PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR STUDIES ON SALT TOLERANCE OF BULGARIAN 6-ROW BARLEY CULTIVARS

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Abstract

Soil salinization is among the major crop yield limiting factors in modern agriculture. Excessive irrigation and climate changes are among the main factors leading to increased salinity in previously unaffected areas, including the Balkan Peninsula and Bulgaria. The identification of salt tolerant cereal crops is essential for utilization of the salinized agricultural areas. Several approaches (physiological, biochemical and molecular) have been considered as important tools for identification of more tolerant cultivars and lines of cereal crops, including barley.

The photosynthetic activity and the membrane stability of 4 Bulgarian 6-row barley cultivars IZ Bori, Aheloj2, Bozhin and Vesletc, grown under 250mM NaCl for 10 days, was estimated by measuring the chlorophyll fluorescence and electrolyte leakage. Salt treatment of barley plants at the third leaf stage showed stability of the photosystem II (PSII), as evaluated from chlorophyll fluorescence data. However, some parameters such as ratio of fluorescence decay (Rfd), *proportion of light* not used for photochemistry (LNU) and non-photochemical quenching (NPQ) changed and these changes depend on the duration of the salt treatment and the genetic potential of barley cultivars. Electrolyte leakage measurement showed genotype variation in the response to salt stress. The photosynthetic apparatus and the membrane stability of the cultivar IZ Bori were found to be much more tolerant to salt treatment, compared with the remaining cultivars, with the lowest one for cv. Vesletc and Aheloj2. Among the biochemical markers used for studying the response to salt stress, the antioxidant enzymes such as peroxidases, ascorbate peroxidases, catalases, superoxide dismutases and glutathione reductases were used as indicators of tolerance to oxidative stress induced during the initial stage of the salt stress in two of barley cultivars (Bozhin and IZ Bori). Enzyme profiles showed several isoforms associated with salt stress response in both cultivars as well as with higher salt stress tolerance of IZ Bori. Molecular markers such as SSRs previously shown to be located near to some QTLs expressed under abiotic stress showed specific grouping of the four cultivars in 2 subclusters of the constructed UPGMA dendrogram which is in a correspondence with the physiological analysis.

The SSR analysis, enzyme profiling, the photochemical activity of PSII and electrolyte leakage measurements revealed considerable differences between the genotypes under salinity stress which suggests the presence of genetic variation and the possibility of selection for salinity tolerant genotypes.

Key words: barley (*H. vulgare* L.), salt stress, Photosystem II, antioxidant enzymes, SSR (Simple Sequence Repeats) markers, genetic variation

INTRODUCTION

Barley (*Hordeum vulgare* L.) is the fourth largest cereal crop worldwide with application in brewing and as a fodder crop. It is one of the most salt tolerant crop species (Jiang et al., 2006). Among the abiotic stresses, salinity limits barley production and it is one of the major abiotic stresses, especially in arid and semi-arid regions where salt concentration is similar to that of the seawater (Pareek et al., 2010). Plants respond to salt stress through modifications of their morphological, physiological and metabolic processes. Salinity affects plant growth due to changes in many physiological processes including photosynthesis (Kalaji & Guo, 2008). Increase of lipid peroxidation, proline content, peroxidase, electrolyte leakage, activity of superoxide dismutase (SOD), ascorbate peroxidase, catalase and glutathione reductase, and decrease in relative water content and pigment content in barley have been also reported under salinity stress (Mian et al., 2011).

Selection of barley cultivars with higher tolerance to salt stress has been considered as economic and efficient means of utilizing salt-prone areas when combined with appropriate management practices (Quisenberry, 1992). Therefore, improving salt tolerance is one of the major objectives in plant breeding programs for crops grown in arid and semi-arid areas. Several tools have been identified as informative, rapid and non-intrusive for identification of barley genotypes with increased tolerance to salt stress: chlorophyll fluorescence, leaf water potential, RWC, etc. (Matin et al. 1989; Maxwell & Johnson, 2000), biochemical (Ashraf & Harris, 2004) and molecular markers. The latest have shown excellent potential to assist selection of quantitative trait loci (QTLs), associated with physiological and agronomical traits expressing under salinity stress at different stages of development (Xue et al., 2009).

