Osmotic adjustment in spring durum wheat pollen grains under induced drought stress

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Osmotic adjustment (OA) is recognized as one of the main mechanisms of drought tolerance in crops. OA is realized by reducing the osmotic potential due to accumulation of organic and inorganic osmolytes in response to a water deficit. This mechanism is manifested in all plant cells, including pollen grains. We carried out pollen analysis to differentiate the genotypes of spring durum wheat in tolerance to drought. Eight wheat assessments of different ecological and geographical origin from the collection of the Altai Research Institute of Agriculture, Russia were studied. They were Omskaya stepnaya, Zhemchuzhina Sibiri, Bezenchukskaya 210, Solnechnaya 573, Ozis, Pamyati Yanchenko, 12S1-14, and 12S2-24. Plants were grown at the field plots in 2016. Drought was simulated by adding 55% PEG 6000 to the culture medium. Pollen samples were incubated in the dark at 24 ± 1°C during 48 hours at 70-80% relative humidity. To assess the efficiency of cation accumulation due to transmembrane migration from the external environment, 10 mM KCl as the osmolyte was added to the pollen culture medium containing 55% PEG 6000. Plasmolytic phenomena were assessed under a microscope by changing the cytoplasm area by scanning the surface of each pollen grain. Quantitative measurements of the projected area of pollen cytoplasm were carried out. We have established significant differences among varieties of spring durum wheat relative to OA mechanisms responsible for dehydration avoidance. The response of pollen grains to in vitro induced osmotic stress reflected various strategies for the behavior of genotypes involving or not transporting K⁺ cations from the cultivation medium. Most of the accessions tested are capable of supporting the cellular turgor, using only intrinsic adjustment mechanism, or combining it with osmolyte induced OA. One line has been shown to exhibit the induced osmotic adaptation, which caused some restoration of the projected cytoplasm area. One line did not resist osmotic stress, reducing the pollen turgor both under stress and in the presence of osmolyte. We succeeded in revealing a good correspondence between the pollen reactions under induced in vitro osmotic stress and the field drought tolerance index of genotypes. This made it possible to test the diversity of the accessions for tolerance to drought by scanning the pollen surface. Assessment of the behavior of the male gametophyte population of wheat under conditions of induced stress allows screening and determining drought-tolerant genotypes.

Keywords: drought, osmotic adjustment, pollen grain, durum wheat, field drought tolerance index
Material and Methods

Eight genotypes of spring durum wheat of different ecological and geographical origin from the collection of the Altai Research Institute of Agriculture, Barnaul, Russia were studied. They were Omskaya stepnaya, Zhemchuzhina Sibiri, Bezencshukskaya 210, Solnechnaya 573, Oazis, Pamjatli Yanichenko, 12S1, Bezenchukskaya 210, Solnechnaya 573, Oazis, Pamyati Yanchenko, 12S1.

2010; Blum, 2011; Farooq et al., 2011; Boopathi, 2013; Nezhadhahmadi et al., 2013; Obata et al., 2015; Fleta-Soriano & Munné-Bosch, 2016). However, the success of any breeding program is determined by the availability of reliable markers, which allow identifying drought-tolerant genotypes. Crops show various morphological, physiological, biochemical, and molecular responses to the problem of water shortage (De Micco & Aronne, 2012; Nezhadhahmadi et al., 2013; Jarzyniak & Jasinski, 2014; Rybka & Nita, 2015; Fleta-Soriano & Munné-Bosch, 2016; Reynolds et al., 2016; Bi et al., 2017). Common strategies for drought adaptation include escape, avoidance, and tolerance. Drought-escaping mechanism allows plants to complete their life cycle before developing a severe dry period. This is realized by active metabolism, rapid growth, and accelerated maturation. Avoidance strategies are based on the maintaining water uptake through the modification of root architecture. Decrease in water loss is also due to reduced stomatal and cuticular conductance and reduced evaporation surfaces. Drought tolerance mechanisms are associated with the ability of cells to counter low water potential. This is achieved via osmotic adjustment and the synthesis of low-molecular weight proteins that protect plants from damage caused by water deficiency (Ashraf & Foolad, 2007; Bacelar et al., 2012). For crops grown in conditions of water shortage in arable soils, the latest two strategies are relevant. The terms “avoiding drought” and “adapting to drought” are fundamental, determining the physiological status of plants in conditions of water scarcity (Rybka & Nita, 2015). It is worth mentioning another term used in the scientific literature, “drought resistance” (Blum, 2011), which is according to Blum, is more appropriate and does a working one main for breeders who create varieties with increased productivity for regions prone to droughts.

