80:20 (v:v) ethanol:water, followed by centrifugation at 3000 × *g* for 10 min. A 1-mL aliquot of diluted sample was added to a test tube with either (i) a TBA solution composed of 20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene, or (ii) a +TBA solution containing the above plus 0.65% TBA. Samples were then mixed vigorously, heated at 95 °C in a hot plate (neoBlock1, 2-2503) for 25 min, cooled, and centrifuged at 3000 × *g* for 10 min. Absorbance values were measured at 440 nm, 532 nm, and 600 nm by an ELISA microplate reader (SpectraMax M2). The equivalents of MDA were calculated by the following equations:

1) [(Abs 532 $_{+TBA}$) – (Abs 600 $_{+TBA}$) – (Abs 532 $_{-TBA}$ – Abs 600 $_{-TBA}$)] = A

2) [(Abs 440 $_{+TBA}$ – Abs 600 $_{+TBA}$) 0.0571] = B

3) MDA equivalents (nmol mL^{-1}) = (A – B / 157,000) 10^6

2.3 RNA extraction and gene expression analysis with real-time quantitative PCR

RNA extraction was performed using the Trizol protocol followed by an RNeasy mini kit (Qiagen, Cat no: 74104) cleanup (Chomczynski and Mackey, 1995). The quantity and quality of RNA was determined by Nanodrop ND-Spectrometer 1000 and confirmed by gel electrophoresis, which contains 1.5% agarose and formaldehyde. A 2-step procedure was used for real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Reverse transcription reactions were performed with 2 µg of RNA, 2.5 µM Anchored-oligo(dT)18, 1X Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer, 20 U Protector Rnase Inhibitor, 1 mM deoxynucleotide Mix, 5 mM DTT, and 10 U Transcriptor High Fidelity Reverse Transcriptase using the High Fidelity cDNA Synthesis Kit (Roche).

Real-time PCR was performed using Light Cycler 480 System (Roche). The sequences of primers and probes (presented in Table 1) of the target gene superoxide dismutase (SOD, NCBI, Entrez Gene ID: 543981) and actin (ACT, NCBI Entrez Gene ID: 543519) used for normalization were designed based on sequences of tomato genes available in the databank (http://www.ncbi.nlm.nih.gov/). These sequences are common sequences of 4 different classes of SODs located in the cytosol, chloroplasts, mitochondria, and peroxisomes of tomato cells. Amplifications of the PCR product were monitored via intercalation of hybridization probes. Copy numbers of genes (SOD, ACT) under stress treatments were determined by using standard curves.

2.4 SOD enzyme activity assay

Whole plants, which were exposed to different concentrations of NaCl and PEG stress, were homogenized with (1:1, w/v) 0.2 M phosphate buffer (pH 7.0) with a cold mortar and pestle. The homogenate was centrifuged at 27,000 × *g* for 20 min. The supernatants were photochemically assayed for SOD activity at 440 nm, using the SOD determination kit, which allows SOD assaying by utilizing Dojindo's highly water soluble tetrazoliun salt, WST-1 (2-3-5-2H tetrazolium salt, monosodium salt), that produces a water soluble formazan dye upon reduction with a superoxide anion. Absorbance at 440 nm is proportional to the amount of superoxide anion, and the SOD activity can be quantified by measuring the decrease in the color development at 440 nm (Sigma Aldrich 19160).

2.5. Statistical methods

The abundance of target gene transcripts was normalized to ACT and set relative to the control plants according to the $2^{-\Delta\Delta CT}$ method (Livak and Scmittgen, 2001). Changes in relative expression levels (REL) of genes were checked for statistical significance according to one-way ANOVA. The results were considered statistically significant if the P value was < 0.05 in Dunnett's test.

Table 1. Primer and probe sequences of SOD (NCBI, Entrez Gene ID: 543981) and actin (NCBI Entrez Gene ID: 543519).

SOD F FW	GAGCTTGAGGATGACCTCG
SOD S Fw	CTTGAGGATGACCTCGGAAA
SOD A Rev	TGTTGCTGCTGCATTTACTTC
SOD R Rev	CCGGAGAGGAGGGTAAATAC
SOD FL	TGCCAATCGTCCACCAGCAT-FL
SOD LC 640	GCCAGTGGTAAGACTGAGTTCATGGCC p
actin F Fw	CATTGTCCACAGAAAGTGCTTCTA
actin S Fw	TCTGTTTCCCGGTTTTGCTATTAT
actin A Rev	AACCACATTAAATGGAAACATGAGAT
actin R Rev	TGCATCAGGCACCTCTCAAG
actin FL	ATTCATAGCCCCCACCACCAAAC-FL
actin LC 640	TCTCCATCCCATCAAAAAAACAAATTGACT p

