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Profile of antioxidant enzymes in two Bulgarian barley cultivars at early growth stage, differing in salt stress response

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Abstract

Soil salinization is among the major crop yield limiting factors in contemporary agriculture. Excessive irrigation and climate changes are among the causes for increased salinity in previously unaffected areas, including the Balkan Peninsula. The development of salt tolerant cultivars of existing crops is essential for meeting the growing food production necessity and for utilization of salinized agricultural areas. Marker assisted selection is among the promising approaches for fast and efficient screening of newly developed cultivars for tolerance to various stresses. The identification of genetic and protein markers for stress tolerance is an essential tool for accelerating of breeding programs and development of modern agriculture. Along with osmotic stress and ion toxicity, high NaCl concentrations negatively affect plant growth and development by promoting burst in reactive oxygen species formation. Antioxidative systems are essential for overcoming of this negative effect. In the present study two Bulgarian barley cultivars – Bozhin and IZ Bori were studied. Four days old seedlings were evaluated for their ability to grow at 0.15 and 0.3 M NaCl and proteins were isolated from roots and leaves. Antioxidant enzymes: peroxidases, ascorbate peroxidases, catalases, superoxide dismutases and glutathione reductases were studied. Enzymatic profiles obtained by zymographic analyses after electrophoretic separation showed several isoforms associated with salt stress response and salt stress tolerance. Further analyses and comparison of zymographic to genetic and metabolomic data would further reveal differences in the two cultivars and establish appropriate molecular markers for salt tolerance.

Keywords: antioxidative enzymes, reactive oxygen species, salt stress, zymographic analysis

Introduction

Soil salinity is a complex worldwide phenomenon suspected to be the main responsible factor for the decreased plant productivity. According to the Food and Agriculture Organization (FAO) of the United Nations up to year 2002 the irrigated croplands have reached 17% and now are growing by 1-2% every year. As these lands were utilized for agricultural needs and are believed to provide almost 40% of the world`s food supply (mostly rice, wheat, maize and barley) there is a certain need for research in the field of stress-tolerant culture plants.

Soil salinity is one of the main stress factors with considerable influence on plants regardless of culture species. The presence of high salt concentrations in the environment is a principle reason for the induction of oxidative stress in plant cells. Oxidative stress may cause irreversible cell damage for the plant as it is defined as the impairment of the balance between the production and deactivation of reactive oxygen species (Munns and Tester, 2008). The cell machinery contains a variety of enzymes implicated in the antioxidant defense system. The enzymes of the ascorbate-glutathione cycle are among the most important players in the maintenance of favorable redox potential (Noctor and Foyer, 1998) by detoxification of reactive oxygen species produced in deleterious stress conditions. In the first step H_2O_2 is reduced to water by ascorbate peroxidase (APX; EC 1.11.1.11), using ascorbate as electron donor thus producing monodehydroascorbate. The regeneration of

ascorbate involves several steps, eventually leading to oxidation of the tripeptide glutathione (GSH) to glutathione disulphide. Glutathione reductase (GR; EC 1.8.1.7) is responsible for the maintenance of high GSH to GSSG ratio by reduction of GSSG produced in result of ascorbate regeneration as well as during direct H_2O_2 detoxification by glutathione peroxidase and in other oxidative processes (Noctor and Foyer, 1998).

Hydrogen peroxide is also detoxified by catalases (CAT; EC 1.11.1.6) and various peroxidases (POX; EC 1.11.1.x), also responsible for the reduction of other peroxides, produced in conditions of oxidative stress (Gill and Tuteja, 2010). Hydrogen peroxide is also produced during the reactions of superoxide (O_2^-) elimination by superoxide dismutases (SOD; EC 1.15.1.1). The above mentioned enzymatic systems comprise a significant part of the intracellular antioxidant machinery and are often associated with abiotic stress responses.

Various studies in recent years show the significance of antioxidant enzymes in salt stress response in cereals as maize (de Azevedo Neto et al., 2006), barley (Kim et al., 2005; Yildiz and Terzi, 2013; Maksimovic et al., 2013; Adem et al., 2014; Fan et al., 2014), wheat (Esfandiari et al., 2007; Bhutta, 2011) etc. The change in enzymatic activity and isoform profiles was established as a suitable approach for discrimination between salt tolerant and salt sensitive cultivars of agriculturally important crops (Kim et al., 2005).

