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Research Article

WHY DO WHEAT SEEDLINGS RESPOND DIFFERENTLY TO DROUGHT SIMULATED BY POLYETHYLENE GLYCOL 6000 OSMOTIC STRESS OR SOIL DRYING

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Summary: This study compares the physiological responses of two wheat varieties to soil drying and treatment with polyethylene glycol (PEG 6000). Soil drought and PEG-generated osmotic stress were applied to seedlings of a modern semi-dwarf variety (carrier of *R*educed *h*eigh*t* or *Rht* genes) and a historic (old) tall variety. Under severe soil drying, the leaves of the old genotype lost larger amounts of water and the cell membranes damage of the leaf cells was higher compared to the semi-dwarf variety. In contrast, under osmotic stress the old variety managed to preserve better water balance and membrane integrity in leaf cells than the modern one. The leaves of the old genotype are more oblong (higher dissection index), while those of the modern variety are more round. In case of a more oblong leaf shape, larger amounts of water evaporate from the leaf surface mainly on the account of additional evaporation from the borderline zones. Under severe soil drought, the water movement practically ceases, leading to more rapid exhaustion of the water reserves and stronger damage of cell membranes in the leaf tissues, observed to a greater extent in the variety with more oblong leaves. Under osmotic stress there is still water, although hardly accessible, in the vicinity of roots, and the evaporation from leaves probably maintains water flow although to a minimal extent. The more intensive evaporation from leaves with a more oblong shape causes more intensive water movement thus aiding the better preservation of water balance and cell membrane stability in the leaf tissues.

Keywords: Evaporation; leaf dissection index; leaf shape; osmotic stress; soil drying; wheat.

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INTRODUCTION

Soil water deficit is considered a major source of risk for plant growth and crop productivity. Research on the dessication effects and plant stress responses involves drought simulation by either soil drying (Demirevska et al., 2008; Vassileva et al., 2009; Petrov et al., 2012), or use of an osmoticum, such as high molecular weight polyethylene glycol (PEG) (Kocheva et al., 2013; Yang et al., 2015). When drought is induced by withholding water to plants grown in

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pots water potential declines at a faster rate compared to natural soil drought leading to non-homogenous stress for plants (Krizek, 1985). Treatment with PEG in water cultures provides more homogeneous and precise stress than soil drying (Kramer, 1983; Krizek, 1985). However, drought effects may vary, depending on the method of stress induction and the species. Thus, Fan and Blake (1997) reported significantly higher membrane injury and reduced net photosynthesis and stomatal conductance in woody species under PEG simulated osmotic stress compared to soil drying. The authors suggest that in addition to the PEG-damage masked drought responses, PEG itself causes injury to leaf tissues (unrelated to dehydration). Although PEG application greatly facilitates experimentation, the cause of this controversy following different treatments to simulate drought remains open for suggestions.

One of the plant strategies to withstand limited water availability relies on morphological traits that confer water saving capacity of stressed tissues. Such traits are high leaf thickness, low leaf area-to-volume ratio, high trichome density, altered stomatal density and stomatal conductance (Lyshede, 1979, Groom and Lamont, 1997). Leaf shape is another trait that is affected by and strongly influences leaf water relations (reviewed in Nicotra et al*.,* 2011). It is also a factor that could contribute to plant drought tolerance. Recently, the relationship between leaf shape and plant capacity to preserve leaf water status and membrane integrity of leaf cells under severe soil drought was demonstrated in a comparative study of modern and historic bread wheat varieties (Petrov et al., 2018). The established correlations indicate that during water deprivation, the shorter and wider or more rounded leaves (i.e. having lower values for the dissection index) evaporate to a lower extent and better preserve cell membrane integrity thus lessening the adverse effects of the stress.

This study was aimed to compare the physiological changes in wheat plants in response to soil drying and osmotic stress generated in a nutrient solution by PEG 6000. Two bread wheat varieties that differ by presence/absence of height reducing (*Rht*) genes were used in the experiments. The high productive modern semi-dwarf variety carries mutant alleles at two *Rht* loci (Landjeva et al., 2012) whereas the old (or historic) variety is tall and carries the wild type alleles. Besides impact on plant stature and productivity, *Rht* genes also affect leaf size (King et al*.,* 1983) and leaf shape towards more rounded one, which in turn contributes to improved desiccation tolerance through less intensive evaporation and better preserved cell membrane integrity (Petrov et al*.,* 2018). In this study, the effects of soil drying and PEG 6000 simulated osmotic stress on physiological functioning in a semi-dwarf genotype (carrier of two *Rht* genes) and a tall one (without *Rht* genes) were evaluated in dependence of the genotype-specific leaf shape.

