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Studying The Responses of *Hordeum vulgare* Seedlings to Salinity and Osmotic Stresses: Oxidative Stress/Antioxidants Play Crucial Roles

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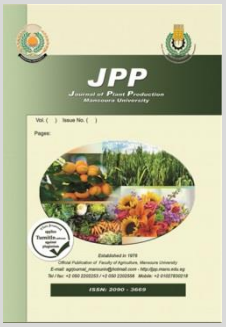

ABSTRACT

Salinity and osmotic stresses are prime reasons of plant growth and productivity reduction in semi-arid regions and cause complex series of physiological, cellular, and molecular changes. Since osmotic and ionic effects are correlated and initiated by salinity, separating both is an important step in understanding the basis of salt tolerance. Barely seedlings(cultivar Giza 134)were treated with either NaCl(150 mM)or iso-osmotic polyethylene glycol 6000 (19.5% PEG). Treatments were applied two times before sampling and collected after two weeks from emergence. Results showed decreasing of fresh matter in treated seedlings, especially those treated with PEG. Furthermore, significant increase of non-enzymatic antioxidants, oxidative markers in addition to enzymatic antioxidants examined (peroxidase (POX), and polyphenol oxidase (PPO)) was detected with PEG treatment. The osmoregulators including proline (Pro) and glycine betaine(GB) increased in the root tissue, in conjunction with enhancement in the antioxidant status of leaves by applying PEG. Based on molecular analysis using real-time RT-PCR, HvNHX gene (coding for Na+/H+ antiporter) was highly expressed after 48 h from treatment in the roots under salinity, but it was expressed in PEG-treated leaves rather than salt-treated ones, and the opposite was true for HvGORK gene (regulate voltage-gated K+-permeable channels).On the other hand, HvDREB gene(coding for dehydration responsive element binding protein)has recorded higher expression in the roots under PEG treatment compared to control. Taken together, the current study suggests that the studied barley cultivar possesses higher tolerance to salt stress than osmotic stress imposed by PEG, so it could be more suitable for cultivation under salinity conditions.

Keywords: Barely, PEG, salinity, osmotic stress, oxidative markers, antioxidants, gene expression

INTRODUCTION

A significant scientific topic of interest is environmental stress, since it affects agricultural production, survival and distribution (Tavakkoli *et al*., 2010). Jamil *et al*. (2011) stated that half of cultivated land is projected to suffer from salt stress by 2050 since 10 percent of arable land is impacted by salinity every year for natural and human causes. The osmotic effect initiated by NaCl can be achieved by iso-osmotic PEG (a non-ionic and water-soluble polymer) as outlined by Sayar *et al*. (2010). Darko *et al*. (2019) reported that the wheat seedlings had a different response to osmotic stress induced by either NaCl or PEG with distinct metabolic regulations. Ultimately, gathering the biochemical, physiological and gene regulating network knowledge is being necessary for reaching a clear overview about mechanisms of salt tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006).

In saline soil, osmotic stress generates effects similar to those of water stress caused by drought stress. Mitsuya *et al*. (2002) added that exposure to salinity causes ionic stress and eventually rises to toxicity (Mitsuya *et al*., 2002). In addition, it was found that salinity stimulates reactive oxygen species (ROS) production which subsequently cause reduction of growth rate and injury to macromolecules in the form of proteins, lipids and nucleic acids (Arif *et al*., 2020). Hydrogen peroxide (H_2O_2) is non-radical ROS that exerts its detrimental effect through generation of highly reactive hydroxyl radicals (Ślesak *et al*., 2007). MDA (the end product of lipid peroxidation) is a common and important index for evaluating the redox status of plants. The lower MDA level reflects a higher antioxidative ability andresistance as suggested by Dhanda *et al*. (2004).

Salt tolerant plants develop enzymatic (POX, CAT, SOD and PPO) as well as non-enzymatic antioxidants (AsA and GSH) to resist the damaging effects caused by ROS. POX catalyzes the oxidation of phenolics into quinones, in addition to its role in the removal of H_2O_2 (Bhardwaj and Yadav, 2012). PPO catalyzes the oxidation of mono-, di-, and polyhydric phenols to produce quinones. CAT is one of the key enzymes that remove H_2O_2 in addition to POX. CAT is different from APX because CAT requires a reductive substrate and has a high affinity for H_2O_2. SOD is an enzyme that could catalyze the dismutation of the superoxide radical into O_2 or H_2O_2, besides it can restrict the production of hydroxyl radical (Wang *et al*., 2018). AsA represents the most powerful non-enzymatic antioxidant which can reduce

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H_2O_2 to water *via* ASA–GSH cycle (Athar *et al*., 2008). GSH serves as an antioxidant, reacting with free radicals. Glutathione also contributes to the elimination of toxic metabolites via acting as a substrate for glutathione S-transferases and as an electron donor for glutathione peroxidases (De pinto *et al*., 2013).

The antioxidant capacity can be estimated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, where DPPH encounters proton donors, such as antioxidants (Islam *et al*., 2012). Plants enhance their resistance through accumulation of Pro and GB which contribute to cellular osmotic adjustments under high salinity and water stress (Szabados and Savouré, 2010).

Also, Pro compared to GB can directly scavenge superoxide or hydrogen peroxide and induce an increase of antioxidant enzyme activities (El Moukhtari *et al*, 2020).

Under stress, multiple signals are transmitted to the cells and act on proteins involved in transcription factors, which regulate the expression of stress-responsive genes, so that plants gradually adapt to stress (Liang *et al*., 2018). The salt-responsive genes are categorized into the following functional classifications: ion transport or homeostasis e.g., vacuolar $Na(^+)$/ $H(^+)$ antiporter (*NHX*), dehydration-related transcription factors e.g., dehydration responsive element-binding protein (DREB), and guard cell outward rectifying K^+ channel (GORK). NHX proteins use Na^+ (and/ or K^+) regulation as a simple and convenient means of maintaining cell osmotic balance, thereby improving the ability of the plant to cope with both salt and drought stresses (Huang *et al*., 2018). DREB proteins are essential transcription factors that influence stress endurance by regulating many abiotic stress-related genes, which are responsible for compatible solutes accumulation as Pro and GB (Xu *et al*., 2009). *GORK* genes possess a role in mediating potassium release from the cell and activated by membrane depolarization as mentioned by Adem *et al*. (2020). Furthermore, accumulation of ROS under stress conditions could activate GORK channels inducing more K^+ efflux, leading eventually to programmed cell death (Demidchik *et al*., 2010). Shabala and Pottosin (2010) suggested that knocking out *GORK* genes would increase salt tolerance.