3. Results

3.1. Estimation of lipid peroxidation

The MDA contents of tomato samples treated for different time periods and with different concentrations of NaCl and PEG stress are shown in Figure 1, Table 2, Figure 2, and Table 3, respectively. A concentration of 100 mM NaCl did not increase lipid peroxidation levels in tomato samples until 9 h after exposure. After 9 h, MDA content gradually increased until it reached maximum levels at 24 h after the addition of NaCl to the growth media. MDA content was not significantly altered in tomato samples

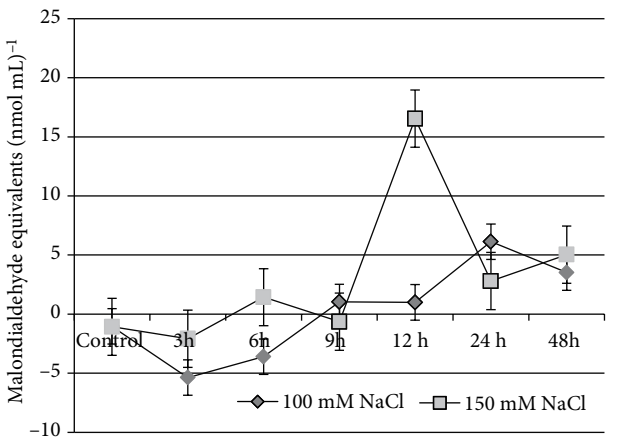


Figure 1. Lipid peroxidation (malondialdehyde = MDA content) in *Lycopersicum esculentum* samples exposed to different periods and concentrations of NaCl. All points represent the averages (n = 6).

Table 2. Absorbances and MDA equivalent levels (nmol mL^{-1} g FW^{-1}) of 100 and 150 mM NaCl-treated tomato plants assayed after processing.

Plant material	Assayed without TBA (–TBA)		Assayed with TBA (+TBA)			A	B	Total MDA equivalents	Average MDA equivalents
	532	600	440	532	600				
100 mM NaCl Control	0.147	0.14	0.136	0.159	0.156	–0.004	–0.00114	–0.0182	–0.01037
	0.101	0.099	0.166	0.161	0.159	0	0.0004	–0.00255	
3rd of 100 mM NaCl	0.103	0.101	0.1	0.101	0.103	–0.004	–0.00017	–0.02439	–0.05369
	0.079	0.078	0.2	0.204	0.217	–0.014	–0.00097	–0.08299	
6th of 100 mM NaCl	0.097	0.091	0.175	0.162	0.157	–0.001	0.001028	–0.01292	–0.03602
	0.109	0.095	0.231	0.198	0.191	–0.007	0.002284	–0.05913	
9th of 100 mM NaCl	0.097	0.09	0.119	0.114	0.102	0.005	0.0009707	0.025664331	0.010286306
	0.121	0.115	0.153	0.145	0.139	–1.38778E–17	0.0007994	–0.00509172	
12th of 100 mM NaCl	0.074	0.07	0.134	0.126	0.119	0.003	0.0008565	0.013652866	0.009922611
	0.101	0.098	0.217	0.204	0.199	0.002	0.0010278	0.006192357	
24th of 100 mM NaCl	0.156	0.142	0.242	0.224	0.203	0.007	0.0022269	0.030401911	0.061251274
	0.188	0.169	0.294	0.269	0.232	0.018	0.0035402	0.092100637	
48th of 100 mM NaCl	0.116	0.107	0.179	0.198	0.185	0.004	–0.0003426	0.027659873	0.035125159
	0.146	0.131	0.196	0.196	0.173	0.008	0.0013133	0.042590446	
150 mM NaCl Control	0.139	0.126	0.138	0.149	0.141	–0.005	–0.0001713	–0.030756051	–0.030756051
	0.115	0.108	0.164	0.169	0.167	–0.005	–0.0001713	–0.030756051	
3rd of 150 mM NaCl	0.111	0.1	0.163	0.16	0.149	1.38778E–17	0.0007994	–0.00509172	–0.020833439
	0.106	0.097	0.187	0.178	0.174	–0.005	0.0007423	–0.036575159	
6th of 150 mM NaCl	0.109	0.105	0.201	0.177	0.167	0.006	0.0019414	0.025850955	0.014296497
	0.105	0.099	0.28	0.244	0.235	0.003	0.0025695	0.002742038	
9th of 150 mM NaCl	0.115	0.107	0.215	0.187	0.174	0.005	0.0023411	0.016935669	–0.006448408
	0.153	0.141	0.279	0.242	0.232	–0.002	0.0026837	–0.029832484	
12th of 150 mM NaCl	0.136	0.133	0.282	0.247	0.226	0.018	0.0031976	0.094282803	0.165344268
	0.261	0.265	0.412	0.414	0.379	0.039	0.0018843	0.236405732	
24th of 150 mM NaCl	0.079	0.074	0.157	0.179	0.166	0.008	–0.0005139	0.054228662	0.027935032
	0.095	0.092	0.168	0.159	0.155	0.001	0.0007423	0.001641401	
48th of 150 mM NaCl	0.078	0.07	0.212	0.186	0.168	0.01	0.0025124	0.04769172	0.050330892
	0.07	0.063	0.217	0.188	0.17	0.011	0.0026837	0.052970064	

exposed to 150 mM of NaCl solution for 12 h. The maximum MDA content was determined in tomato plants that were subjected to 150 mM PEG stress for 12 h. MDA content decreased after 24 h of NaCl stress but remained above the control level (Figure 1; Table 2).