Soil salinisation is one of the major soil degradation problems in Southern European countries including Bulgaria and global warming is expected to increase the threat of secondary salinisation. The most problematic salt-affected areas in Bulgaria are regions like Sliven, Yambol, Burgas, Plovdiv and V. Tarnovo, encompassing more than 35000ha (http://www.eea.government.bg/). The main reasons for soil salinity are high level of underground water mineralisation and drying of wetlands. In the latest years soil salinity is supported by irregular and changeable irrigation. Up to now little studies were conducted for determining salt stress tolerance of Bulgarian barley cultivars. Considering the growing impact of this problem on the modern agriculture, it is of high importance to determine the salt tolerance capacity of barley genetic resources.

In the present pilot study we focused on assessment of some physiological parameters, antioxidant enzymes in response to salt stress of 4 Bulgarian feed barley cultivars and on the genetic diversity available in 6-row germplasm in Bulgaria. This study aims to provide an efficient and reliable monitoring system which in combination with genetic markers would further expand our knowledge on the local cultivars potential. It can be further used for development of successful breeding strategy for improvement of salt tolerance of Bulgarian barley.

MATERIALS AND METHODS

Plant material

Four 6-row barley cultivars (Aheloj2, Vesletc, IZ Bori and Bozhin) developed in the Institute of Agriculture, Karnobat, Bulgaria were used. Most of the genotypes showed tolerance to drought.

Chlorophyll fluorescence measurements

For synchronization of germination, seeds were placed on wet (H_2O) filter paper in glass dishes at 4°C in the dark for 3 days. Then they were transferred to hydroponic system using modified Hoagland solution. The seedlings were grown in a phytotron chamber (FYTOSCOPE FS130) for a period of 14 days (until 3rd leaf was emerged) under the following conditions: 16/8h (light/dark), 22/18°C, 250mmol $m^{-2}s^{-1}$. After this period the plants of each cultivar were divided into 2 groups: non salt-treated (control) and salt-treated by adding of NaCl to the nutrient solution (250mM NaCl). At 0h (day 15th), the group of control plants was measured while the second one was subjected to salt stress for 10 days. During this period, two parallel chlorophyll fluorescence measurements of the control (3 plants) and salt treated plants (3 plants) were performed at 5 time points: 24h, 96h, 120h, 168h and 240h, respectively. The measurements at each time point were performed in a 3 replicates/plant using a portable chlorophyll fluorometer PAM-2500 (Heinz Walz GmbH, Effeltrich, Germany) and the obtained data were processed in Excel (Microsoft Office) and statistically verified (ANOVA). The analyzed parameters were as follow: quantum yield of Φ_{PSII} (Y_{PSII}), electron transport rate (ETR), ratio of fluorescence decay (Rfd), *proportion of light* not used for photochemistry (LNU), photochemical quenching (qP), non-photochemical quenching (NPQ), etc.

Electrolyte leakage

Electrolyte leakage from leaf tissue was measured with a conductivity meter after 24 h incubation of leaf disks (total weight 0.25 g) in 25 ml double-distilled water. The conductivity (μS) of the floating solution was measured and normalized against double-distilled water. Following each measurement the maximum leakage of the tissue was determined by boiling the leaves 15 min at 100°C. The results are expressed as percentage of maximum leakage.