Barley (*Hordeum vulgare* L.) is rated as salt tolerant crop among plants; however, a great genetic variation for salt tolerance exists between its cultivars. Nonetheless, barely is still vulnerable to yield loss because of salinization (Munns and Tester, 2008). Hence, exploring salt tolerance responsive genes remains a mean for barley breeding programs for improving its salt tolerance (Mian *et al*., 2011). Filek *et al*. (2012) mentioned the deleterious effects caused by PEG which appear as leaf wilting and rolling as a result of stomatal closure. Therefore, the present study aimed to compare between salt and osmotic stresses, caused by NaCl and PEG, respectively through morphological, physiological and molecular attributes.

MATERIALS AND METHODS

Growth conditions and stress treatments

Barely grains (*Hordium vulgare* L.) cultivar- Giza 134 (moderate salt tolerant), were obtained from the Agricultural Research Center of Egypt. Sterilized barely grains (5% Clorox for 10 min) were grown in 12.5 cm diameter and 3.5 cm depth plastic pots, containing 100 g sandy soil. The pots were kept in dark until the radical initiation. The plants were irrigated as required for 20 days under controlled greenhouse conditions (23±3°C, 12 h day length, humidity ~65%). After 7 days from sowing, seedling were watered with NaCl (150 mM) or PEG 6000 (19.5%). For all measurements, samples were collected 3 days post treatment, except for real time PCR experiments, samples were collected after 12, 24 and 48 hours from salinity treatment.

Measurement of growth criteria

Seedlings were washed with tap water several times to remove sand particles then with distilled water and separated into shoots and roots. Fresh weights (F.wt.) of them were recorded and dried in an oven at 60º C to a constant weight. Fresh and dry samples were preserved for further analysis.

Estimation of oxidative stress markers

Lipid peroxidation level as malondialdehyde (MDA) and the concentration of H_2O_2 in plant samples were estimated. The MDA concentration was measured using the Heath and Paker (1968) technique. A sample of 0.5 g fresh leaves in 10 ml of 5% trichloroacetic acid (TCA) has been collected. After extraction, the homogenate was centrifuged for 10 minutes at 4000 rpm. The supernatant (2 ml) was combined with 2 ml of thiobarbaturic acid (TBA) and incubated in a water bath at 100 ºC for a period of 20 minutes and cooled immediately. At 532 nm and 600 nm, the absorbance was read. The concentration of MDA was estimated with the extinction coefficient (155 mM^{-1} cm^{-1}) and expressed as µmol/g f.wt. The content of H_2O_2 was measured using Velikova *et al*. (2000) technique. 100 mg of fresh leaves was extracted with 0.1 % TCA and centrifuged at 12,000 rpm for 15 min. 0.5 ml of supernatant has subsequently been combined with 0.5 ml of 10 mM potassium phosphate buffer (K-PBS) of 7.0 pH and 1 M potassium iodide and the absorbance was determined at 390 nm by using UNICO Vis Spectrophotometer (Model 1200, USA). The amount of H_2O_2 was calculated using an extinction coefficient (0.28 μM^{-1} cm^{-1}) and expressed as µmol g^{-1} f.wt.

Quantification of non-enzymatic antioxidants

Ascorbic acid was estimated according to Oser (1965) method. An amount of 0.1 g of leaf tissue was homogenized in 5 ml of 5% (w/v) sulfosalicylic acid (SSA) then centrifuged at ten thousand rpm for 10 min. It was incubated for 40 minutes at 60º C in a water bath, cooled and centrifuged at 3000 for 10 minutes. The absorbance was measured at 660 nm and the AsA content estimated using a constructed calibration curve as mg/g d.wt. The content of GSH was determined using Anderson (1985) technique. In 5 ml of 3 percent SSA, fresh material (0.1 g) was homogenized under cold conditions. The homogenate was centrifuged for 10 minutes at 10,000 rpm. An amount of 0.5 ml aliquot, 0.5 ml reaction buffer (0.5 ml potassium phosphate buffer (K-PBS), pH 7.0) and 50 µl of 3 mM 5, 5'-dithio-bis 2-nitrobenzoic acid (DTNB) were added. After 5 min, the absorbance was measured at 412 nm and the GSH level was estimated using the standard GSH curve reported as µmol/ml.

Assay of antioxidant enzymes

Four antioxidant enzymes, including peroxidase (POX), polyphenol oxidase (PPO), catalase (CAT), and superoxide dismutase (SOD), were assayed in leaf tissue. POX was measured spectrophotometrically through guaiacol/H_2O_2 as substrate according to the method of Egley *et al*. (1983). The reaction was initiated by adding 100 µl of crude enzyme extract to 2.4 ml of substrate buffer (100 mM potassium phosphate buffer, pH 6.8). The increase in absorbance was recorded spectrophotometrically after one min at 470 nm. Peroxidase specific activity was expressed as µM/ g.f.wt. min^{-1}. The spectrophotometric activity testing of PPO was conducted by detecting the absorbance rise at 420 nm for 4-methylcatechol according to Oktay *et al*. (1995). The test was carried out at room temperature using a buffer of 100 mM sodium phosphate (pH 7.0), 5 mM 4-methylcatechol and 500 µl crude extract. CAT activity was assayed as outlined by Aebi (1983) by measuring the decline in absorbance at 240 nm resulting from the degradation of H_2O_2. The reaction mixture composed of 3.0 ml of 100 mM sodium phosphate buffer (pH 7.0), 30 mM H_2O_2 and 100 µl raw extract. The activity was calculated by an extinction coefficient (40 mM^{-1} cm^{-1}). The activity of SOD was measured by measuring the photo-reduction inhibition of nitroblue tetrazolium (Kumar *et al*., 2012). The reaction mixture included 50 mM sodium phosphate buffer (pH 7.6), 0,1 mM EDTA, 50 mM sodium carbonate, 12 mM L-methionine, 50 µM NBT, 10 µM riboflavine, and 100 µl of crude extract. The SOD reaction was performed by exposing the reaction mixture for 15 minutes at room temperature to white light. Absorbance was measured at 560 nm after 15 min incubation.