Osmotic adjustment (OA) is wide recognized as one of the key mechanisms of drought tolerance (or “dehydration avoidance” according to Blum) in crop plants. OA is realized by decreasing the osmotic potential due to accumulation of organic and inorganic osmolytes in response to a water deficit (Zhang et al., 1999). It should be emphasized that this term is used when the response of pollen grains to induced osmotic stress. To do this, freshly cut ears were delivered to the laboratory just b plots in 2016. Mechanisms of osmotic adaptation were evaluated in vitro using pollen grains exposed to different levels of osmotic stress. To do this, freshly cut ears were delivered to the laboratory just before the start of the experiment. Drought was simulated by adding a high molecular weight osmotic agent, polyethylene glycol 6000 (PEG 6000), to the culture medium. Pollen grains from ripened but not cracked anthers were immersed over microscope slide in a drop of 30% PEG 6000 (control) as well as 55% PEG 6000 and incubated in the dark at 24 ± 1°C during 48 hours at 70-80% relative humidity. PEG is a non-penetrating osmotic that causes the collapse of cell walls and protoplast compression imitating wet the water balance of the cell under conditions of osmotic stress. Also, a variant of cultivating pollen grains in 55% PEG 6000 containing 10 mM KCl solution as an osmolyte was studied. The use of 30% PEG 6000 as a non-stressing control is due to the fact that at lower osmotic concentrations the pollen grains showed bursting (Banica et al., 2008; Patil, Ravikumar, 2011). Cytological analysis was carried out under the Olympus BX-S1 microscope at a magnification of 10×15×40. Plasmolytic phenomena were assessed
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Results and Discussion

The revealed high-level of overlapping in the genes expressed during the gametophytic and the sporophytic generations (Mascarenhas, 1990) and the similarity in their behavior under the influence of various external factors give a basis for studying the response of plants to different stresses by screening the pollen produced by those plants (Hormaza & Herrero, 1996). Besides to reducing the cost of screening, it becomes possible to analyze the stress reaction of pollen produced by a single plant without destroying this plant. To differentiate homozygous and heterozygous lines of bread wheat for use them in backcross programs, Morgan (1999) designed an experiment to assess the possibility that the gene responsible for osmotic adjustment in leaves (or) is also expressed in pollen. He managed to find the corresponding allelic differences in the pollen grains subjected to osmotic stress induced by PEG solution. We applied Morgan's pollen test to identify durum wheat genotypes that are capable of OA, with a view to their further use in breeding programs for the creation of drought tolerant varieties.

The cytoplasm area of durum wheat pollen grains subjected to different levels of osmotic stress differentiates depending both on the genotype and on the conditions of pollen cultivation (Table 1). This is confirmed by two-way analysis of variance ($F_{\text{genotypes}} = 13.02 > F_{0.05} = 2.76; F_{\text{conditions}} = 3.98 > F_{0.05} = 3.74$).