Enzyme analysis

The experiment was performed with two 6-row cultivars: IZ Bori and Bozhin. The seeds from both cvs were surface sterilized and germinated for 3 days in dark on moisture (H_2O) paper rolls. Then the seedlings were transferred to another paper rolls soaked in boxes with tap water containing 150 and 300 mM NaCl and were additionally grown for 72h period in a diurnal growth chamber (Forma Scientific) at 23°C and 12/12h photoperiod. At the end of this period root and leaf tissues of stressed and non-stressed plants were immediately frozen into liquid nitrogene (N_2). For protein isolation plant material was grinded to powder in liquid N_2, resuspended in phosphate buffered saline (PBS), centrifuged and the supernatant containing soluble intracellular proteins was separated for further analyses. All electrophoretic separations were conducted according to

Laemmli's protocol (Laemmli, 1970), while the zymographic analyses of superoxide dismutase was done according to the procedure described by Misra & Fridovich (1977).

Isolation of genomic DNA, PCR amplification, electrophoresis, data scoring and statistical analysis

DNA was isolated from leaf material of bulked samples (5 plants/cultivar) using a modified CTAB method of Murray & Tompson (1980). The amplification of 18 genomic- and EST-SSR loci was performed according to the conditions published in http://bioinf.scri.ac.uk/ssr/barley_s.html and http://wheat.pw.usda.gov/cgi-bin/graingenes/. The electrophoresis of PCR products was done on an automatic sequencer (AFL Express II). Fragment sizes of the PCR products were calculated using Fragment Manager (Pharmacia) software by comparing with internal size standards added to each lane in the loading buffer. Amplification profiles of the 4 barley genotypes were compared with each other and bands of DNA fragments were scored as a binary data with presence (1) or absences (0), for all accessions and then converted to a genetic similarity (GS) matrix. The data were used to estimate genetic similarity (GS) on the basis of the number of shared amplification products (Nei & Li, 1979). Phylogenetic trees were constructed based on similarity matrix obtained with neighbor joining (NJ) method using Jaccard formula. The relationships among genotypes were displayed as dendrogram using the NTSYSpc 2.01 software package (Rohlf, 1998).

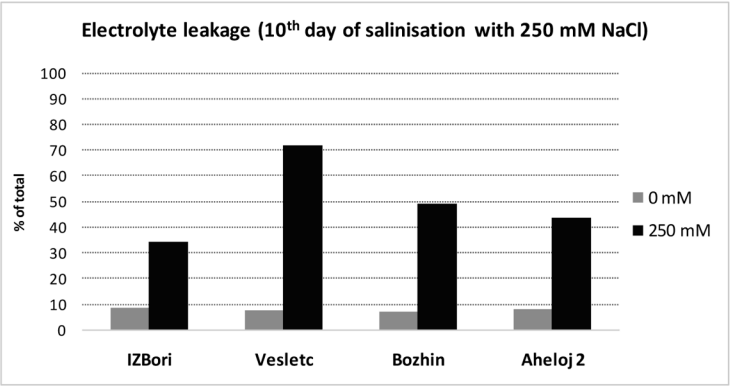
RESULTS AND DISCUSSION

Large efforts were devoted in the last decade to identify and select barley cultivars and lines having high degree of salt-tolerance which enable them not only to survive during their growth in salty soil and newly reclaimed desert lands but also having high productivity and yield. Solving of such problem by selection of highly tolerant barley genotypes will assist in plant breeding programs through identification of molecular, physiological and genetic markers that determine salt tolerance capacity (Munns & Tester, 2008). In this study an assessment of genetic variation in response to salinity stress of 4 Bulgarian feed barley cultivars was initiated using an integrated approach based on physiological, biochemical and molecular analyses.