All periods of 100 mM PEG stress led to increases in MDA content in tomato plants, compared to the control. MDA levels started to increase over an initial time span of 100 mM PEG stress up to 6 h. This level decreased at 6 h and then increased up until 24 h of exposure. Minimum MDA content was observed in tomato plants subjected to 100 mM of PEG stress for 6 h, while the maximum level was determined after 24 h of application. A similar MDA data pattern with 100 mM PEG stress was observed with 150 mM PEG stress application. There were differences between 100 and 150 mM PEG treatments at 6 and 48 h (Figure 2; Table 3).

3.2. Real-time quantitative PCR (RT-PCR)

The expression levels of SOD and ACT genes were analyzed by real-time PCR (Light Cycler 480 System, Roche) in *L. esculentum* samples that were exposed to different periods and concentrations of NaCl and PEG (Figures 3 and 4). Real-time RT–PCR data are normalized with ACT as a housekeeping and an internal control gene and also

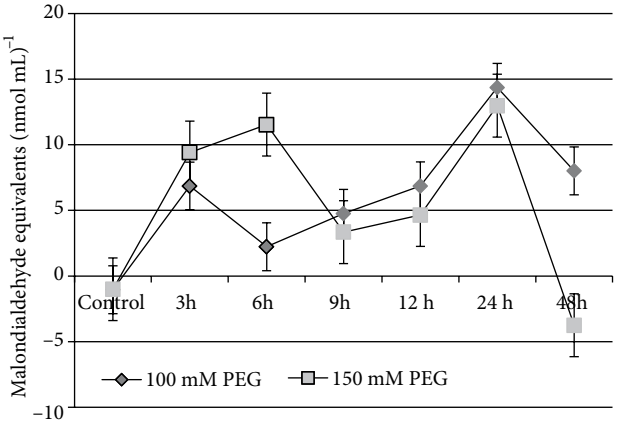


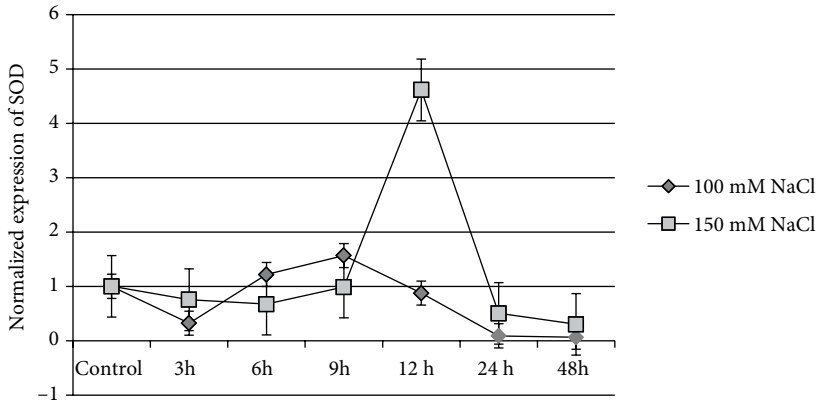
Figure 2. Lipid peroxidation (malondialdehyde = MDA content) in *Lycopersicum esculentum* samples exposed to different periods and concentrations of PEG. All points represent the averages (n = 6).

with the control samples. To evaluate the stability of the results, SOD and ACT transcript levels of all samples were measured 3 times for each length of exposure time.

With regard to the control and to each other, different expression levels were recorded in all lengths of both 100 and 150 mM NaCl exposure. In the first time period (3 h), expression level was suppressed under both 100 and 150 mM NaCl. Subsequently, expression recovered until 9 h for 100 mM NaCl. The same recovery was shown until 12 h for 150 mM NaCl. At this time period, the maximum SOD expression level, which was 4.6-fold above control level, was recorded. Following the recovery period, a decrease was observed again. During the same length of time, plasmolysis started in the leaves of the tomato samples. Results obtained under almost all of the NaCl treatment conditions were statistically significant (Figure 3).