Table 1. The projected area of spring durum wheat pollen grains under conditions of in vitro osmotic stress, pxl$^2$ (mean ± SE)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>30% PEG (A)</th>
<th>55% PEG (B)</th>
<th>55% PEG + 10 mM KCl (C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solnechnaya 573</td>
<td>56824.47±4467.5</td>
<td>60323.32±3019.3</td>
<td>56036.94±723.2</td>
</tr>
<tr>
<td>Pamyati Yanchenko</td>
<td>55861.76±2198.2</td>
<td>56305.41±4452.7</td>
<td>56489.54±507.7</td>
</tr>
<tr>
<td>Bezenchukskaya 210</td>
<td>55595.15±832.7</td>
<td>64724.56±9801.9</td>
<td>56042.11±128.2</td>
</tr>
<tr>
<td>Oazis</td>
<td>50342.31±533.6</td>
<td>54818.37±726.9</td>
<td>57016.49±626.3</td>
</tr>
<tr>
<td>Zhemchuzhina Sibiria</td>
<td>50206.27±2328.9</td>
<td>50268.12±838.0</td>
<td>51399.17±395.4</td>
</tr>
<tr>
<td>Omskaya stepnaya</td>
<td>48386.25±553.3</td>
<td>54114.92±355.0</td>
<td>51082.20±606.9</td>
</tr>
<tr>
<td>12S2-24</td>
<td>46364.25±534.3</td>
<td>40386.25±1013.3</td>
<td>40956.21±2021.2</td>
</tr>
<tr>
<td>12S1-14</td>
<td>45943.25±423.3</td>
<td>41869.25±343.3</td>
<td>40023.25±335.8</td>
</tr>
<tr>
<td>LSD$_{0.05}$</td>
<td>7220.9</td>
<td>12946.8</td>
<td>1596.8</td>
</tr>
</tbody>
</table>

To avoid the influence of the initial pollen size, we calculated the relative index for estimating the pollen grain areas under conditions of induced stress, expressed as a percentage of the cytoplasm area of pollen exposed to 30% PEG 6000 solution. As it turned out, the studied genotypes are characterized by different behavior scenarios under different models of osmotic stress. So in Zhemchuzhina Sibiria and Pamyati Yanchenko, the cytoplasm area of pollen after immersion in 55% PEG 6000 has not changed. Four genotypes showed an increase in the index, whereas the lines 12S1-14 (Fig. 1A) and 12S2-24 did a decrease by more than 10% in the cytoplasm area of stressed pollen (Fig. 2).

Morgan (1999) found that the mechanism of osmotic adjustment in wheat pollen grains under induced stress conditions occurred only after the addition of a small amount of potassium chloride. The greatest response was observed at a concentration of 0.2 to 10 mM. He suggested that the before identified gene $or$, which conditions differences in the OA of wheat leaves, is responsible for the potassium transport. In our experiment, the reaction of pollen to the addition of potassium chloride as an osmolyte varied in different genotypes. In some varieties, like Pamyati Yanchenko and Zhemchuzhina Sibiria, the projected area of the cytoplasm in pollen subjected to osmotic stress remained at the control level, while in others we observed a decrease in the index relative to non-stressed pollen (Solnechnaya 573). The surface of the cytoplasm has changed. Clear cavities have appeared (Fig. 1B). In contrast, the Oazis variety showed active accumulation of osmolyte, increasing the area of pollen grains by 13%. Besides, we did not observe any plasmatic phenomena, and the pollen remained round with a uniformly distributed cytoplasm in the cell (Fig. 1C). Various durum wheat genotypes probably use different types of osmotic adaptation with different levels of inorganic cation involvement.

We tried to determine the mechanisms of OA, working in the accessions we studied. The identification of such mechanisms is based on the reasoning presented by Patil & Ravikumar (2011) and by David (2012). The detection of the type of pollen...
reaction on osmotic stress was carried out according to the ratio of the parameters of the cytoplasm (A, B, C). The so-called “intrinsic” OA is defined as the ratio B/A $\geq$ 1. When B/A < 1, there is no intrinsic OA. The reaction of the pollen cytoplasm to the external supply of an osmolyte in the culture medium is treated as an “induced” OA, which is found at the ratio C/B $\geq$ 1. The ratio C/B < 1 indicates the absence of induced OA. The total OA is composed of internal and induced ones. It is determined by the ratio C/A.