MATERIAL AND METHODS

Plant material

Two Bulgarian bread wheat (*Triticum aestivum* L.) varieties, Enola and Slomer, were used. Enola is a semi-dwarf variety, carrier of the gene combination *Rht8*+*Rht-B1b/d*, the two *Rht* genes being mutant

alleles at two loci, on chromosomes 2D and 4B, respectively (Landjeva et al. 2012), while Slomer is an old local variety of tall stature, carrier of the wild type alleles at both loci.

PEG induced osmotic stress

Seeds were germinated at 22°C in the dark on moistened filter paper. Three-day-old seedlings were placed to half Hoagland nutrient solution for 4 days, then transferred to full Hoaglad for another 3 days, and grown in a climatic chamber with 12 h day/night cycles at 22/18°C, 100 μmol $m^{-2}s^{-1}$ light intensity and 70% relative humidity.

Osmotic stress was imposed on 10-day-old seedlings by transferring them to 25% polyethylene glycol (PEG 6000 Ph. Eur.) dissolved in full Hoagland solution for 72 h. The osmotic potential of PEG solution (– 0.7 MPa) was determined according to Michel et al. (1973). Plants grown in nutrient solution served as controls. All analyses were performed on 2nd fully developed leaves.

Soil drying

Seeds were soaked for 4 h in tap water and planted in 800-g pots with alluvial meadow soil (pH 6.2). Plants were grown in a climatic chamber with 22/18°C day/night temperature, 14-h photoperiod, irradiance of 250 μmol $m^{-2}s^{-1}$ and 70% relative humidity. Tap water was supplied daily sustaining 60% of full soil moisture capacity. For drought experiment, 14-day-old plants (3-leaf stage) were left without watering for 6 days, resulting in 10% of soil full moisture capacity. Regularly watered plants served as untreated controls. All analyses were performed on 2nd fully developed leaves.

Relative water content of leaves

Leaf relative water content (RWC) was estimated according to Turner (1981) using the equation:

$$RWC\ (\%) = [(FW - DW) / (TW - DW)] \times 100$$

where FW is the fresh weight of the leaves, TW is the weight at full turgor, measured after floating the leaves for 24 h in water in light at ambient temperature, and DW is the weight estimated after drying the leaves at 80°C for 4 h or until a constant weight is achieved.

Leaf anatomy

Samples were taken from the central area of the leaf blade. Leaf pieces were fixed in 3% lutaraldehyde 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). Leaf thickness was evaluated in cross sections. Microscopic images were captured and saved on a digital image processor (International Micro-Vision Inc., Redwood City, CA, USA). Measurements were made with 3D DOCTOR Software (Able Software Corp., Lexington, MA, USA). Leaf pieces from three different plants per variant were fixed. Stomata density was measured in leaf epidermal impressions collected from non-stressed plants. The abaxial and adaxial epidermis of the leaf was carefully smeared with collodium in the mid-area of the leaf for few seconds. The replicas were peeled with

a clear scotch tape and observed under a light microscope. Microscopic images were captured and saved on a digital image processor (International Micro-Vision Inc.). Counting was made with 3D DOCTOR Software (Able Software Corp.).

Cell membranes Injury index

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

$$\text{Injury index (\%)} = [1 - (1 - t_1 / t_2) / (1 - c_1 / c_2)] \times 100$$

where t_1 and t_2 are the initial and final (after boiling) readings of the conductivity of the solutions in which treated samples were immersed, and c_1 and c_2 are the corresponding values of controls (Premachandra, 1992).

Leaf dissection index

Leaf dissection index (DI) as a function of leaf perimeter and area was calculated by Fourier transformation (Kincaid and Schneider, 1983):

$$DI = \text{perimeter} / [2\sqrt{(\text{area} \times \pi)}]$$

Statistical analysis

For the statistical analysis, leaf thickness was evaluated in cross-sections obtained from 8 leaves per variant from two independent experiments. Stomata and trichome density were evaluated in epidermal impressions obtained from 10 leaves per variant from two independent experiments. The RWC and Injury index were determined using 10 and 8 leaves per variant, respectively, from two independent experiments. Leaf area and perimeter were evaluated in leaf scans obtained from 10 leaves per variant from two independent experiments. Comparison of means was performed by the Fisher LSD test ($p < 0.05$) after performing ANOVA analysis.