Barley production in Bulgaria comprises approximately 8% of total grain yield (National Grain Producers Association, 2012). A number of local malting and feed barley cultivars were developed through classical selection and chemical mutagenesis in the last 30 years in the Institute of Agriculture, Karnobat (Agricultural Academy, 2014). However most of the efforts were directed toward improvement of winter and cold hardiness as well as disease resistance to powdery mildew, covered Smut, brown and black stem rust etc. Little or no studies were conducted for characterization of salt tolerance of the existing cultivars. Considering the growing importance of soil salinity in contemporary agriculture, it would be of particular interest to monitor the tolerance potential of the existing barley genetic resources in the country in order to direct selection toward development of salt tolerant cultivars. In the present pilot study we focused on the profiling of antioxidant enzymes in response to salt stress of two Bulgarian feed barley cultivars, characterized by the highest yield within the group of 6-row cultivars. This study aims to provide an efficient and reliable monitoring system which in combination with genetic markers and physiological traits would further improve our knowledge on the local cultivars potential. In addition it will outline a successful breeding strategy for further improvement of salt tolerance of Bulgarian barley.

Materials and Methods

Seeds from IZ Bori and Bozhin winter feed barley cultivars were generously provided by the Institute of Agriculture, Karnobat, Bulgaria. After surface sterilization for 10 min with 5% sodium hypochlorite seeds were germinated in tap water soaked paper rolls for three days in the dark in diurnal growth chamber, Forma Scientific at 23^{o}C. Roots and shoot lengths were measured and subsequently seedlings (a total of 15 per NaCl concentration) were transferred to paper rolls, soaked with 0, 0.15 and 0.3 M NaCl in tap water solution for additional 72 hours at 23^{o}C and 12/12 photoperiod. The concentrations of Na and Cl ions in the tap water were not taken into account. The average figures in Sofia region for a six month period in 2014 are as follow: pH 7.4, electro conductivity 97.3 µs cm^{-1}, Na^+ concentration less than 0.0002 mol l^{-1} and Cl^- concentration 0.008 mol l^{-1} (public information by Laboratory Testing Complex in Sofiyska Voda AD). Upon termination of the stress conditions roots and leaves lengths were measured again and plant material was immediately frozen into liquid nitrogen. Growth was estimated as final length (in cm) minus initial length of roots and leaves for each individual seedling, than averaged and expressed as percentage of controls.

For protein isolation plant material was grinded to powder in liquid nitrogen, resuspended in phosphate buffered saline (PBS), pH 7.2, centrifuged at 10 000 x g at 4^{o}C for 15 minutes and supernatants, containing soluble intracellular proteins were separated for further analyses. Protein concentrations were determined with BCA protein estimation kit (Pierce) according to producer's protocol with bovine serum albumin used as standard protein solution.

All electrophoretic separations were conducted according to Laemmli`s protocol (Laemmli, 1970) in 12.5% T resolving gels and 4% T stacking gels. For native electrophoreses SDS was omitted from gels, sample buffer and running buffer. After denaturing electrophoreses proteins were renatured in 2.5% Triton X 100 for 30 min at room temperature. All zymographic analyses were

performed according to protocols, available in Handbook of detection of enzymes on electrophoretic gels (Manchenko, 2003).

Glutathione reductase activity assay is based on the reduction of NADPH. A replica gel, containing 0.1 M TRIS.HCl buffer pH 8.0, 0.2% NADPH, 1.5% glutathione disulphide (GSSG) in 1% w/v agarose solution was poured over the polyacrylamide gel. The gels were incubated 37°C for 30 min and observed under UV light. Glutathione reductase isoforms appeared as dark bands on light background.

For catalase activity electrophoretic gels were supplemented with 0.5% soluble starch. After electrophoresis gels were incubated for 3 min in 60 mM $Na_2S_2O_3$ in 20 mM H_2O_2 at room temperature, washed briefly in distilled water and stained in 90 mM KI. Catalase isoforms were visualized as clear bands on dark background.

Peroxidase activity assay is based on the oxidation of diaminobenzidine (DAB) in the presence of hydrogen peroxide. The gels were incubated in 10 mM potassium phosphate buffer pH 7.2, containing 4 mg ml^{-1} DAB, 20 mM H_2O_2 and 2 mg ml^{-1} $CoCl_2$ as enhancer at room temperature until brown bands appeared.