Evaluation of antioxidant activities (DPPH and reducing power)

The leaf extracts' electron donating capacity was evaluated by the bleaching of the purple 2,2- diphenyl-1-picrylhydrazyl radical (DPPH) solution using the method of Brand-Williams *et al*. (1995) and Bondet *et al*. (1997). The dried shoot sample of young seedlings (0.1 g) was extracted in 95% ethanol, and then an aliquot of extract was mixed with 3.9 ml of a DPPH solution (0.03 g/L). The mixture was rapidly mixed and left to stand in the dark for 1 h at room temperature. The absorbance was then read at 517 nm against a blank. The Oyaizu (1986) technique was used to evaluate the reducing power of *H. vulgare*. One ml of ethanolic extracts of leaves was combined with sodium phosphate buffer and incubated to 50°C for 20 min in a water bath. The supernatant was then combined with TCA and ferric chloride solution. The intensity of the blue green color was measured at 700 nm.

Evaluation of osmolytes

For proline (Pro) measurement, method outlined by Bates *et al*. (1973) was applied. In 5 ml of a 3 percent aqueous SSA, 0.1 g of fine dried tissues (shoot/root) was homogenized. Centrifugation removed the residues. The reaction of one ml of supernatant was allowed with 2 ml of ninhydrin acid and 2 ml of acetic acid for 1 hour at 100°C. The content of the GB in the leaves and roots was estimated by the method designed by Grieve and Grattan (1983). The dried tissues were agitated mechanically for 24 h at room temperature with 20 ml distilled water. Then the samples were filtrated and the filtrates were diluted with 2 N HCl. The 0.1 ml cold per-iodide reagent dissolved in 9 ml of 1, 2-dichloroethane. The absorbance was measured at 365 nm after 2 h. The content of GB was measured using a standard curve and reported as mg/g d.wt.

Gene expression analysis

For qRT-PCR analysis, barley roots and leaves were harvested and snap frozen with liquid nitrogen at time points 12, 24 and 48 before being stored at -80 °C for real-time RT-PCR analysis. Total RNA was extracted using TRIzol reagent using Chomczynski (1993) technique. Ambion's DNA-free reagent was used to eliminate genomic DNA contamination. For the synthesis of cDNA, Invitrogen's superscript III Reverse Transcriptase kit with an oligo $(dT)_{20}$ primer was applied, following the manufacturer's instructions. Each sample was executed three times. Data acquisition was performed during the extension stage. qRT-PCR was conducted as described by Burton *et al*. (2008) using a RG6000 Rotor-Gene real time thermal cycler (QIAGEN, ABI System, USA) and SYBR® green PCR reagent (Fermentas, USA). Primers shown in Table 1 were designed to investigate the expression of number of key genes involved in Na^+ sequestration, mediating potassium efflux besides dehydration and included members of the *NHX*, *GORK* and *DREB* family of genes. The test gene transcript normalization was relative to the control gene (*Actin*).

Real-time-PCR assay:

Each reaction consisted of a mixture of 20 µl, containing primer pairs. The reaction was done using Rotor-Gene 6000 (QIAGEN, ABI System, USA). The amplification program included: initial denaturation step at 95°C for 10 min, 40 cycles of 95°C for 15 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 sec. β -actin gene was used as a reference gene (Saleha, 2010).

Table 1. Sequences of primers used in this study

Primer	Direction	Sequences 5-3
DREB	Forward	*ATGGAGACCGGGGGTAGC*
	Reverse	*GCAGACTCAAACTCATCCTTGG*
NHX	Forward	*TGCATATCTACCAGTGCTTAT*
	Reverse	*GGTTCAAGACACAAGTTCAGT*
GORK	Forward	*CCACACGAGGCGAAGAAG*
	Reverse	*GAGGAATCCACAGCATCACC*
ß- actin	Forward	*AGACCTTCAACACCCCTGCTATGT*
	Reverse	*CCAATCCAGACACTGTACTTCCTT*

Real time RT-PCR data analysis

The relative expression was counted by Livak and Schmittgen (2001) method. Consequently, for each sample, the difference (Δ) in quantification cycle value (CT) between the target (CT_{target} averaged from three technical replicates) and the reference ($CT_{reference}$, a fixed CT value used for all samples) was converted into relative quantities (RQ) using the exponential function with the efficiency (E) of the PCR reaction. The CT (threshold of cycle) value for each gene was established through an automated threshold analysis on the Applied Biosystems Integrated (ABI) System. The CT value of each target gene was normalized to $CT_{reference}$ to obtain CT_{target} where:

$$\Delta CT_{target} = (CT_{target} - CT_{reference}) \text{ and}$$
$$\Delta CT_{control} = (CT_{control} - CT_{reference}).$$

The relative expression quantity of the target gene was determined as follows:

$$\Delta\Delta CT = (\Delta CT_{target} - \Delta CT_{control}).$$

Statistical analysis

For means comparison and the statistical significance levels, pairwise t-test in all possible combination of the treatments was employed using SPSS software version 23 (IBM support portal, USA).

Results and discussion

Inhibitory effects of PEG on seedling growth exceeds NaCl effects

Results shown in Figure 1 indicated that the negative effects of PEG on fresh weights of the examined salt tolerant barley cultivar were more pronounced than the effects of NaCl. This was consistent with Alam *et al*. (2020) who suggested that the inhibitory effects of NaCl were much lower than PEG effects on *Senna italic* growth. This difference may be due to antagonistic effects on mass flow through the capillary pores in root cell walls and membranes caused by higher viscosity of PEG 6000 solution. Moreover, such negative effects of PEG on plant development have previously been related to inhibitory effects on oxygen availability to the roots and/or to the presence of phytotoxic contaminants such as heavy metals (Plaut and Federman, 1985). In addition, greater relative growth rates were ascribed by Lokhande *et al*. (2010) at NaCl stressed calli of *Sesuvium portulacastrum* than iso-osmotic PEG stressed calli, primarily via osmotic adjustment achieved by buildup of saline ions in the form of osmoregulators. The PEG-stressed calli relies exclusively on the production of organic osmolytes (Pro, GB, and sugars).