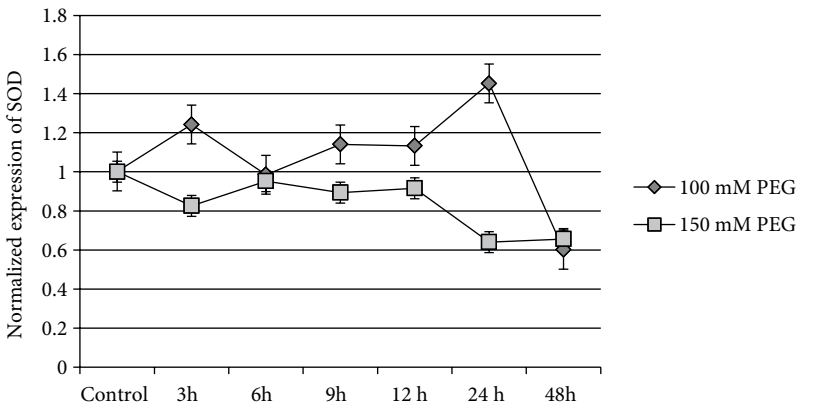
Table 3. Absorbances and MDA equivalent levels (nmol mL^{-1} g FW^{-1}) of 100 and 150 mM PEG treated tomato plants assayed after processing.

Plant material	Assayed without TBA (–TBA)		Assayed with TBA (+TBA)			A	B	Total MDA equivalents	Average MDA equivalents
	532	600	440	532	600				
100 mM PEG Control	0.16	0.158	0.176	0.2	0.196	0.002	–0.001142	0.020012739	0.059325159
	0.126	0.122	0.181	0.192	0.172	0.016	0.0005139	0.09863758	
3rd of 100 mM PEG	0.143	0.138	0.169	0.18	0.164	0.011	0.0002855	0.068245223	0.068520382
	0.129	0.122	0.204	0.202	0.183	0.012	0.0011991	0.068795541	
6th of 100 mM PEG	0.13	0.12	0.213	0.204	0.182	0.012	0.0017701	0.065158599	0.022302548
	0.156	0.138	0.28	0.258	0.241	–0.001	0.0022269	–0.020553503	
9th of 100 mM PEG	0.142	0.136	0.165	0.146	0.129	0.011	0.0020556	0.056970701	0.047598408
	0.109	0.098	0.208	0.192	0.173	0.008	0.0019985	0.038226115	
12th of 100 mM PEG	0.139	0.132	0.161	0.15	0.134	0.009	0.0015417	0.047505096	0.068525159
	0.142	0.14	0.218	0.202	0.184	0.016	0.0019414	0.089545223	
24th of 100 mM PEG	0.172	0.165	0.256	0.234	0.199	0.028	0.0032547	0.157613376	0.143601592
	0.205	0.184	0.336	0.317	0.272	0.024	0.0036544	0.129589809	
48th of 100 mM PEG	0.176	0.167	0.205	0.23	0.211	0.01	–0.0003426	0.065876433	0.080074841
	0.158	0.155	0.231	0.229	0.21	0.016	0.0011991	0.094273248	
150 mM PEG Control	0.179	0.169	0.211	0.205	0.196	–0.001	0.0008565	–0.011824841	0.009558917
	0.147	0.146	0.208	0.195	0.188	0.006	0.001142	0.030942675	
3rd of 150 mM PEG	0.136	0.133	0.196	0.199	0.176	0.02	0.001142	0.12011465	0.094091401
	0.15	0.141	0.225	0.223	0.202	0.012	0.0013133	0.068068153	
6th of 150 mM PEG	0.149	0.144	0.235	0.218	0.191	0.022	0.0025124	0.124124841	0.115298089
	0.162	0.161	0.244	0.224	0.204	0.019	0.002284	0.106471338	
9th of 150 mM PEG	0.154	0.147	0.245	0.213	0.188	0.018	0.0032547	0.093919108	0.033409554
	0.158	0.139	0.311	0.272	0.254	–0.001	0.0032547	–0.0271	
12th of 150 mM PEG	0.205	0.184	0.311	0.264	0.247	–0.004	0.0036544	–0.04875414	0.046423567
	0.262	0.251	0.455	0.426	0.389	0.026	0.0037686	0.141601274	
24th of 150 mM PEG	0.189	0.181	0.209	0.225	0.19	0.027	0.0010849	0.165064331	0.129762102
	0.162	0.143	0.24	0.238	0.202	0.017	0.0021698	0.094459873	
48th of 150 mM PEG	0.184	0.162	0.272	0.219	0.199	–0.002	0.0041683	–0.039288535	–0.037558599
	0.159	0.143	0.292	0.226	0.211	–0.001	0.0046251	–0.035828662	



(All points represent the averages (n=6).SOD gene expression changes in 100 mM NaCl treated tomato plants at 3h, 6h, 9h, 24h and 48h were found statistically significant at p<0.001. Gene expression changes in 3h of 150 mM NaCl was found statistically significant at p<0.05 while 6h and 24h were found statistically significant at p<0.01. 12h and 48h of 150 mM NaCl led to statistically signifi cant changes at p<0.001.)