Superoxide dismutase activity protocol is based on the oxidation of o-dianisidine in the presence of O_2^-. Electrophoretic gels were incubated with staining solution comprised of 10 mM potassium phosphate buffer pH 7.2, 2mM o-dianisidine and 0.1 mM riboflavin in the dark for 60 min, then exposed to light until brown bands appeared.

Ascorbate peroxidase activity was visualized with staining solution containing 2.45 mM nitroblue tetrazolium (NBT) and 28 mM TEMED after incubation in 4 mM L-ascorbate, 2 mM H_2O_2.

All zymograms were photographed, digitally enhanced and analyzed on ImageJ software. The intensity of each band was measured and compared on the same software.

Results

Four days seedlings of two feed Bulgarian barley cultivars were subjected to salt stress experiments in order to identify polymorphisms in the izoenzyme profiles that can be used as potential markers in breeding programs for salt stress tolerance. The applied stress treatments (0.15 and 0.3 M NaCl) induce reduction in the length of both shoots and roots in comparison to control in the studied 2 barley cultivars. This reduction is most pronounced in roots than in leaves. The latter is manifested better in cv. Bozhin (fig. 1). These data are in accordance with the observed differences in some physiological parameters of the studied here cultivars (data not shown).

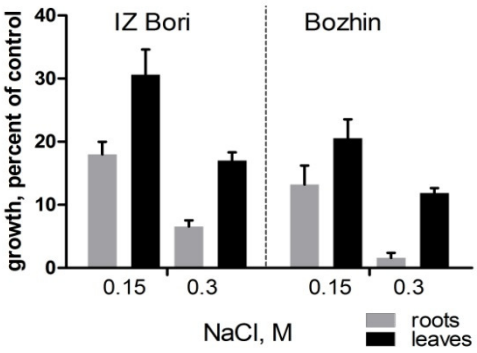

Figure 1. Average roots and leaves growth after 72h salt treatment.

Profiling of several enzymes involved in antioxidant defense system such as glutathione reductase, catalase, peroxidase superoxide dismutase and ascorbate peroxidase was performed.

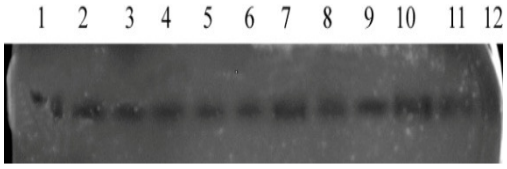

Figure 2. Glutathione reductase zymogram after 12.5 % T Native PAGE. Samples are in the following order: IZ Bori, roots: 1 – control; 2 – 0.15 M NaCl; 3 – 0.3 M NaCl; Bozhin, roots: 4 – control; 5 – 0.15 M NaCl; 6 – 0.3 M NaCl; IZ Bori, leaves: 7 – control; 8 – 0.15 M NaCl; 9 – 0.3 M NaCl; Bozhin, leaves: 10 – control; 11 – 0.15 M NaCl; 12 – 0.3 M NaCl.

One isoform of GR was identified in both cultivars in roots as well as in leaves at all NaCl concentrations (fig. 2). Zymographic profile was obtained after electrophoresis in native conditions. No significant change in activity in response to salt stress was detected densitometrically. Similarly the APX zymographic profile after denaturing electrophoresis (fig. 3) did not show significant changes in APX activity in any of the four isoforms detected.

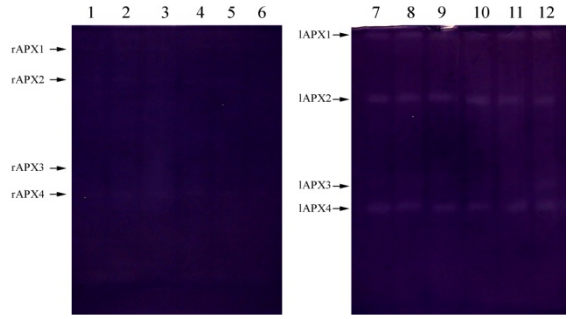

Figure 3. Ascorbate peroxidase zymogram after 12.5 % T SDS PAGE. Samples are in the following order: IZ Bori, roots: 1 – control; 2 – 0.15 M NaCl; 3 – 0.3 M NaCl; Bozhin, roots: 4 – control; 5 – 0.15 M NaCl; 6 – 0.3 M NaCl; IZ Bori, leaves: 7 – control; 8 – 0.15 M NaCl; 9 – 0.3 M NaCl; Bozhin, leaves: 10 – control; 11 – 0.15 M NaCl; 12 – 0.3 M NaCl.