RESULTS

Leaf morpho-anatomy

In seedlings grown on dry soil, leaves of the old variety Slomer were thicker compared to those of the modern semi-dwarf variety Enola (Table 1). No difference in the leaf thickness was observed between the two genotypes following PEG-induced osmotic stress in water culture. Leaves of Slomer grown under soil drought conditions were thicker than those grown as water culture. The leaf stomata density was higher in variety Enola under both droughtened soil and PEG-induced water deficit. In both stress conditions, no difference in leaf area between the two genotypes was recorded. Leaf area was of greater values in soil-grown seedlings than in those grown in water culture. Despite the drought stress simulation approach, the leaf dissection index (DI) was higher in the old variety Slomer. On average over

Table 1. Comparison of leaf morpho-anatomical parameters in seedlings of two bread wheat varieties (Enola, a modern semi-dwarf variety, carrier of two *Rht* genes, and Slomer, an old tall variety) grown on drying soil or PEG-6000 simulated osmotic stress in water culture. Mean values (± standard error) are presented. Mean values in each column followed by different letters differ significantly at $p<0.05$.

Variety / drought induction approach	Leaf thickness (μm)	Stomata density upper + lower epidermis (number per mm^2)	Leaf area (cm^2)	Leaf dissection index
Enola / dry soil	157.2 ± 3.7^{b}	80.2 ± 2.7^{a}	7.09 ± 0.17^{a}	7.80 ± 0.19^{c}
Slomer / dry soil	169.2 ± 2.2^{a}	67.7 ± 3.9^{bc}	7.13 ± 0.33^{a}	8.70 ± 0.15^{a}
Enola / PEG	151.8 ± 8.4^{bc}	68.3 ± 1.7^{b}	6.20 ± 0.29^{b}	6.87 ± 0.14^{d}
Slomer / PEG	137.8 ± 6.3^{c}	59.5 ± 3.6^{c}	6.36 ± 0.15^{b}	8.11 ± 0.16^{b}

the two genotypes, the leaf DI was higher in seedlings grown on droughtened soil.

Water status and cell membrane stability in leaves

In controls, the leaf relative water content (RWC) was higher in plants grown in water culture (Fig. 1). Under soil drought, higher leaf RWC was observed in variety Enola, while under PEG-simulated stress it was variety Slomer that had higher RWC in leaves (Fig. 1). Soil drying also resulted in less electrolyte leakage from damaged leaf tissues of variety Enola. On the contrary, Slomer was less prone to PEG-induced injury of the leaf cell membranes as evidenced by the lower injury index (Fig. 2).

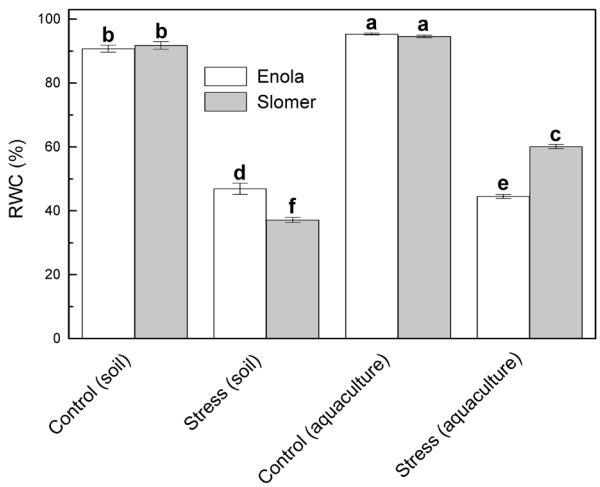

Figure 1. Leaf relative water content (RWC) of the 2nd leaf of seedlings of two bread wheat varieties (Enola, a modern semi-dwarf variety, carrier of two *Rht* genes, and Slomer, an old tall variety), grown either in drying soil or subjected to PEG-6000 simulated osmotic stress in water culture.

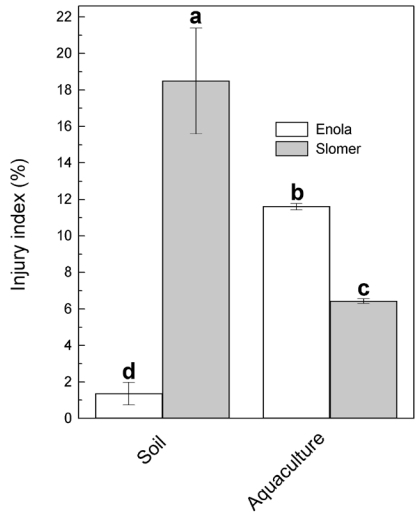

Figure 2. Membrane stability assessed by the Injury index of the 2nd leaf of seedlings of two bread wheat varieties (Enola, a modern semi-dwarf variety, carrier of two *Rht* genes, and Slomer, an old tall variety), grown either in drying soil or subjected to PEG-6000 simulated osmotic stress in water culture.