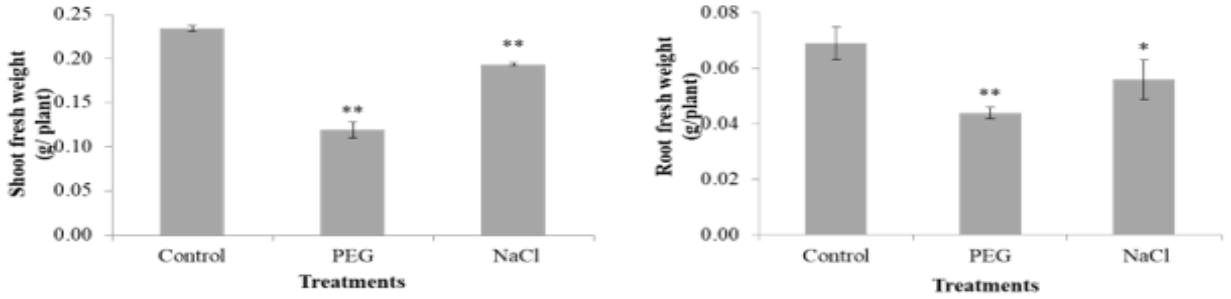

Fig. 1. Effect of NaCl (150 mM) and iso-osmotic PEG (19.5%) on shoot and root fresh weights of barely seedlings, where: ** = Highly significant (P≤ 0.01), * = Significant (0.01 ≤P≤ 0.05), and ns= non-significant (P> 0.05).

Osmotic stress exaggerates the production of hydrogen peroxide and malondialdehyde contents more than salinity stress

Results in this investigation showed that salt-treated seedlings possessed the ability to hamper H_2O_2 and MDA over-production, with regard to drought-treated ones (Fig. 2). The higher levels of H_2O_2 and MDA recorded under PEG treatment suggested that the extent of oxidative injury is much higher than defense mechanisms. According to Sahoo *et al*. (2020), PEG mediated osmotic stress resulted in oxidative burst in Chinese potato genotypes as evident by accumulation of MDA as well as H_2O_2 *in vitro*. Such results is consistent with Lan *et al*. (2020), who found high induction of MDA in PEG treatments, whilst stable MDA levels in NaCl-treated seedlings were recorded. These results confirmed the requirement of barely seedlings to low ionic stress as environmental signal for mechanism activation to stabilize membrane system. That was supported by Tahjib-UI-Arif *et al.* (2019), who confirmed the role of moderate salt stress in the induction of salt adaptation in plants.

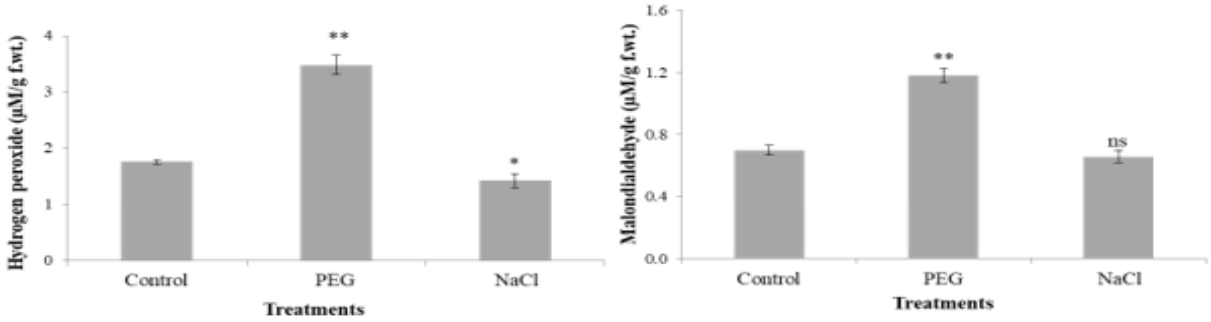

Fig. 2.Effect of NaCl (150 mM) and iso-osmotic PEG (19.5%) on hydrogen peroxide and malondialdehyde contents of barely seedlings, where: ** = Highly significant (P≤ 0.01), * = Significant (0.01 ≤P≤ 0.05), and ns= non-significant (P> 0.05).

Induction of ascorbic acid (AsA) and reduced glutathione (GSH) under PEG application

AsA as well as GSH represent key non-enzymatic antioxidant component for tolerance achievement in plants. Data presented in Figure 3 showed enhanced levels of both antioxidant molecules under PEG treatment. Interestingly, PEG application has stimulated AsA production by 61.7% compared to control. This phenomenon suggests that osmotic stress by PEG served as an environmental signal factor for induction of the non-enzymatic antioxidant mechanism. Likewise, the application of PEG has increased AsA and GSH in tolerant genotypes of Chinese potato (Sahoo *et al*., 2020). On the other hand, application of PEG possessed a stimulatory effect on GSH content by 113.5%,

whilst NaCl has provoked GSH by 32.0% compared to control. As outlined by Huang *et al*. (2005), glutathione plays a potential role in tolerance by reacting with ROS and helping AsA regeneration. The mentioned enhancement effect may indicate the high activity of glutathione reductase (GR) which led to more reduction of oxidized glutathione (GSSH) to reduced form (GSH). In addition, GSH content is proportional to the level of oxidative stress because GSH tried to maintain redox balance in the cell (Hossain *et al*., 2017). In this experiment, PEG-induced ROS imbalance is more severe than NaCl, which indicates the additional role of PEG in redox disturbance in the cell.

