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ION LEAKAGE AND LEAF ANATOMY OF BARLEY PLANTS SUBJECTED TO DEHYDRATION

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Summary. In the present study the physiological status of two barley (*Hordeum vulgare,* L.) cultivars subjected to dehydration was evaluated. Ten-days-old plants were exposed to soil drying by withholding irrigation. The degree of stress was assessed by comparing leaf water content and the levels of malondialdehyde and hydrogen peroxide of treated and control plants. Cell membrane stability was evaluated based on electrolyte leakage from leaf tissues. It was ascertained that besides the commonly used Injury index leakage kinetics gave additional information about the changes in ion concentrations in leaf symplast and apoplast which could be used for preliminary stress evaluation of contrasting genotypes. The examined cultivars differed in their water content which corresponded to the extent of membrane injury caused by dehydration. Exposure to drought caused a significant decrease both in leaf thickness and total area of mesophyll cells. The most obvious change in leaf anatomy was the disappearance of vacuoles in mesophyll cells which contributed to the leakage of the major part of electrolytes during the fast phase of the kinetics. The involvement of the cell wall and the vacuole in osmotic stress response are briefly discussed.

Key words: barley; drought stress; electrolyte leakage; hydrogen peroxide; leaf anatomy; malondialdehyde; vacuoles.

INTRODUCTION

Drought causes various morphological, physiological and biochemical changes in plants. One of the harmful effects of desiccation is connected with the generation of reactive oxygen species such as hydrogen peroxide and products of lipid peroxidation, such as malondialdehyde, whose primary targets of damage are cell membranes (Kuzniak and Urbanek, 2000; Fu and Huang, 2001; Király and Czövek, 2002). Electrolyte leakage from plant tissues is widely used for the assessment of stress impact on cell membrane stability (Bajji et al., 2002; Repo et al., 2004; Farooq and Azam, 2006; Roy et al., 2009). Solutes leak both from the symplast (cytosol) and the apoplast (including cell walls, xylem elements and extracellular spaces). The largest compartment in the cytosol of a mature plant cell is the vacuole which may occupy more than 80% of the total cell volume. Due to their

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large dimensions, vacuoles normally account for the dominant proportion of the total intracellular ionic and water content (Karley et al., 2000) and are essential to cellular strategies of plant growth and development. Vacuoles function as a pool for ions and metabolites and are involved in the processes of detoxification, homeostasis and cellular responses to stress factors. In plant vegetative organs vacuoles act in combination with cell walls to generate turgor which is the driving force for hydraulic stiffness and growth (Marty, 1999). Maintaining normal cell metabolism often requires water transport from the vacuole into the cytosol through specialized water channels called aquaporins (Luu and Maurel, 2005). The apoplast, on the other hand, is considered a site for short-term nutrient storage and solute exchange (Hoson, 1998). As a transient ion reservoir the cell wall has some advantages over the vacuole such as high cation exchange capacity and ease with which ions can be taken up (Sattelmacher, 2001). Cell walls may alter ion diffusion rate due to adsorption/desorption interactions of their components acting as ion-exchangers (Marschner, 1995). Accumulation of ions in the leaf apoplast has been reported for salt-grown barley leaves (Cuin et al., 2003). Moreover, a significant part of the water in leaves and other organs is present in the apoplast, outside the cell plasma membrane which is important for the transfer of metabolites and ions between cells (Wardlaw, 2005). Conductometric measurement of electrolytes leaking from plant tissues is a widely used technique for assessment of cell membrane stability. Injury index is an important parameter especially for plants subjected to stress (Repo et al., 2004; Farooq and Azam, 2006). However, the involvement of different cell compartments such as cell walls, vacuoles or other cell organelles in the integral cell membrane stability remains unclear. The diffusion model proposed by Kocheva et al. (2005) differentiated the contribution of ions leaked from the cytosol and the extracellular matrix based on the rates of the fluxes in these two compartments. However, the role of leaf anatomy in this process was not considered.

On the basis of the above considerations we aimed to find how soil drought influenced parameters of electrolyte leakage, leaf anatomy and some physiological markers of water stress such as water content, accumulation of lipid peroxidation products and hydrogen peroxide content in the leaves of young barley plants with different levels of stress tolerance.