1. Physiological analysis

Cell membrane stability under saline conditions (250mM NaCl) was determined through measurement of electrolyte leakage from leaf tissues. This method is widely used for assessment of stress impact on cell membrane stability in cereals (Bajji et al., 2002). The examined 4 cultivars differed in their electrolyte leakage values which correspond to the extent of membrane injury caused by the salt stress (Fig. 1). The lowest injury was observed for cv. IZ Bori (34%), while the highest one for cv. Vesletc (72%). The data showed that IZ Bori is the most tolerant cultivar as regards to salinization. Similar changes in the% of electrolyte leakage among tolerant and sensitive cereals in response to salt stress were reported by various authors (Ashraf & Harris, 2004; Plazek et al., 2013). Chaum et al. (2010) have found that this parameter is in direct relation with salt

Figure 1. Effect of NaCl on cell membrane stability of four Bulgarian 6-row barley cultivars.



stress in oil palm seedlings. The authors have established that the ratio of electrolyte leakage in pea and bean plants increased with the increment of NaCl concentration.

Chlorophyll fluorescence is a good, rapid and non invasive measurement of the changes in PSII photochemistry under abiotic stresses. The unaffected (stable) values of Fv/Fm in the studied here 6-row barley mean that there is no loss in the yield of PSII photochemistry and confirms the resistance of their photosynthetic machinery to salt stress. However, variation in two parameters (Rfd and LNU) determining some changes in this machinery (Fig. 2a) was observed. Both parameters showed better tolerance of IZ Bori to the stress. In addition, a variation in the NPQ was also observed. It is known that salt stress correlates with NPQ and this parameter increases substantially under saline conditions (Dongsansuk et al., 2013). The increase of NPQ may reflect a reduced demand for products of electron transport and hence increased heat dissipation. Our study shows that NPQ has different values at each time point of sampling of cultivars. The lowest and most constant NPQ rates during the prolonged stress experiment (10 days) was found in IZ Bori, thus meaning that this barley variety

Figure 2a. Effect of NaCl on the ratio of fluorescence decay (Rfd) of 6 row barley cultivars

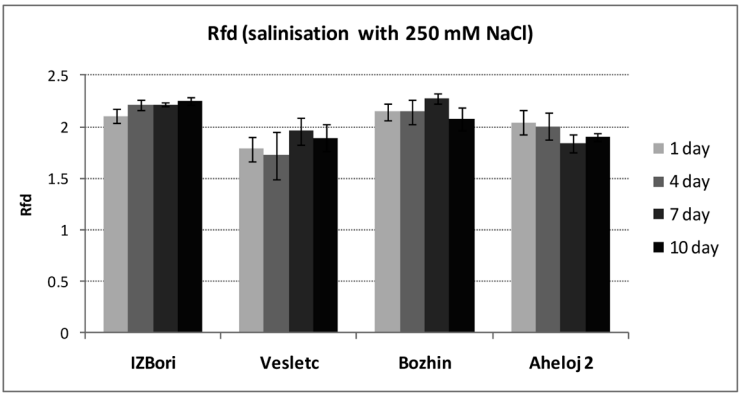
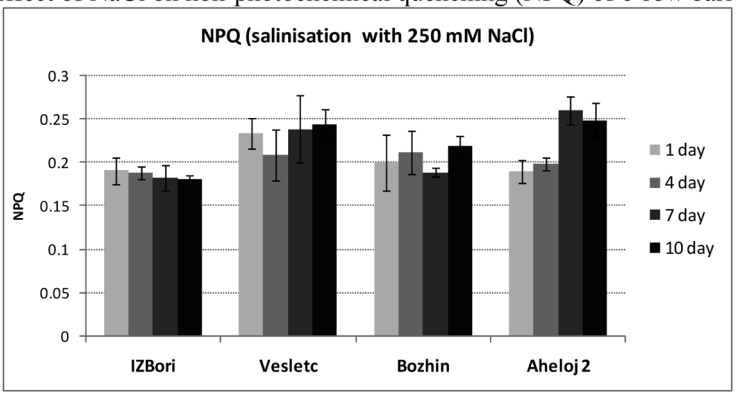


Figure 2b. Effect of NaCl on non-photochemical quenching (NPQ) of 6-row barley cultivars.