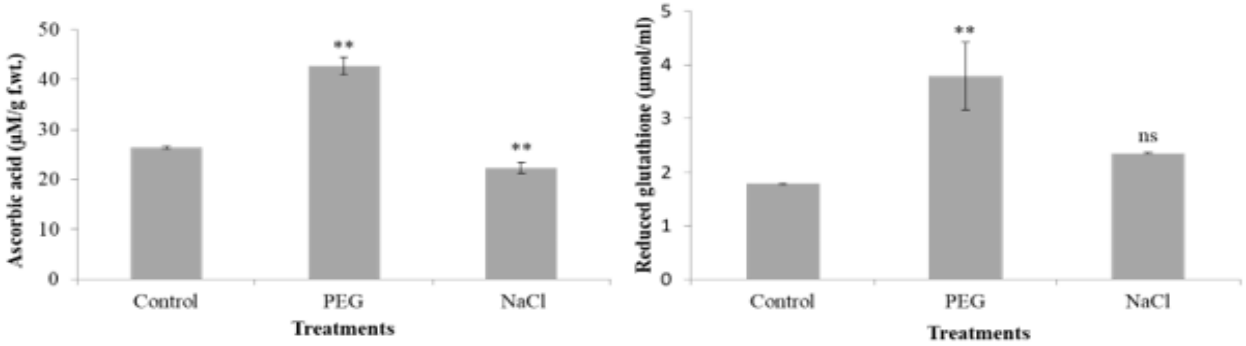

Fig. 3. Effect of NaCl (150 mM) and iso-osmotic PEG (19.5%) on ascorbic acid and reduced glutathione contents of barely seedlings, where: ** = Highly significant (P≤ 0.01), * = Significant (0.01 ≤P≤ 0.05), and ns= non-significant (P> 0.05).

Variations in the activity of antioxidant enzymes depends on stress type

Data shown in Figure 4 demonstrated higher activities of POX and PPO under PEG treatment more than NaCl, whilst higher CAT and SOD under salt treatment, compared to control. According to Radic and Pevalek-Kozlina (2010), drought-induced oxidative stress led to enhancement in the activity of anti-oxidative POX in duckweed. It was shown that CAT works with SOD to better protect plants against cell damage under various types of stresses (Scandalios, 2005). According to Foyer *et al*. (2011), the enhancement of CAT activity can conserve chloroplasts from sustained electron flows by targeting ROS action under salinity stress. The reported increase in SOD specific activity (Fig. 4) was suggested to be due to the transcriptional activation of the SOS (sodium overlay sensitive) genes in the presence of superoxide; that is *de novo* synthesis of the enzyme (Caverzan *et al*., 2016). This was compatible with Kim *et al*. (2018) who confirmed high activity of SOD in rice plants exposed to NaCl for preventing toxic accumulation of active oxygen molecules. Thus, in the present study, H_2O_2 might have up-regulated antioxidative system under NaCl-induced stresses, since CAT activity was enhanced to such an extent that they in turn ensured lower H_2O_2 levels as well as less MDA content. In view of the enzymes studied, the activation of SOD and CAT may be sufficient to prevent or minimize the detrimental consequences of oxidative stress caused by salt stress. Since the species is significantly more salt tolerant, extra antioxidant enzymes such as PPO or POX may not be activated.

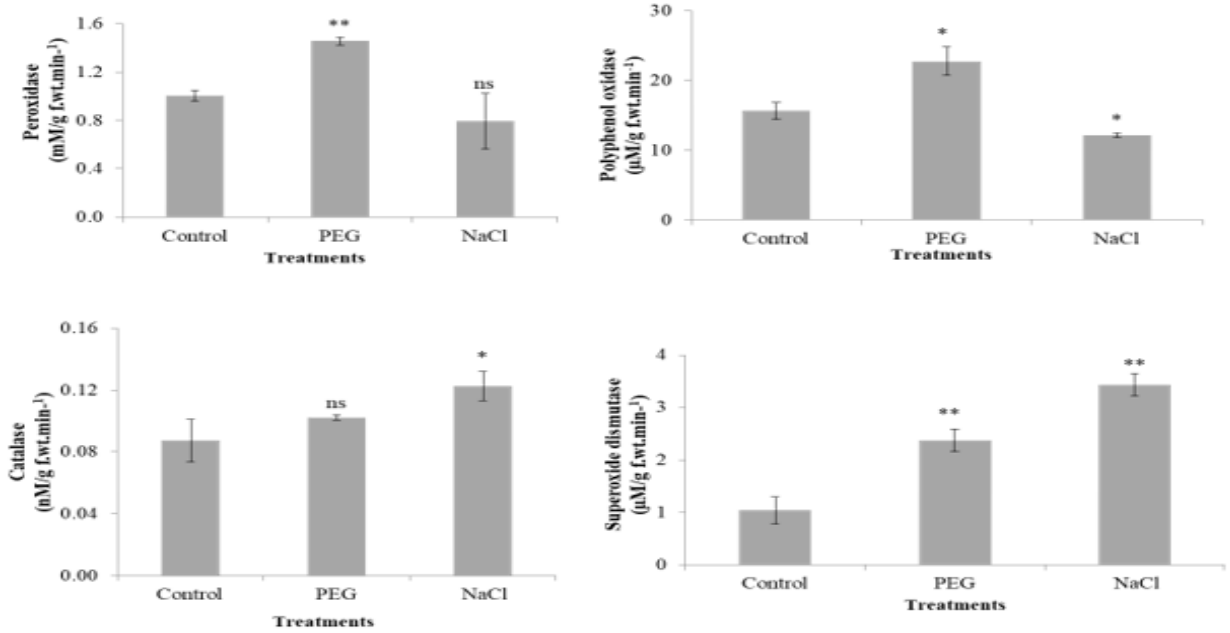

Fig. 4. Effect of NaCl (150 mM) and iso-osmotic PEG (19.5%) on antioxidant enzymes: POX (peroxidase), PPO (polyphenol oxidase), CAT (catalase) and SOD (superoxide dismutase) of barely seedlings, where: ** = Highly significant (P≤ 0.01), * = Significant (0.01 ≤P≤ 0.05), and ns= non-significant (P> 0.05).

Compared to salinity, osmotic stress enhances antioxidant capacity of barely seedlings

Results presented in Fig.5 demonstrated that seedlings supplemented with PEG had higher reducing power and DPPH (2,2- diphenyl-1-picrylhydrazyl radical) radical scavenging activity over those treated with NaCl. It may express the severity of osmotic stress initiated by PEG that led to the increment for antioxidant needs. This could be supported through the increment in GSH as well as AsA levels. Such enhancing effect may correlate with transcript abundance augmentation of key enzymes involved in antioxidative capacity such as GR and betaine aldehyde dehydrogenase, as well as choline monooxygenase (Sathee *et al*., 2021). This was compatible with Sarker and Oba (2018) who recorded higher levels of DPPH in *Amaranthus tricolor* under drought stress. The antioxidant activity based on reducing power may reflect the higher content of phenolics, which represent major contributor of antioxidant activity due to their scavenging ability on radicals. In addition, phenolics may enhance the formation of photochemical compounds which resulted in an increase in total antioxidant capacity (Wojdylo *et al*., 2007).