MATERIAL AND METHODS

Plant material and experimental design

Barley (*Hordeum vulgare*, L.) plants from two cultivars Odesskii and Houters differing in their performance in field conditions were used in the experiments. Seeds were planted in small pots containing 450 g leached meadow cinnamon soil (pH 6.2) and grown in a phytostat chamber with day/night temperature 24/17°C, 150 $\mu molm^{-2}s^{-1}$ PAR and 14-h photoperiod. Relative soil moisture was maintained at 60% of the maximal soil water capacity measured gravimetrically according to Evett, 2008. Ten-days-old plants were subjected to desiccation for 7 consecutive days by withholding water. Control plants were supplied with tap water daily during

the whole period of investigation. All experiments were performed on the first fully expanded leaf.

Water content (WC), malondialdehyde (MDA) and hydrogen peroxide measurements

Water content was calculated as:

$$WC\ (\%) = [(FW - DW)/FW].100$$

where FW is the fresh weight of the leaves and DW is the weight estimated after drying the leaves for 4 h at 80°C or until a constant value was achieved. Accumulation of malonyldialdehyde (MDA) was determined according to the method of Cakmak and Horst (1991). For the analyses, 0.3 g leaves were homogenized in 3 ml 0.1% trichloroacetic acid and extract was clarified by centrifugation (10,000 x g for 20 min at 4°C). An aliquot of 0.5 ml of supernatant was added to 1.5 ml 0.5% (w/v) thiobarbituric acid dissolved in 20% (w/v) trichloroacetic acid. The mixture was kept in a boiling water bath for 30 min and then quickly cooled in an ice bath. Absorbance was measured spectrophotometrically at 532 nm and corrected for non-specific absorption at 600 nm. MDA content was calculated using a molar extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as μmol g^{-1}DW. Hydrogen peroxide was measured spectrophotometrically according to Alexieva et al. (2001). The reaction mixture contained 0.5 ml leaf extract in 0.5% trichloroacetic acid (TCA), 0.5 ml 100 mM K-phosphate buffer (pH 7.4) and 2 ml reagent (0.5M KI w/v in fresh distilled water). The blank probe contained 0.5% TCA in the absence of leaf extract. The reaction was developed for 1 h in darkness and absorbance was measured at 352 nm. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations of H_2O_2.

Injury index and electrolyte leakage kinetics

For determination of membrane Injury index 15 leaf pieces (2 cm in length) were cut from stressed and control plants. They were washed with distilled water to remove the solution from injured cells and were then immersed in 15 ml of distilled water at room temperature. After 24 h incubation conductivity of the solutions was measured with a conductometer Elwro 5721 (Poland). Finally, samples were boiled for 30 min, cooled at room temperature and conductivity was read again. Injury index was estimated from the formula:

$$I\ (\%) = [1 - (1 - t_1/t_2)/(1 - c_1/c_2)].100$$

where t_1 and t_2 are the first and second (after boiling) measurements of the conductivity of the solutions in which treated samples were immersed and c_1 and c_2 are the respective values of controls (Premachandra et al. 1992). For estimation of electrolyte leakage kinetics conductivity of the solutions was measured repeatedly during the 24-h-period. Results were expressed as relative conductivity ratio κ/κ_{max} where κ is conductivity of samples at a particular moment and κ_{max} is total electrolyte content determined after killing the tissues. When relative conductivity values were presented for each measuring point of the incubation time a two-phase kinetics was evidenced. Fit of experimental results was performed

by exponential associate function (NLSF procedure, Origin 5.0) with four variable parameters:

$$C_o(t) = b_1(1 - e^{-a1t}) + b_2(1 - e^{-a2t}) + C_o^{\,o}$$

where $C_o(t)$ describes ion concentration change in time. A previously introduced by Kocheva et al. (2005) and more convenient parameter T is used which represents a combination of the four function parameters (a_1, b_1, a_2, b_2) and is defined as:

$$T = \frac{\ln[a_1 b_1 / a_2 b_2]}{a_1 - a_2}$$

T describes the first, fast phase of the kinetics which is attributed to ion leakage from the apoplast compartment, while the slower second phase is ascribed to the membrane. T represents the moment at which the two phases reach equal rates.