Figure 3. SOD mRNA levels in the *Lycopersicum esculentum* seedlings exposed to different time periods and concentrations of NaCl. All points represent the averages (n = 6). SOD gene expression changes in 100 mM NaCl-treated tomato plants at 3 h, 6 h, 9 h, 24 h, and 48 h were found statistically significant at P < 0.001. Gene expression changes in 3 h of 150 mM NaCl were found to be statistically significant at P < 0.05, while 6 h and 24 h were found statistically significant at P < 0.01; 12 h and 48 h of 150 mM NaCl led to statistically significant changes at P < 0.001.



(All points represent the averages (n=6). SOD gene expression changes in 100 mM PEG treated tomato plants at 24h and 48h were found statistically significant at p<0.001. Gene expression changes in 3h, 24h and 48h of 150 mM PEG were found statistically significant at p<0.001 and 9h of 150 mM PEG showed statistically significant changes at p<0.05).

Figure 4. SOD mRNA levels in the *Lycopersicum esculentum* seedlings exposed to different periods and concentrations of PEG. All points represent the averages (n = 6). SOD gene expression changes in 100 mM PEG-treated tomato plants at 24 h and 48 h were found statistically significant at P < 0.001. Gene expression changes in 3 h, 24 h, and 48 h of 150 mM PEG were found to be statistically significant at P < 0.001, and 9 h of 150 mM PEG showed statistically significant changes at P < 0.05.

The SOD expression level achieved after application of 100 mM PEG was analyzed statistically, and it was observed that the alteration was not significant until 24 h of 100 mM PEG treatment. Subsequently, in tomato samples exposed to 100 mM PEG stress for 24 h, the SOD expression level exhibited a 1.14-fold increase compared to the control. Following the recovery period, again a decrease was observed in the samples exposed to 100 mM PEG stress for 48 h. On the other hand, SOD expression levels were under control levels in all lengths of exposure under 150 mM PEG stress. It was remarkable that SOD gene expression levels were similar in tomato samples

exposed to 6 h and 48 h treatment of both 100 and 150 mM PEG stress.

3.3. SOD enzyme activity assays

To verify the responses of the tomato plant to different periods and concentrations of NaCl and PEG stress, differentiation in the enzyme activity of SOD, based on inhibition of WST-1, was determined. The results are shown in Figures 5 and 6. Short exposure times of 3 h and 6 h to 100 mM NaCl led to significant increases in the inhibition rate of WST-1, indicating SOD enzyme activity was strongly increased. After 6 h, the inhibition rate of WST-1 was similar to up to 48 h of 100 mM NaCl stress. For the 150 mM NaCl treatment, the SOD enzyme activity pattern determined by the inhibition rate of WST-1 was slightly similar to the 100 mM pattern. There was an initial increase in WST-1 inhibition; however, between 12 and 24 h, a stationary period was observed before an increase at 48 h. The difference in the inhibition rate of WST-1 between the 2 NaCl treatments was the absence of a stationary period in SOD activity in the tomato samples exposed to 100 mM NaCl (Figure 5).

For all concentrations of the PEG treatment, SOD enzyme activity showed similar patterns. Tomato plants exposed to 3 h of PEG stress had a significant increase in the inhibition rate of WST-1, indicating a strong increase in SOD enzyme activity. This increase was higher in the 150 mM PEG stress than in the application of 100 mM PEG stress. After these periods, while the inhibition rate of WST-1 decreased at 6 h treatment of 100 mM PEG, this decrease continued until 9 h treatment of 150 mM PEG. Between the time periods of 6 h and 9 h for 100 mM PEG stress and 9 h and 12 h for 150 mM PEG stress, a stationary period was shown in the inhibition rate of WST-1. For the 100 mM PEG treatment, SOD enzyme activity pattern determined over the inhibition rate of WST-1 increased

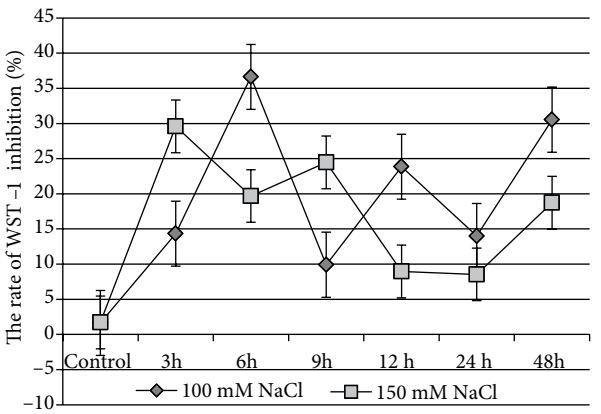


Figure 5. SOD enzyme activity in the *Lycopersicum esculentum* seedlings exposed to different periods and concentrations of NaCl. All points represent the averages (n = 6). All changes were found to be statistically significant at P < 0.001 compared to the control.