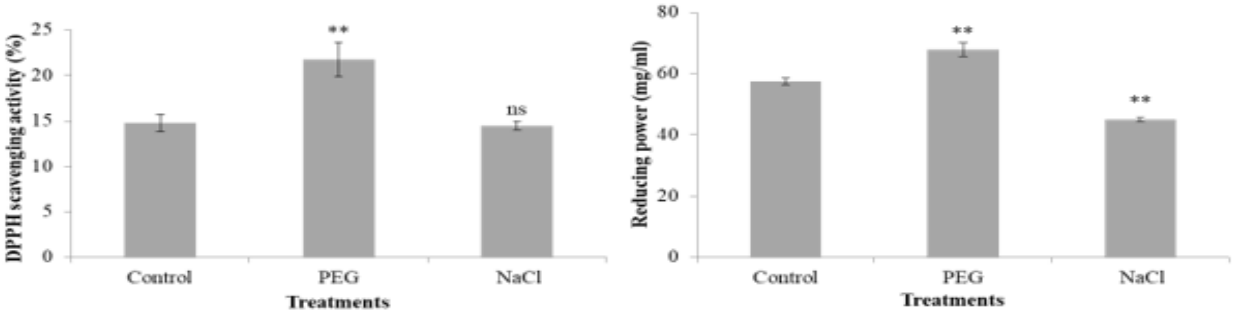

Fig. 5. Effect of NaCl (150 mM) and iso-osmotic PEG (19.5%) on DPPH (2,2- diphenyl-1-picrylhydrazyl radical) scavenging activity and reducing power of barely seedlings, where: ** = Highly significant (P≤ 0.01), * = Significant (0.01 ≤P≤ 0.05), and ns= non-significant (P> 0.05).

PEG enhances the induction of proline (Pro) and glycine betaine (GB) in roots

Data shown in Figure 6 reported a significant decrease in Pro amount in leaves by the exposure to either salt or osmotic stress. On the other hand, high increment in Pro and GB in the root tissues under osmotic stress, compared to control was detected. There is an evidence that Pro accumulation is a symptom of salt stress-induced metabolic disorders rather than being involved in its alleviation (Silveira *et al*., 2009). Likewise, Darko *et al*. (2019) reported that PEG stimulated accumulation of GB in wheat seedlings. In the salt-treated *Suaeda aegyptiaca*, there has been an enhancement in relative abundance of enzymes involved in GB biosynthesis in the form of choline monooxygenase, S-adenosyl methionine synthetase and betaine aldehyde dehydrogenase (Askari *et al*. 2006). As mentioned by Pang *et al*. (2010), salinity enhanced Pro accumulation in *Thellungiella* through augmenting enzymes responsible for Pro biosynthesis, and in the same time suppressing enzymes catalyzing hydrolysis of Pro.

Also, Ahmad *et al*. (2007) stated that Pro accumulation was lower in NaCl treated rice callus than PEG. Although Pro and GB contents were augmented under osmotic stress conditions, they were not sufficient to reduce the destructive effects caused by the accumulation of hydrogen peroxide and MDA (Figures 2 and 6). Jain *et al*. (2001) reported that considerable Pro accumulation observed under PEG-induced stress could not ameliorate consequent oxidative damage on duckweed minor growth, though it might have contributed to more efficient antioxidant enzyme activity. Results of the current study suggested the roots to be highly sensitive organs towards stress, making them in need for Pro and GB accumulation. The sensitivity was specific for roots treated with PEG, which make them preserving higher amount of Pro and preventing translation to leaves.

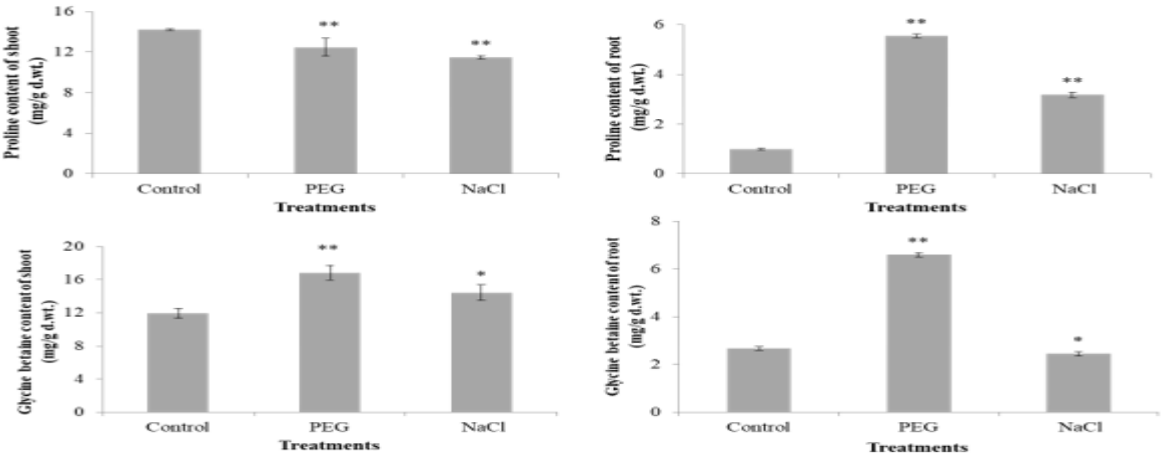

Fig. 6. Effect of NaCl (150 mM) and iso-osmotic PEG (19.5%) on proline and glycine betaine contents in both roots and leaves of barely seedlings, where: ** = Highly significant (P≤ 0.01), * = Significant (0.01 ≤P≤ 0.05), and ns= non-significant (P> 0.05).