A number of CAT isoforms were visualized after electrophoresis in native conditions (fig. 4) that appeared as large diffuse bands in the beginning of the resolving gel. The more pronounced change was observed in Bozhin where the activity gradually decreased in roots and increased in leaves in response to salt treatment.

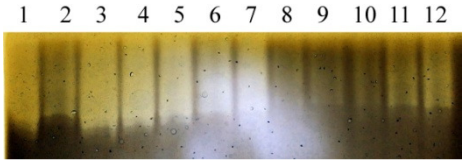

Figure 4. Catalase zymogram after 12.5 % T Native PAGE. Samples are in the following order: IZ Bori, roots: 1 – control; 2 – 0.15 M NaCl; 3 – 0.3 M NaCl; Bozhin, roots: 4 – control; 5 – 0.15 M NaCl; 6 – 0.3 M NaCl; IZ Bori, leaves: 7 – control; 8 – 0.15 M NaCl; 9 – 0.3 M NaCl; Bozhin, leaves: 10 – control; 11 – 0.15 M NaCl; 12 – 0.3 M NaCl.

The most significant profile changes were observed in POX (fig. 5) and SOD (fig. 6) zymograms after denaturing electrophoreses. Seven POX isoforms were visualized in roots and 5 in leaves of both barley cultivars (fig. 5).

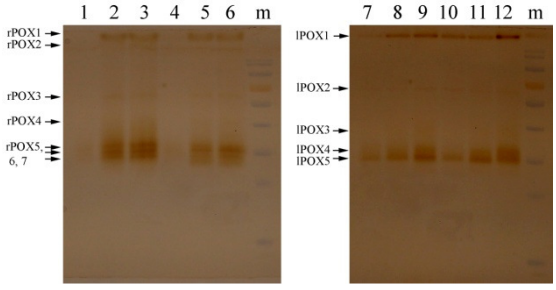

Figure 5. Peroxidase zymogram after 12.5 % T SDS PAGE. Samples are in the following order: IZ Bori, roots: 1 – control; 2 – 0.15 M NaCl; 3 – 0.3 M NaCl; Bozhin, roots: 4 – control; 5 – 0.15 M NaCl; 6 – 0.3 M NaCl; IZ Bori, leaves: 7 – control; 8 – 0.15 M NaCl; 9 – 0.3 M NaCl; Bozhin, leaves: 10 – control; 11 – 0.15 M NaCl; 12 – 0.3 M NaCl; m – protein standards.

The activity of all of them increased in response to salt treatment. In roots all of the POX isoforms were barely visible in controls and showed equal increase both at 0.15 and 0.3 M NaCl. The activity of POX isoforms in leaves gradually increase from controls to 0.3 M NaCl treatment. Similar observations were recorded for the SOD isoforms in roots and leaves (fig. 6). However one of the SOD isoforms in roots (37 kDa) showed increase in activity in response to salt treatment only in IZ Bori cultivar and two SOD isoforms in leaves (40 and 70 kDa) showed significant increase in activity only in Bozhin. The results of the zymographic profiling of all five antioxidant enzymes are summarized in table 1.

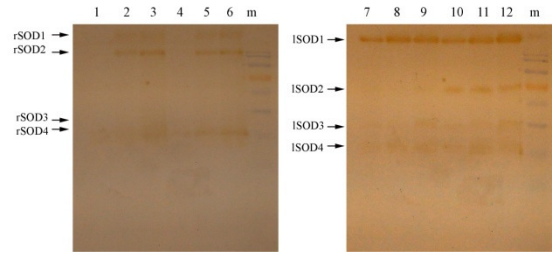

Figure 6. Superoxide dismutase after 12.5 % T SDS PAGE. Samples are in the following order: IZ Bori, roots: 1 – control; 2 – 0.15 M NaCl; 3 – 0.3 M NaCl; Bozhin, roots: 4 – control; 5 – 0.15 M NaCl; 6 – 0.3 M NaCl; IZ Bori, leaves: 7 – control; 8 – 0.15 M NaCl; 9 – 0.3 M NaCl; Bozhin, leaves: 10 – control; 11 – 0.15 M NaCl; 12 – 0.3 M NaCl; m – protein standards.