Light microscopy and image processing

Samples were taken from the central area of the leaf blade, avoiding the main vein. Leaf pieces were fixed in 3% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) and embedded in low viscosity Spurr's epoxy-resin. Semi-thin cross-sections cut on an ultramicrotome Tesla BS 490 (Czech Republic) were stained with 0.01% (w/v) toluidine blue and observed under a light microscope Carl Zeiss, Jena (Germany). Microscopic images were captured and saved on a digital image processor (International Micro-Vision Inc., Redwood City, CA, USA). Leaf thickness and total area of palisade cells between veins were evaluated in cross sections, obtained from 6 leaves per variant from two independent experiments. Measurements were made with 3D Doctor Software (Able Software Corp., Lexington, MA, USA).

Statistical analysis

Two independent experiments were conducted and all parameters were measured in at least 3 replications. Data are presented as mean values ± SE.

RESULTS AND DISCUSSION

When barley plants were desiccated for 7 days (until soil moisture reached 35-40%) leaf water content (WC) decreased significantly (Table 1). Of the two studied cultivars, cv. Odesskii showed lower WC (59%) as compared to cv. Houters (71%). Regarding control plants cv. Houters again had higher WC than cv. Odesskii. This is indicative of a better water retention ability in the leaves of cv. Houters.

Tissue dehydration was accompanied with certain anatomical changes (Fig. 1 and Fig. 2). Regarding control plants cv. Houters had greater leaf thickness than cv. Odesskii (Fig. 2A). Upon desiccation this parameter was reduced significantly in both cultivars. Total area of mesophyll cells in cv. Houters showed greater changes after drought as compared to cv. Odesskii. This parameter decreased to 40% of respective control in cv. Odesskii and as low as 29% in cv. Houters (Fig. 2B). The significantly larger mesophyll area in cv. Houters controls presumed larger size of vacuoles. This assumption was supported by the higher WC measured in cv. Houters leaves under normal and stress conditions (Table 1).

Monocotyledonous plants (such as wheat) have a specific leaf anatomy

Table 1. Water content (WC, %), period T of the prompt phase of ion leakage kinetics and Injury index in leaves of barley plants subjected to dehydration. Data are means ± SE.

Variant	WC [%]	Period T [min]	Injury index [%]
Odesskii control	88.81±0.31	59.3±4.8	
Houters control	90.75±0.10	36.4±2.0	
Odesskii stress	58.67±4.46	25.4±1.5	77.40±3.84
Houters stress	71.06±1.31	23.6±2.0	53.14±1.16

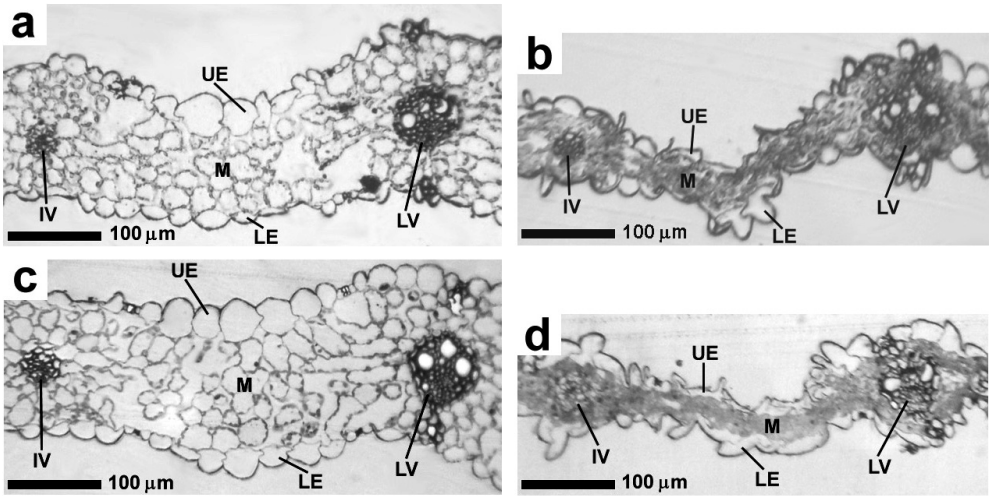

Figure 1. Micrographs of barley leaves before and after exposure to drought. Cross-sections at the central area of the leaf blade, avoiding the main vein: (a) cv. Odesskii control; (b) cv. Odesskii stress; (c) cv. Houters control; (d) cv. Houters stress. UE = upper epidermis; LE = lower epidermis; M = mesophyll; IV = intermediate vein; LV = lateral vein.