Changes in expression of selected genes in treated barely seedlings

Identification of components of salt/drought tolerance including their ability to alter the expression of stress-responsive genes was demonstrated in Figure 7. Data showed that at the time point 48 h, *HvNHX* (Na^+/H^+ antiporter) gene was expressed greatly in the roots under salt stress, whilst it recorded the highest expression in the leaves treated with PEG. This was in accordance with Wu *et al*. (2011), who revealed that *ZxNHX* encodes a tonoplast Na^+/H^+ antiporter and plays important roles in sodium accumulation and homeostasis under salt and drought conditions in *Zygophyllum xanthoxylum*. The high expression of *ZxNHX* occurred as a result of the ability of *Z. xanthoxylum* to accumulate larger amounts of sodium than potassium in the leaves under drought conditions, even in low salt soil. As outlined by Zahran *et al*. (2007), expression levels of four *NHX* genes in the wild legume, *Melilotus indicus* were studied, where salinity induced *MtNHX1* expression at the roots. In addition, Zörb *et al*. (2005) stated that *ZmNHX* expression displayed an organ-specific pattern with no *ZmNHX* response in the shoot. However, up-regulation of *ZmNHX* expression in roots resulted in enhanced exclusion of sodium in xylem parenchyma cells that had exposed to high NaCl levels, suggesting ZmNHX genes may promote sodium accumulation in the root cell vacuoles of *Zea mays*. The previous results suggested two different mechanisms, sodium avoidance or cellular accumulation into compartments, have been developed in order that salinity may be coped with, and that expression of *NHX* antiporters is linked to the accumulator phenotype.

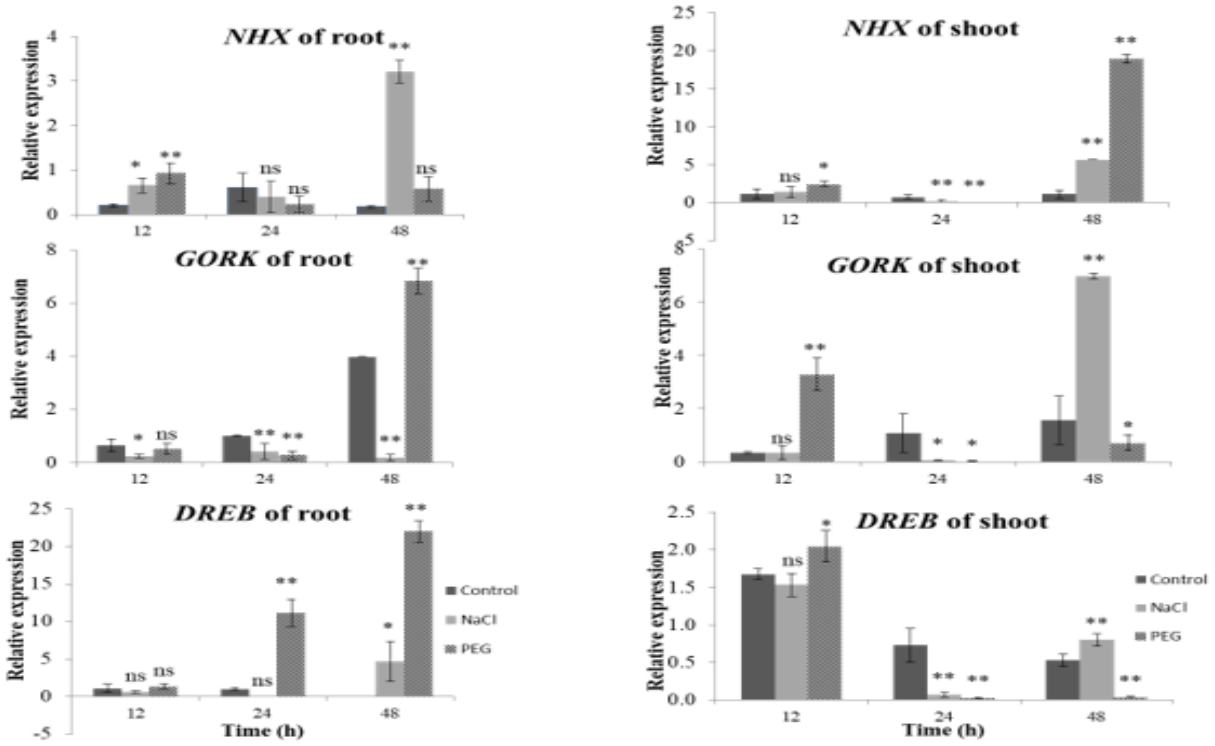

Fig. 7. Effect of NaCl (150 mM) and iso-osmotic PEG (19.5%) on relative expression of *HvNHX*, *HvDREB* and *HvGORK* genes in roots and leaves of barely seedlings at different time points, where: ** = Highly significant (P≤ 0.01), * = Significant (0.01 ≤P≤ 0.05), and ns= non-significant (P> 0.05).

K^+ release channel gene *GORK* was up-regulated upon exposure to drought, salt and cold stresses (Becker *et al*., 2003). Given the fact that NaCl-induced K^+ efflux is mediated predominantly by *GORK* channels, it would be very tempting to suggest that knocking out *GORK* genes would increase salt tolerance (Shabala and Pottosin, 2014). Our results demonstrated a strong correlation between GORK and NHX transcripts in both roots and leaves (Fig. 7). For example: At time point 48 h, *HvGORK* was highly expressed in roots under water stress, whilst expressed in shoot greatly under salinization. Such results were in an opposite manner with *HvNHX*. The paradoxical role of both genes could reflect the explanation; *NHXs* possess the ability to compartmentalize more sodium in vacuole, whilst *GORK* encodes potassium efflux from cytoplasm. Furthermore, low expression of *HvGORK* in salt-stressed roots confers salt tolerance, where Chen *et al*. (2007) stated a positive correlation between the overall salt tolerance and the ability of root tissue to retain K^+ in barely. This was compatible with Becker *et al*. (2003), who outlined that the potassium release channel *GORK* was expressed in roots under water stress in the model plant Arabidopsis. The impact of salinity on K^+ homeostasis is induction of potassium efflux from both root and leaf cells as reported by Demidchik *et al*. (2014). This efflux has been clearly established to be the result of influx of extra sodium into the cytoplasm losing compartmentation into vacuoles, with a consequential activation of *GORK*, through which K^+ is extruded. Thus, stress tolerance mechanisms can be achieved through blocking membrane depolarization by reaching a more negative inside potential, to maintain intracellular K^+ retention enhancement (Falhof *et al*., 2016).