Discussion

The Institute of Agriculture in Karnobat, Bulgaria currently offers seeds of four winter feed barley (*Hordeum sativum* Jess. subsp. vulgare L.) cultivars, originally developed there. Three of them – Vesletc, Aheloy 2 and Bozhin were released back in 1994 and one – IZ Bori is newly developed and released to the market in 2010. IZ Bori and Bozhin cultivars are characterized by the highest yield – 840 – 900 kg da^{-1} compared to 800 kg da^{-1} in Aheloy 2 and 720 kg da^{-1} in Vesletc. All of the cultivars showed good cold tolerance and good to very good disease resistance (Agricultural Academy, 2014). IZ Bori and Bozhin cultivarswere chosen for the present study based on these characteristics.

Based on the acquired results the newly developed cultivar IZ Bori showed better growth than Bozhin in response to salt treatment. Based on

some physiological parameters (unpublished data) these two cultivars were less affected by salinity treatment than the other two feed barley cultivars. The deleterious effect of NaCl was more pronounced on roots length rather than leaves. Considering that plant roots are under the direct impact of high soil salinity it is expected that the growth inhibition is more pronounced especially during the first days of salt stress.

Table 1. List of the visualized isoforms of antioxidant enzymes withotchange or with increased activity. Different isoforms are labeled as on the zymograms. Molecular weight in kDa is shown in brackets.

Antioxidant enzymes isoforms	
leaves	
no change	increased
GR 1	CAT (in Bozhin)
lAPX1 (>200 kDa)	lPOX1(>200kDa)
lAPX2 (65 kDa)	lPOX2(65kDa)
lAPX3 (35 kDa)	lPOX3(39kDa)
lAPX4 (>30 kDa)	lPOX4(36kDa)
lSOD1 (>200 kDa)	lPOX5(35kDa)
	lSOD2(70kDa) in Bozhin
	lSOD3(40kDa) in Bozhin
roots	
no change	increased
GR1	rPOX1(>200kDa)
rAPX1(190kDa)	rPOX2(>200kDa)
rAPX2(95kDa)	rPOX3(70kDa)
rAPX3(35kDa)	rPOX4(65kDa)
rAPX4(30kDa)	rPOX5(36kDa)
	rPOX6(35kDa)
	rPOX7(32kDa)
	rSOD1(>200kDa)
	rSOD2(170kDa)
	rSOD4(35kDa)
	rSOD3(37kDa)–in IZ Bori

Previous reports showed that an overall increase in activity of all antioxidant enzymes is observed in conditions of salt stress and that the increase is more consistent in roots (Kim et al., 2005). This response is already significant in the very first stages of salt stress response (24 h). However the activity of any of the enzymes did not show significant changes after reaching its peak in the first hours (Kim et al., 2005).

As shown in recent reports (Kim et al., 2005) the response is different in roots and shoots either as total activity in assay and as isoenzymes detected. Catalase is the only of the five enzymes with higher activity in shoots rather than roots. The major organ-specific differences in the isoenzyme profiles were observed for SOD and POX while CAT and APX showed comparatively similar isoforms in both shoots and roots (Kim et al., 2005). Similarly in our experiment the major isoenzyme differences were observed for POX (Fig. 5) and SOD (Fig. 6).

Yildiz and Terzi (Yildiz and Terzi, 2013) showed differential response in activity of antioxidant enzymes in leaves of two contrasting in salt tolerance Turkish barley cultivars. While POX and APX activities were higher in the salt-tolerant cultivar at all NaCl concentrations, CAT and SOD tend to be higher in the salt-sensitive cultivar at higher salt molarity but lower at 0.1 M NaCl. Similary CAT and SOD isoforms showed increased activity in leaves of Bozhin (Fig. 4 and 6). Leaves peroxidases were increased in both Bulgarian cultivars (Fig. 5).

Maksimovic et al (Maksimovic et al., 2013) established some significant correlation between antioxidant enzymes activity in roots and salt stress response of barley genotypes. In summary, APX did not prove indicative for salt stress tolerance. In contrast CAT and SOD activities were constitutively higher in salt-sensitive cultivar and CAT activity increased in salt-tolerant cultivar. Our results further show that the changes in activity of particular isoforms successfully discriminate between cultivars with similar but still differing salt stress response. While APX profile and activity did not change (Fig. 3), CAT activity decreased in the more sensitive cultivar (Fig. 4). Superoxide dismutase isoforms activity in roots increased correlatively to NaCl concentration (Fig. 6).