comprising only of spongy mesophyll tissue and a lack of palisade mesophyll. In our experiments the most apparent alteration in leaf anatomy was shrinking of the cytoplasm and total disappearance of vacuoles in the desiccated tissues (Fig. 1). It is established that vacuoles are the principal sites of solute accumulation and transport across the tonoplast membrane is essential for this process (Karley et al., 2000). Generally, osmotic stress causes ion efflux from this cellular compartment into the cytosol in order for homeostasis to be maintained (Cuin et al., 2003). As a consequence of the drought stress applied in our study vacuoles in both cultivars virtually disappeared (Fig. 1). Most likely, during the period of dehydration, their contents were emptied into the extracellular matrix. The ions were probably electrostatically bound to the charged components of the cell wall and during the incubation of the samples they were easily given away and leaked rapidly

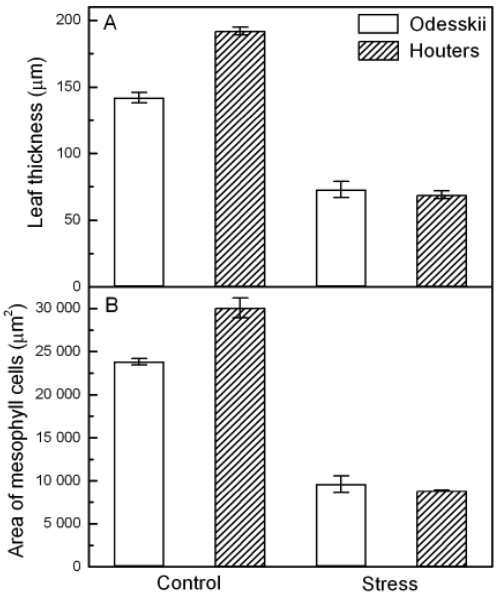

Figure. 2. Leaf thickness (A) and total area of mesophyll cells between bundle sheaths (B) measured on light microscopy photographs of semi-thin cross-sections from leaf tissue in two barley cultivars grown on soil under normal water conditions (control) and desiccation (stress).

into the outer solution. Electrolyte leakage from damaged tissues was commonly used to assess cell membrane stability (Farooq and Azam, 2006; Sikder and Paul, 2010). Bajji et al. (2002) have revealed that electrolyte leakage correlated well with drought resistance level in durum wheat with the sensitive cultivar having a higher membrane injury index. In addition to the well-known Injury index, we applied a model describing the kinetics of electrolyte leakage which gave more information about ion diffusion process through different cell compartments (Kocheva et al., 2005). It was shown that ion leakage kinetics from various plant tissues could be described by an exponential curve in which two phases were distinguished. The initially observed prompt phase was attributed to processes of absorption/desorption of ions or passive diffusion through the cell wall, while the slower phase was associated with cell membranes. Ion leakage kinetics (Fig. 3) showed that stressed barley plants had a faster rate of electrolyte efflux in the first leakage phase compared with control plants. The k/k_{max} ratio expressed the relative change in electrolyte concentration in the outer solution. It increased with the development of stress which indicated greater ion efflux from damaged tissues. This relative conductivity was considerably higher in stressed cv. Odesskii versus cv. Houters plants which signified greater injury in the former cultivar. Furthermore, the reduction of period T in cv. Odesskii was 57%, whereas in cv. Houters only 35% decrease in comparison with respective control was evidenced (Table 1). The faster rate of electrolyte leakage in cv. Odesskii (Fig. 3) correlated with a higher degree of dehydration assessed by WC in the leaves (Table 1). This was in agreement with the observation that stressed plants from cv. Odesskii had a higher Injury index (77%) than plants of cv. Houters (53%). The kinetic model of ion leakage introduced by Kocheva et al. (2005) offered precise evaluation of stress impact on different genotypes and allowed the contribution of apoplast and symplast to tissue effusate to be differentiated based on the rates of concentration change. In the fast first phase ions leak promptly through the apoplast into the outer solution since the cell wall represents no significant barrier to them. The slower second phase reflects electrolytes passage through the plasmalemma whose elevated permeability could imply damage or altered competence. Membrane damage