DREB (dehydration responsive element-binding) transcription factors could be considered the first line to

achieve tolerance against desiccation. As stated by Khan (2011), most of the *DREB*-transgenic plants accumulated higher osmoprotectants in the form of Pro, GB and soluble sugar content as the main defense strategy against dehydration and salt stress, compared to that of corresponding wild plants. Our results showed high induction of *HvDREB* at the time point 48 h in the roots under both salt and drought stresses, specifically drought (Fig. 7). Nevertheless, the induction of *DREB* in leaves was decreased gradually by time, where the time point 12 has recorded the highest gene expression level among all treatments, especially PEG treatment. Likewise, the transcription of *AmDREB3* was mainly induced in roots under drought and salt stresses, while in shoots it was mainly induced by heat, UV-B, and ABA treatments in *Ammopiptanthus mongolicus* (Ren *et al*., 2019). As outlined by Li *et al*. (2005), the expression of *GmDREBc* was not induced by salt, drought, low temperature or even ABA in leaves of *Glycine max* L., whilst induced in the roots. This was incompatible with Xu *et al*. (2009) who mentioned that high levels of *HvDREB1* transcripts accumulated in leaves in response to high salinity and drought. Nevertheless, *HvDREB* mRNA began to accumulate at 1 h and reached a maximum at 2 h after salt treatment. At 24 h, no mRNA was detected. Under drought conditions, the expression pattern of *HvDREB* was similar to that with salt treatment, but the initial time of drought-induced transcription was later than with salt treatment.

CONCLUSION

NaCl and drought are among the most remarkable abiotic factors limiting crop growth and plant production. Although PEG and NaCl possess similar osmotic potentials, barely plants responded to them by different physiological and molecular mechanisms. PEG was responsible for more damage more than NaCl; where plants supplemented with PEG confer more antioxidants and osmolytes to counteract the deleterious effects of oxidative stress verified by higher hydrogen peroxide level and malondialdehyde content. The presented data suggests that both physiological drought and salinity lead to changes in the regulation of a basic set of salt tolerant genes (*HvNHX*, *HVGORK and HvDREB*), but with different adaptive changes. It could be concluded that salt tolerance of barely (cultivar Giza 134) is almost determined through their ability to withstand excessive Na^+ and Cl^- ions rather than their ability of osmotic stress tolerance.

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دراسة إستجابات بادرات الشعير للإجهاد الملحي والأسموزي: الإجهاد بالأكسدة ومضاداتها يلعبان دورا هاما

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يعتبر كل من الإجهاد الملحي والأسموزي سببين رئيسين لانخفاض نمو وإنتاجية النبات وذلك في الأراضي شبه القاحلة يتبعها مجموعة من الاستجابات علي المستويات الفسيولوجية والخلوية والجزيئية. ونظرا لأن كلا من التأثيرات الاسموزية والأيونية يرتبطان معا وينتجان تحت ظروف الإجهاد الملحي, فإن فصل هذين المكونين يعد خطوة هامة لفهم أساسيات التكيف مع الملوحة. في الدراسة الحالية تم معاملة صنف الشعير (جيزة 134) إما بكلوريد الصوديوم (150 مل مول) أو البولي إيثلين جليكول المساوي له أسموزيا (19.5%) وقد تم ترتيب الأوعية طبقا لتصميم القطاعات الكاملة العشوائية بوضع كل عشرة نباتات في وعاء.وتم تطبيق المعاملات مرتين قبل التجميع والذي تم بعد أسبوعين من خروج البادرات. وقد أوضحت النتائج حدوث تقلص في الوزن الطازج في البادرات المعاملة وخاصة تلك التي تم معاملتها بالبولي إيثلين جليكول. وقد تم تسجيل مستويات عالية من علامات الأكسدة ومضاداتها الإنزيمية واللا إنزيمية وذلك تحت الإجهاد الأسموزي. وقد حدثت زيادة واضحة في علامات الأكسدة المتمثلة في المالون داي ألديهيد وفوق أكسيد الهيدروجين وذلك عند المعاملة بالبولي إيثلين جليكول.علاوة علي ذلك حدثت زيادة واضحة في مضادات الأكسدة اللاإنزيمية في صورة حمض الأسكوربيك والجلوتاثيون المختزل بالإضافة إلي الكشف عن نشاطات عالية من مضادات الأكسدة الإنزيمية المختبرة مثل البيروكسديز والبولي فينول أكسديز وذلم بالمعاملة بالبولي إيثلين جليكول. وعلي الجانب الاخر فقد حدث تراكم لنشاطات عالية من إنزيمي السوبر أوكسيد ديسميوتيز والكاتاليز وذلك في النباتات التي تم ريها بالملح أكثر من البولي إيثلين جليكول.وقد حدثت زيادة في المنظمات الأسموزية مثل البرولين والجليسين بيتاين في نسيج الجذر وذلك بالتزامن مع زيادة نظام مضادات الأكسدة في الأوراق وذلك بتطبيق البولي إيثلين جليكول.واعتمادا علي التحليل الجزيئي باستخدام تفاعل البوليميراز المتسلسل, وجد أن الجين إن إتش إكس فقد زاد تعبيره الجيني بعد مرور 48 ساعة من معاملة الجذور بالملح في حين برز تعبيره الجيني في الأوراق عنها في الجذور تحت ظروف الجفاف والعكس كان صحيحا بالنسبة ل جين جورك. وعلي الجانب الاخرفقد سجل جين الدريب أعلي تعبير جيني له أثناء معاملة الجذور بالبولي إيثلين جليكول وذلك بمقارنته مع المعاملة الضابطة. وتشير الدراسة الحالية إلي أن صنف الشعير المستخدم تحت الدراسة يملك مقاومة أعلي للإجهاد الملحي عن الإجهاد الأسموزي بالبولي إيثلين جليكول ولذلك فإنه يكون أكثر ملائمة للزراعة تحت ظروف الملوحة.

كلمات البحث: الشعير- الأصناف- البولي إيثلين جليكول- الملوحة- الضغط الأسموزي- علامات الأكسدة- مضادات الأكسدة- التعبير الجيني.