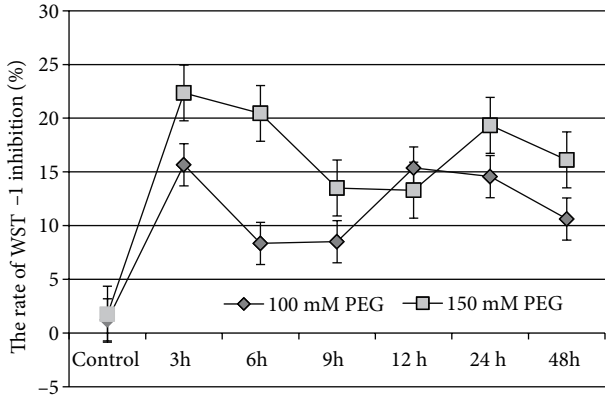


Figure 6. SOD enzyme activity in the *Lycopersicum esculentum* seedlings exposed to different time periods and concentrations of PEG. All points represent the averages (n = 6). All changes were found to be statistically significant at P < 0.001 compared to the control.

at the 12 h application of 100 mM PEG stress and again decreased at the 48 h treatment. A slight increase was observed at 24 h, which was followed by a decrease at 48 h (Figure 6).

4. Discussion

When plants are exposed to salinity and drought stress, a rapid and temporary drop is observed in the growth rate of plants. After the stress is alleviated, there is a gradual recovery to a new reduced rate of growth. There are rapid, essentially instantaneous changes in leaf expansion rates with a sudden change in salinity (Munns, 2002). On the other hand, PEG, which generates drought stress, has the same effect as salt stress on a plant by exerting the same osmotic pressure (Yeo et al., 1991; Munns and Tester, 2008). The similarity between water and salt stress also apply to most metabolic processes: there can be strong correlations between increases in leaf ion concentrations and reductions in photosynthesis or stomata conductance (Munns, 2002).

Chlorophylls are excited in excess of the metabolic capacity for energy use and dissipation (Asada, 2006) and ROS accumulation (Apel and Hirt, 2004). ROS generation can be initiated by the univalent reduction of O_2, or by the transfer of excess excitation energy to O_2. The transfer of electrons leads to the generation of superoxide radicals (O^{-2}), hydrogen peroxide (H_2O_2) or a hydroxyl radical (HO^-) (Mittler, 2002). In the current study, enhancement of lipid peroxidation in tomato plants, treated with different concentrations of NaCl and PEG stress for different periods, may be attributed increased accumulations of ROS varieties (Figures 1 and 2). It is known that many effects of stress, such as DNA mutation, protein denaturation, physiologic disorders, and lipid peroxidation, are reversible; however, if stress is excessive,

irreversible damage can occur and lead to cell death (Lester, 1985). According to these facts, ROS-induced lipid peroxidation levels depend on stress types, concentrations of stress, and imposed time stress condition. The reduction in the amount of lipid peroxidation in the current study indicates the inductions of ROS-scavenging (antioxidant) enzymes are the most common mechanisms for detoxifying ROS during stress responses (Mittler, 2002).

Plants have several antioxidant enzymes and metabolites located in different cell compartments; the main ones are SODs, a family of metalloenzymes catalyzing the dismutation of O_2^- to H_2O_2, which is in turn detoxified by CAT, APX, or PRX (Smirnoff, 1993, 1998; Leprince et al., 1994). The effects of SOD under NaCl and PEG stress have been demonstrated by many researchers. Hernández et al. (2000) reported enhanced Mn-SOD and Cu/Zn-SOD transcript abundances in maize and in tomato plants. Numerous studies reported increased oxidative stress, which was correlated with SOD activity during PEG and NaCl stress (Scandalios, 1993; Fadzilla et al., 1997; Dionisio-Sese and Tobita, 1998; Kaya et al., 2013; Gao et al., 2013). Shalata et al. (2001) reported increased SOD activity in salt-tolerant *Lycopersicon pennelli* L. Sekmen Esen et al. (2012) reported increased SOD activity in the xerophytic plant *Gypsophila aucheri* under salt and drought stress.