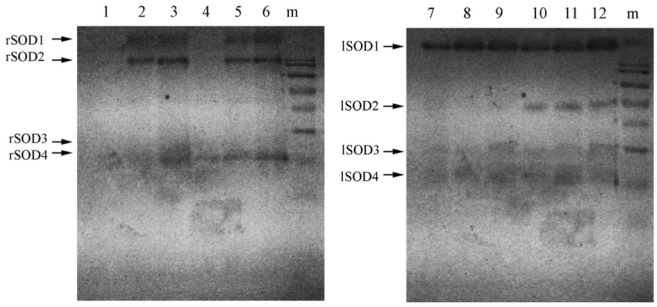


is the most resistant to salinisation. Among the rest of cultivars, Aheloj2 and Vesletc showed the highest NPQ values and therefore they are more influenced by salt treatment (Fig. 2b). Abdeshahian et al. (2010) reported that during salinisation various PSII parameters in wheat (*T. aestivum* L.) were influenced and these parameters (including NPQ) are reliable for the diagnostics of salt stress in wheat. Tavakkoli et al. (2011) also reported similar dependence on NPQ by comparing 4 barley cultivars with different salt-resistance.

2. Biochemical analysis

Salinity causes **oxidative stress** which affects the biochemical and enzymatic components in the plant cell under stressed condition. Profiling of several enzymes involved in antioxidant defense system such as glutathione reductase (GR), catalase (CAT), peroxidase (POX), superoxide dismutase (SOD) and ascorbate peroxidase (APX) was performed in four days seedlings of 2 of the selected barley cvs. Iz Bori and Bozhin that were subjected to different strength of the salt stress (150mM and 300 mM NaCl). The zymographic profiles of GR and APX did not demonstrate any considerable changes in the observed izoforms of the enzymes in leaves and roots in both cultivars. The rest of enzymes showed changes in their activity during the applied stress. The most significant changes were observed in the zymograms of POX and SOD enzymes under denaturing gel electrophoresis conditions. Seven POX isoforms were visualized in roots and 5 in leaves of both barley cultivars (data not shown). Their activity increased in response to salt stress. In roots, the POX isoforms were barely visible in controls but their activity increased equally under both applied stress doses (150 and 300mM NaCl). In contradiction, in leaves the activity of POX isoforms increase gradually from 0mM to 300mM NaCl stress treatment. Similar observations were recorded for the SOD isoforms in roots and leaves (Fig. 3a, b). However, one of the SOD isoforms in roots (37 kDa) showed increase in activity in response to salt treatment only in cv. IZ Bori while in leaves two SOD isoforms (40 and 70 kDa, respectively) showed significant increase in activity only in cv. Bozhin. These results demonstrate that some isoforms are associated with better performance under salt stress. They are consistent with the work of other researchers. For example, Kim et al. (2005) have found that SOD isoform 4 showed increased expression in the roots of salt-stressed barley plants, while higher levels of isoform

Figure 3 a,b. Effect of different NaCl concentrations on SOD activity in roots and shoots of Bulgarian 6- row cultivars IZ Bori and Bozhin.



1 was found in the leaves. Gao et al. (2013) have reported increase of SOD and POX enzyme activities in salt-tolerant barley cultivar by comparing two contrasting genotypes under salinity stress during seedling stage.