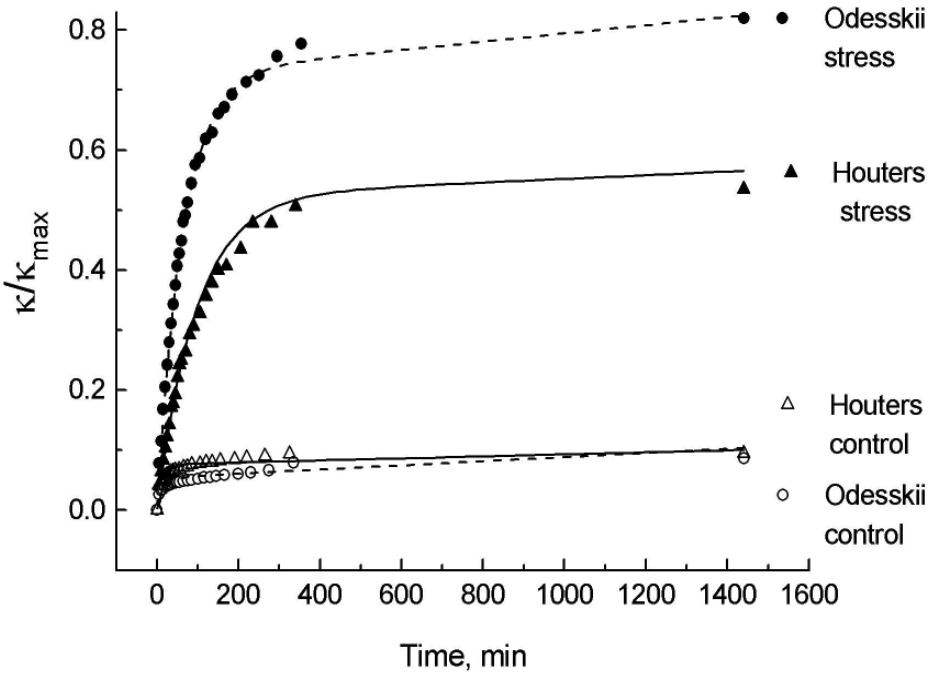

Figure 3. Kinetics of electrolyte leakage from leaves of barley cv. Odesskii and cv. Houters before (control) and after (stress) imposition of drought stress.

might be a result of initiated oxygen stress and the accumulation of reactive oxygen species leading to disturbances in membrane configuration (Hoekstra and Golovina, 1999; Foyer and Noctor, 2005). In the present study, drought caused the formation of active oxygen species (Fig. 4). MDA content increased 5 times in cv. Odesskii and only 2 times in cv. Houters as compared with respective controls (Fig. 4A). This could reflect an increased lipid peroxidation leading to higher leakage of solutes and loss of membrane competence in cv. Odesskii. Hydrogen peroxide levels changed in both cultivars under stress (Fig. 4B). It showed a greater increase in cv. Odesskii (3 times the value of the control) than in cv. Houters (2.4 times the respective control) which could reveal more effective ROS (reactive oxygen species) scavenging mechanisms in cv. Houters.

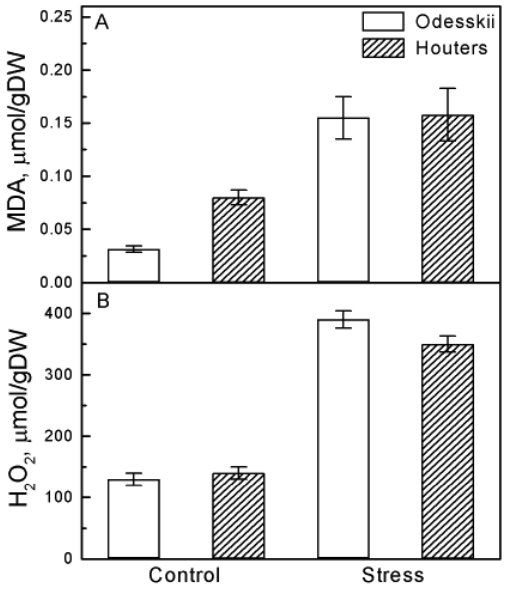

Figure 4. Changes in MDA (A) and H_2O_2 (B) in leaves of control and stressed barley plants of cv. Odesskii and cv. Houters. Data are means ± SE.

CONCLUSIONS

In conclusion, the use of the kinetic model for electrolyte leakage from plant tissues can give additional information about the processes of ion efflux from the apoplast and the symplast. These phenomena are closely related to changes in leaf anatomy and participate in the stress response of plants. In our opinion the roles of the vacuole and the cell wall should be studied more profoundly in future. Moreover, they are significant and distinctive components of the plant cell.

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