DISCUSSION

Leaf morpho-anatomy, water status and cell membrane stability after severe soil drying

According to a recent study of ours, leaf area and thickness, as well as stomata and trichome density did not affect significantly the leaf water balance and the integrity of leaf cell membranes (Petrov et al., 2018). In addition, a negative correlation between the leaf water content and the membrane stability of leaf cells was established (Petrov et al., 2018). The old variety Slomer had higher dissection index in control plants compared to variety Enola (Table 1). Following soil drying, Slomer showed lower leaf relative water content and higher electrolyte leakage from injured leaf tissues than Enola (Fig. 1, 2). In compliance with the model, described in Petrov et al. (2018), we suggest that the more oblong leaf shape in the old variety Slomer could contribute to the additional water evaporation from the marginal leaf zones, thus leading to additional reduction of leaf water reserves and further damaging of leaf cell membranes.

Leaf morpho-anatomy, water status and cell membrane stability after PEG-induced osmotic stress

The genotypes did not differ with respect to leaf area and thickness in control seedlings grown as water culture (Table 1). The leaf stomata density was lower and the leaf dissection index was significantly greater in Slomer compared to Enola (Table 1). In both genotypes, the length of leaf trichoma was considerably reduced making the impact of trichome density on stress tolerance negligible. Contrary to the observations after soil drying, in PEG stressed seedlings the water loss from leaves was smaller in Slomer compared to Enola, and the leaf cell membranes were less injured (Fig. 1, 2).

Differential response to severe soil drying and PEG-induced osmotic stress

Under microscope, no apparent differences between the two genotypes or between the two stress simulation methods were observed (Fig. 3). In case of normal water supply the stomata are open so the evaporation occurs mainly through them and much less from the rest of the leaf blade. Under water deficit when RWC values are less than 65%, the stomata are almost or fully closed (Brodribb et al., 2003). When leaf RWC drops down to 40%, some of the vacuoles are still observable signifying that the water preserved in the vacuoles has not been fully exhausted (Kocheva et al., 2013). After severe soil drying, the leaf RWC in variety Enola was slightly higher than 40%, while in variety Slomer it was just below 40% (Fig. 1) thus indicating that in both genotypes the vacuoles of leaf cells still contained water, though stomata were closed. Leaves of the PEG-treated seedlings of the two genotypes differed more considerably by the shape and insignificantly by stomata density. Leaf RWC in both genotypes was within the range 40-60% (Fig. 1), suggesting that

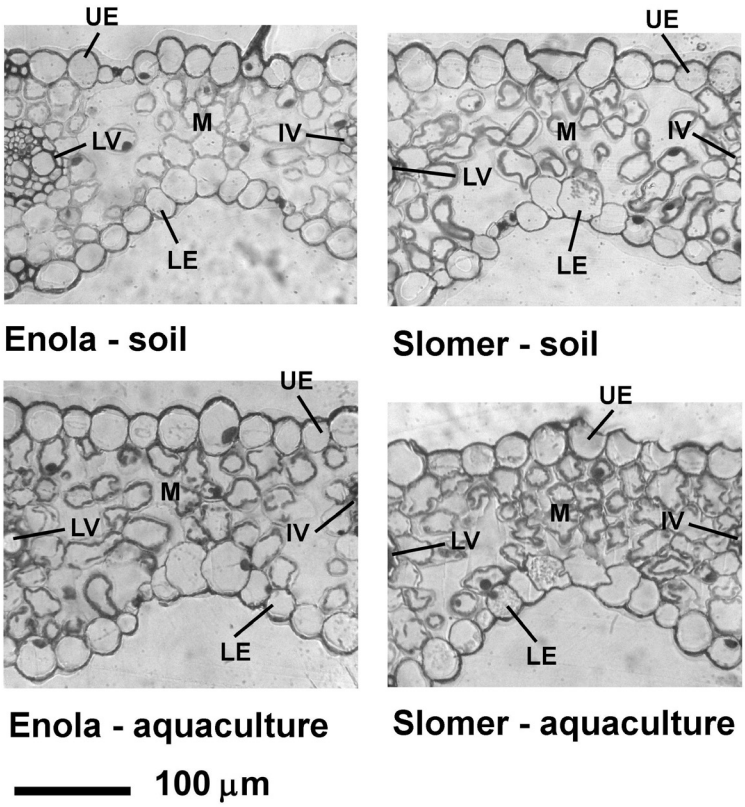

Figure 3. Micrographs of cross sections and replicas of the upper and lower leaf epidermis of two bread wheat varieties grown on soil or in nutrient solution as water culture: **a.** Enola, soil; **b.** Slomer, soil; **c.** Enola, water culture; **d.** Slomer, water culture.