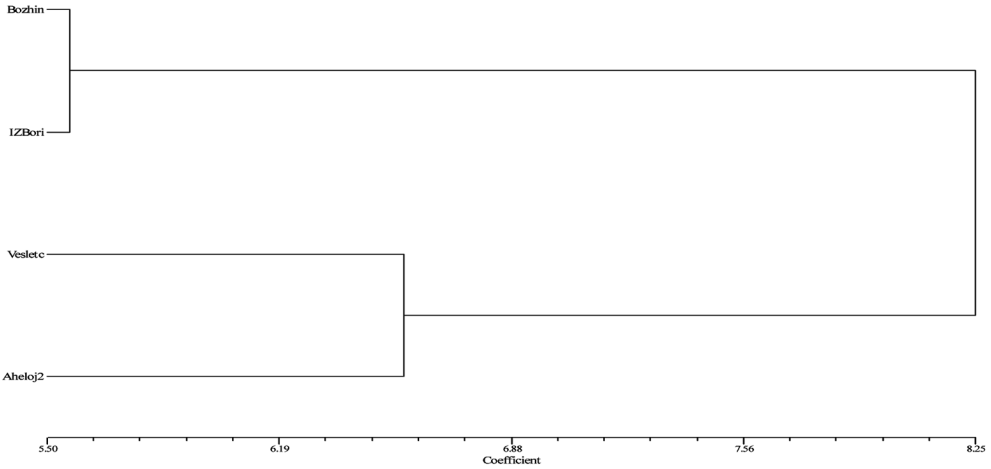
3. Molecular analysis

Among different types of molecular markers available for barley, microsatellite or simple sequence repeats (SSRs) have proven to be the markers of choice for marker-assisted selection (MAS) in breeding and genetic diversity studies. In barley, more than 775 SSRs have been published and genetic maps based on these markers for all seven barley chromosomes are publicly available (Varshney et al. 2007). The characterization and assessment of genetic diversity among the barley genotypes is important at the short term for determining genetic diversity and differentiation among the studied genotypes and at the long term for designing breeding strategies for quantitative and qualitative traits expressing under salt stress. Out of the 18 used SSR primer pairs, only four primers amplifying loci on chromosomes 1 (GBM1042), 2 (Bmag0140 and Bmag0125) and 3 (HVM60) generated monomorphic band profiles in the studied here cultivars. The rest of primers generated between 2 (most of primers) to 5 alleles (HVM03 – 4chr). The highest level of genetic distance was observed between IZ Bori and Aheloj2 (0.375) and between IZ Bori and Vesletc (0.361) while the lowest one between IZ Bori and Bozhin (0.111) (Table 1). This was confirmed also by the constructed dendrogram using UPGMA clustering (Fig. 4). Now it is known that 4HL in addition to chromosomes 2 and 6 is involved in salt stress tolerance in both barley and wheat. Some ion homeostasis traits including shoot Na+, K+, Cl– exclusion were found to be mainly under the genetic control of a region on chromosome

Table 1. Genetic distances between four Bulgarian 6- row barley cultivars as estimated by 18 SSR markers.

GD	Aheloj2	Bozhin	IZ Bori	Vesletc
Aheloj2	0,000			
Bozhin	0,333	0,000		
IZ Bori	**0,375**	**0,111**	0,000	
Vesletc	0,153	0,306	0,361	0,000

Figure 4. UPGMA dendrogram of four 6-row cultivars constructed on the base of 18 SSR markers



4HL of barley as it was found by association mapping using SNP chip analysis (Long et al. 2012). The observed allele variation in locus HVM67 on 4HL, located closely to the mapped QTL for this trait clearly differentiates the studied cultivars into two groups, the first one including IZ Bori and Bozhin (101/105 bp alleles) and the second one comprising of Aheloj2 and Vesletc (105/112 bp alleles). This differentiation is in correspondence with the physiological discrimination of the studied cvs. However, salt tolerance is a complex inherited trait and it is likely that several QTLs and most likely several different mechanisms are involved. Therefore, additional studies are needed to confirm this hypothesis. Solving such a problem will allow the identification of genetic and functional markers for determining the natural variation in salinity tolerance in barley and further, the differentiation between the pathways involved in salt and drought stress response in this important for agriculture cereal crop.

CONCLUSION

For better understanding of the response of cereal crops to salinity stress and mechanisms involved in the stress tolerance it is necessary to study both the physiology and the molecular net– works involved in it. To our knowledge, this study is the first one, utilizing an integrated approach to reveal the genetic variation in Bulgarian barley germplasm in response to salinity stress. It is also a base for identification of salinity tolerant genotypes with application in barley breeding.

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