In addition CAT activity in leaves tend to increase in salt-tolerant cultivars and decrease in salt-sensitive ones after prolonged treatment – 10 days (Fan et al., 2014). An opposite tendency was observed in our experiments although the results are not directly comparable due to the shorter period of treatment and the earlier stage of seedling development. However it should be noted that while APX is not indicative for salt tolerance differences, the changes in the other antioxidant enzymes could

be cultivar specific and misleading especially for salt sensitive cultivars (Fan et al., 2014). Such differences may be useful for differentiation between cultivars with similar phylogenic history.

However the above mentioned (Maksimovic et al., 2013; Fan et al., 2014) reports concluded that salinity induced changes in activity of antioxidant enzymes in either roots or leaves could not be used as biochemical markers in breeding for salt tolerance in barley. Therefore our efforts were directed toward the possibility that specific isoforms could be associated with better growth in high salinity conditions. It is obvious that significant differences in profiles could be observed in a tissue and cultivar dependent manner (Kim et al., 2005). Our preliminary results established CAT, POX and SOD as potential targets for further investigations. It should be also noted that the salt tolerance evaluation of particular cultivar is a complicated and multifactor dependent task (Munns, 2002) and various treatment time and growth conditions should be also applied to acquire a more complete characterization of the existing barley cultivars.

Conclusion

In conclusion, the preliminary results showed that APX and GR profiles are not suitable for evaluation of the salt stress response of the studied Bulgarian barley cultivars at early growth stage. Specific CAT, POX and SOD isoforms may be useful as a supplementary tool to various genetic and physiological analyses for better characterization of salt stress response of Bulgarian barley cultivars and to establish an effective breeding strategy for improvement of salt stress tolerance of Bulgarian barley.

References

Adem, G.D., Roy, S.J., Zhou, M., Bowman, J.P., Shabala, S. 2014. Evaluating contribution of ionic, osmotic and oxidative stress components towards salinity tolerance in barley. *BMC Plant Biol* 14:113

Agricultural Academy, Institute of Agriculture Karnobat. 2014. Catalog of varieties of barley, oat and wheat, bred in the Institute of Agriculture - Karnobat

Bhutta, W.M. 2011. Antioxidant activity of enzymatic system of two different wheat (*Triticum aestivum* L.) cultivars growing under salt stress. *Plant Soil Environ* 57: 101-107.

de Abreu, C.E.B., Gomes-Filho, E. 2006. Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environ Exp Bot* 56: 87–94.

de Azevedo Neto, A.D., Prisco, J.T., Eneas-Filho, J., Esfandiari, E., Shekari, F., Shekari, F., Esfandiari, M. 2007. The effect of salt stress on antioxidant enzymes' activity and lipid peroxidation on the wheat seedling. *Not Bot Hort Agrobot Cluj* 35: 48 – 56.

Gill, S.S., Tuteja, N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem* 12: 909 – 930.

Fan, Y., Zhu, M., Shabala, S., Li, C.D., Johnson, P., Zhou, M.X. 2014. Antioxidant activity in salt-stressed barley leaves: evaluating time- and age-dependence and suitability for the use as a biochemical marker in breeding programs. *J Agro Crop Sci*

Food and Agriculture Organization of the United Nations. www.fao.org

Kim, S.Y., Lim, J.H., Park, M.R., Kim, W.J., Park, T.I., Seo, Y.W., Choi, K.G., Yun, S.J. 2005. Enhanced antioxidant enzymes are associated with reduced hydrogen peroxide in barley roots under saline stress. *J Biochem Mol Biol* 38: 218-224.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680 – 685.

Maksimović, J.D., Zhang, J., Zeng, F., Živanović, B.D., Shabala, L., Zhou, M., Shabala, S. 2013. Linking oxidative and salinity stress tolerance in barley: can root antioxidant enzyme activity be used as a measure of stress tolerance? *Plant Soil* 365:141–155.

Manchenko, G.P. 2003. Handbook of detection of enzymes on electrophoretic gels. *CRC Press*

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

Munns, R. 2002. Comparative physiology of salt and water stress. *Plant Cell Environ* 25: 239-250.

National Grain Producers Association. 2012. www.grain.bg

Noctor, G., Foyer, C.H. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Molec Biol* 49:249–279.

Yildiz, M., Terzi, H. 2013. Effect of NaCl stress on chlorophyll biosynthesis, proline, lipid peroxidation and antioxidative enzymes in leaves of salt-tolerant and salt-sensitive barley cultivars. *J Agri Sci* 19: 79-88.