The current study revealed that the antioxidant responses of tomato to NaCl and PEG stress could be reflected as changes in gene transcripts and the enzyme activities of SOD. The changes in SOD gene transcript levels and enzyme activities in tomato samples under NaCl and PEG stress are shown in Figures 3–6. Analysis of gene expression patterns during NaCl and PEG stress treatments showed a complex profile. The expression level of SOD in tomato samples exposed to 150 mM NaCl stress for 12 h was the maximum (statistically significant at $P < 0.05$, when compared to the control) among all stress conditions, while lipid peroxidation also reached a maximum level at the same length of duration (Figures 1 and 3). Until the 24th time point of 100 mM PEG stress, changes in the gene expression levels of SOD were not found to be statistically significant (Figure 4). Although the samples that were treated with 150 mM of PEG revealed increased MDA contents (except for 48 h) and enzyme activities, gene expression levels were decreased. SOD gene expression patterns at mRNA level and changes in SOD enzyme activities under different periods and concentrations of NaCl and PEG stress revealed no positive correlation, which might be explained by the regulation of genes at the transcriptional, posttranscriptional, and also translational or posttranslational levels. Although the current study highlights some of the points in the regulation of gene expression of SOD under NaCl and PEG stresses in tomato plants, further analysis at protein levels is still necessary to explain this complex connection.

A proper understanding of the molecular mechanisms of plants under stress conditions is a vital process in providing the necessary tools for exploiting plant species with enhanced antioxidant activities in sustainable agricultural systems and regions with adverse environmental conditions. To this end, we aimed to reflect the effects of NaCl and PEG in tomato plants at the enzymatic and mRNA levels. We concluded that stressful conditions led to stress in tomato plants, reflected by increased MDA levels, and triggered the expression levels of the SOD gene, which is responsible for synthesis of the SOD enzyme. Antioxidative response of tomato plants to PEG stress was found to be more vigorous than the response to NaCl stress. All these results pointed to the importance of SOD for stress defense in tomato plants. The overexpression and silencing of the SOD gene remain to be identified in tomato plants under salt and drought stresses in future studies.

References

Alscher RG, Erturk N, Heath LS (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J Exp Bot 53: 1331–1341.

Apel K, Hirt H (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373–399.

Asada K (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol 141: 391–396.

Baloğlu MC, Kavas M, Aydın G, Öktem HA, Yücel AM (2012). Antioxidative and physiological responses of two sunflower (*Helianthus annuus*) cultivars under PEG-mediated drought stress. Turk J Bot 36: 707–714.

Behnamnia M, Kalantari KM, Ziaie J (2009). The effects of brassinosteroid on the induction of biochemical changes in *Lycopersicon esculentum* under drought stress. Turk J Bot 33: 417–428.

Bowler C, Montagu MV, Inze D (1992). Superoxide dismutase and stress tolerance. Annu Rev Plant Physiol 43: 83–116.

Chelly J, Kahn A (1994). RT-PCR and mRNA quantification. In: KB Mullis, Ferre F, Gibs RA, editors. The Polymerase Chain Reaction. Boston, MA, USA: Birkhauser, pp. 97–109.

Chomczynski P, Mackey K (1995). Short technical reports. Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. Biotechniques 19: 942–945.

DalCorso G, Farinati S, Maistri S, Furini A (2008). How plants cope with cadmium: staking all on metabolism and gene expression. J Integr Plant Biol 50: 1268–128.

Del Rio LA, Lyon DS, Olah I, Glick B, Salin ML (1983). Immunocytochemical evidence for a peroxisomal localization of manganese superoxide dismutase in leaf protoplasts from a higher plant. Planta 158: 216–224.

Dionisio-Sese ML, Tobita S (1998). Antioxidant responses of rice seedlings to salinity stress. Plant Sci 135: 1–9.

Donahue JL, Okpodu CM, Cramer CL, Grabau EA, Alscher RG (1997). Responses of antioxidants to paraquat in pea leaves. Plant Physiol 113: 249–257.

Droillard MJ, Paulin A (1990). Isozymes of superoxide dismutase in mitochondria and peroxisomes isolated from petals of carnation (*Dianthus caryophyllus*) during senescence. Plant Physiol 94: 1187–1192.

El-Saht HM (1998). Responses to chilling stress in French bean seedlings: antioxidant compounds. Biol Plant 41: 395–402.

Fadzilla NM, Finch RH, Burdon RH (1997). Salinity, oxidative stress and antioxidant responses in shoot cultures of rice. J Exp Bot 48: 325–331.

Fridovich I (1995). Superoxide radical and superoxide dismutases. Ann Rev Biochem 64: 97–112.

Gomez JM, Jimenez A, Olmos E, Sevilla F (2004). Location and effects of long-term NaCl stress on superoxide dismutase and ascorbate peroxidase isoenzymes of pea (*Pisum sativum* cv. Puget) chloroplasts. J Exp Bot 55: 119–130.

Halliwell B (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 142: 312–322.

Hernandez JA, Jimenez A, Mullineaux P, Sevilla F (2000). Tolerance of pea (*Pisum sativum* L.) to long term salt stress is associated with induction of antioxidant defences. Plant Cell Environ 23: 853–862.

Hodges DM, DeLong JM, Forney CF, Prange RK (1999). Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207: 604–611.