UE - upper epidermis; LE - lower epidermis; M - mesophyll; LV – lateral vein; IV - intermediate vein; T - trichome; S - stomata.

the stomata were closed and the vacuoles of the leaf cells still preserved water. In addition to the negligible difference in stomata density between the two genotypes (Table 1), it is not worth considering their impact for overcoming the osmotic stress. Although the PEG-triggered osmotic force declines water accessibility, some water is still available around the roots. The cohesion theory describes continuous columns of apoplastic water under tension from the roots through the xylem to the evaporating surfaces (Strugger et al., 1943). The leaf mesophyll cells are bathed in the water columns, but the water does not pass through the cells. Following the discovery of water-specific protein channels embedded in cell membranes (i.e., aquaporins), the plausibility of symplastic water movements has been studied (Kaldenhoff et al., 1998; Frange et al., 2001). Some authors advocate three parallel water movements: symplastic movement through plasmodesmata, transcellular movement across cell membranes (aquaporins) and apoplastic flow in cell walls that are not suberized (Steudle et al., 1993; Barbour et al., 2004). We suggest that under severe soil drying, the practical absence of water in the soil disrupts the water movements through the plant. In this case, the more intensive the evaporation from the leaf surface, the more rapid is the reduction of leaf water reserves. When the stomata are closed the evaporation from the leaves is insignificant and occurs mainly from the epidermis. Under PEG-simulated osmotic stress, water although hardly accessible is still available in the vicinity of roots; so, even small amounts of evaporation from the leaves may contribute to the maintenance of water movements through the plant (Fig. 4). The difference in the leaf shape and dissection index might account for the evaporation pattern. More oblong leaves such as those of the old variety Slomer (i.e. with higher dissection index) evaporate larger amounts of water through additional evaporation from the marginal zones thus probably maintaining more intensive water flows (Fig. 4). The more intensive water transport provides more water to the leaf cells thus better preserving the water balance and membrane stability of cells.

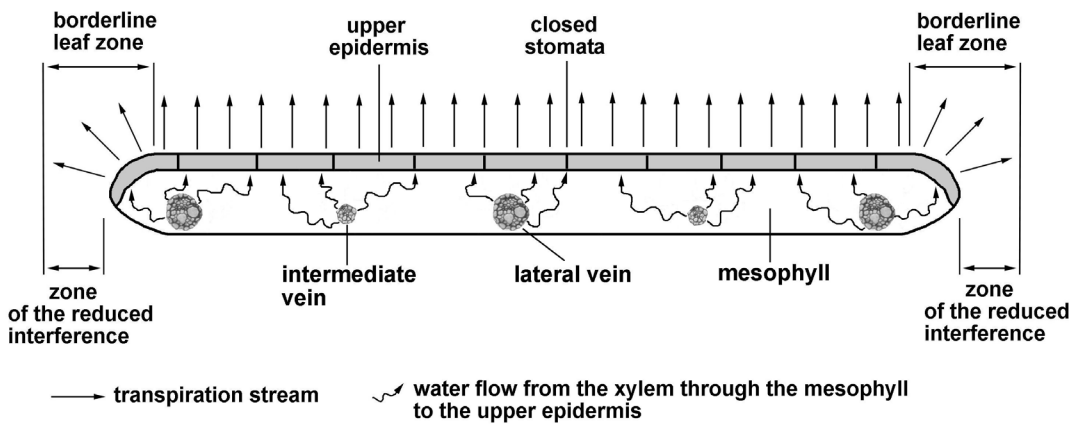

Figure 4. Schematic presentation of the transpiration from the upper leaf epidermis with closed stomata.

CONCLUSION

This study confirms that the desiccation effects on plants may vary in dependence of the drought induction approach. The results also suggest that the genetically determined difference in leaf shape contributes to plant capacity to withstand water deprivation. Caution is warranted in the choice of treatment to mimic drought in cases of significant difference in the leaf shape.

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