Jaleel CA, Gopi R, Manivannan P, Panneerselvam R (2007). Antioxidative potentials as a protective mechanism in *Catharanthus roseus* (L.) G.Don. plants under salinity stress. Turk J Bot 31: 245–251.

Kaya C, Ashraf M, Dikilitaş M, Tuna AL (2013). Alleviation of salt stress-induced adverse effects on maize plants by exogenous application of indoleacetic acid (IAA) and inorganic nutrients – A field trial. Aust J Crop Sci 7: 249–254.

Leprince O, Atherton NM, Deltour R, Hendry GAF (1994). The involvement of respiration in free radical processes during loss of desiccation tolerance in germinating *Zea mays* L.: an electron paramagnetic resonance study. Plant Physiol 104: 1333–1339.

Lester GE (1985). Leaf cell membrane thermostabilities of *Cucumis melo*. J Amer Soc Hort Sci 110: 506–509.

Livak KJ, Scmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta C(T))}$ method. Methods 25: 402–408.

Maathuis FJM, Filatov V, Herzyk P, Krijger GC, Axelsen KB, Chen S, Green BJ, Madagan KL, Sanchez-Fernandez R, Forde BG et al. (2003). Transcriptome analysis of root transporters reveals participation of multiple gene families in the response to cation stress. Plant J 35: 675–692.

Mahesh B, Satish S (2008). Antimicrobial activity of some important medicinal plant against plant and human pathogens. World J Agric Sci 4: 839–843.

Mazzucotelli E, Mastrangelo AM, Crosatti C, Stanca AM, Cattivelli L (2008). Abiotic stress response in plants: when post-transcriptional and post-translational regulations control transcription. Plant Sci 174: 420–431.

Miller AF, Sorkin DL (1997). Superoxide Dismutases: A Molecular Perspective. Comment Mol Cell Biophys 9: 1–48.

Mittler R (2002). Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7: 405–441.

Munir N, Aftab F (2009). The role of polyethylene glycol (PEG) pretreatment in improving sugarcane's salt (NaCl) tolerance. Turk J Bot 33: 407–415.

Munns R (2002). Comparative physiology of salt and water stress. Plant Cell Environ 25: 239–250.

Munns R, Tester M (2008). Mechanisms of salinity tolerance. Annu Rev Plant Biol 59: 651–681.

Plaut Z, Edelstein M, Ben-Hur M (2013). Overcoming salinity barriers to crop production using traditional methods. Crit Rev Plant Sci 32: 250–291.

Salin ML, Bridges SM (1980). Isolation and characterization of an iron-containing superoxide dismutase from a eucaryote, Brassica campestris. Arch Biochem Biophys 201: 369–374.

Scandalios JG (1993). Oxygen stress and superoxide dismutases. Plant Physiol 101: 7–12.

Sekmen Esen AH, Özgür R, Uzilday B, Tanyolaç ZÖ, Dinç A (2012). The response of the xerophytic plant *Gypsophila aucheri* to salt and drought stresses: the role of the antioxidant defence system. Turk J Bot 36: 697–706.

Shalata A, Mittova V, Volokita M, Guy M, Tal M (2001). Response of cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennelli* to salt-dependent oxidative stress: the root antioxidative system. Physiol Plantarum 122: 487–494.

Smirnoff N (1993). The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol 125: 27–58.

Smirnoff N (1998). Plant resistance to environmental stress. Curr Opin Biotechnol 9: 214–219.

Sturzenbaum SR, Kille P (2001). Control genes in quantitative molecular biological techniques: the variability of invariance. Comp Biochem Phys B 130: 281–289.

Tardideu F (2013). Plant response to environmental conditions: assessing potential production, water demand, and negative effects of water deficit. Front Physiol 4: 17.

Tester N, Davenport R (2003). Na+ tolerance and Na+ transport in higher plants. Ann Bot 9: 1–25.

Van Camp W, Willekens H, Bowler C, Van Montagu M, Inzé D (1994). Elevated levels of superoxide dismutase protect transgenic plants against ozone damage. Biotechnology 12: 165–168.

Verma S, Dubey RS (2003). Lead toxicity induced lipid peroxidation and alters the activities of antioxidant enzyme in growing rice plants. Plant Sci 164: 645–655.

Verma SS, Verma U, Tomer RPS (2003). Studies on seed quality parameters in deteriorating seeds in Brassica (*Brassica campestris*) seeds. Seed Sci Technol 31: 389–396.

Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu JK (2006). Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J 45: 523–539.

Yeo AR, Lee KS, Izard P, Boursier PJ, Flowers TJ (1991). Short and long-term effects of salinity on leaf growth in rice (*Oryza sativa* L.). J Exp Bot 42: 881–889.

Zhu JK (2001). Plant salt tolerance. Trends in Plant Sci 6: 66–71.