Figure 1. Modification of projected area of pollen grain cytoplasm in durum wheat genotypes under different models of in vitro osmotic stress induced by PEG 6000: A – 12S1-14; B – Solnechnaya 573; C – Oazis

Figure 2. The reaction of durum wheat pollen cytoplasm under different models of in vitro osmotic stress induced by PEG 6000, % to control

Table 2 represents the parameters described above which we calculated for an individual accession. Statistically significant differences between genotypes for various types of osmotic regulation have been established. The range of variation of the
intrinsic osmotic adjustment was from 0.871 in 12S2-24 to 1.164 in Bezenchukskaya 210. Under induced osmotic stress, the cytoplasm area of the two wheat lines (12S1-14 and 12S2-24) significantly decreased relative to the control value (B/A = 0.911 and 0.871, respectively). The remaining varieties retained the indicator in comparison with non-stress conditions, revealing intrinsic mechanism of OA. The observed retention of the cytoplasm sizes as well as the absence of appreciable cytoplasm plasmolysis in pollen cells incubated in 55% PEG 6000 solution are probably due to synthesis of organic solutes and (or) accumulation of inorganic cations through their release from membranes. In wheat, soluble sugars and K⁺ are shown to play an important role in this process (Morgan, 1999; Eivazi et al., 2007). The efficiency of cation accumulation due to transmembrane migration from the external environment can be assessed by adding osmolyte (KCl) to the pollen cultivation medium containing 55% PEG 6000. According to Table 2, the indices of induced OA (C/B) in four accessions (Bezenchukskaya 210, Omskaya stepnaya, Solnechnaya 573, and 12S1-14) were less than one, indicating that their adaptation to osmotic stress is not related to the external uptake of K⁺. In Pamyati Yanchenko, the pollen cytoplasm has not changed, and the remaining genotypes either partially restored, or even increased this parameter. The index of total osmotic adjustment (C/A) is an integration coefficient, summarizing the final effect of “partnership” of various OA mechanisms. It should be concluded that the only variety Oazis, among the studied ones, increased this parameter in comparison with the intrinsic OA index.

Table 2. Types of osmotic adjustment in durum wheat pollen grains under osmotic stress in vitro induced by PEG 6000

<table>
<thead>
<tr>
<th>Genotype</th>
<th>intrinsic OA (B/A)</th>
<th>Types of osmotic adjustment</th>
<th>induced OA (C/B)</th>
<th>total OA (C/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bezenchukskaya 210</td>
<td>1.164</td>
<td></td>
<td>0.866</td>
<td>1.008</td>
</tr>
<tr>
<td>Omskaya stepnaya</td>
<td>1.118</td>
<td></td>
<td>0.944</td>
<td>1.056</td>
</tr>
<tr>
<td>Solnechnaya 573</td>
<td>1.062</td>
<td></td>
<td>0.929</td>
<td>0.986</td>
</tr>
<tr>
<td>Oazis</td>
<td>1.089</td>
<td></td>
<td>1.041</td>
<td>1.133</td>
</tr>
<tr>
<td>Pamyati Yanchenko</td>
<td>1.008</td>
<td></td>
<td>1.003</td>
<td>1.011</td>
</tr>
<tr>
<td>Zhemchuzhina Sibiri</td>
<td>1.001</td>
<td></td>
<td>1.023</td>
<td>1.024</td>
</tr>
<tr>
<td>12S1-14</td>
<td>0.911</td>
<td></td>
<td>0.956</td>
<td>0.871</td>
</tr>
<tr>
<td>12S2-24</td>
<td>0.871</td>
<td></td>
<td>1.014</td>
<td>0.883</td>
</tr>
</tbody>
</table>

Comparison of different types of osmotic adjustment in pollen grains by regression analysis showed no correlation between induced and total adaptation (Fig. 3A). As a result, 50% of varieties possessing total OA lacked the induced one. However, total osmotic adjustment was found to be highly correlated with intrinsic OA, which was confirmed by a high coefficient of determination equal to 58% (Fig. 3B). In this case, the presence of total osmotic adjustment, as a rule, was accompanied by the presence of the intrinsic OA mechanism. We came to the conclusion that the mechanism of intrinsic adaptation to the osmotic stress induced by PEG 6000 prevails in the studied plant material.

Figure 3. The relationship between induced (A), intrinsic (B) and total osmotic adjustment in spring durum wheat genotypes

The conclusion is confirmed by Fig. 4, which indicates a statistically significant negative correlation between the intrinsic and induced OA. Such a negative correlation can create certain difficulties for combining these mechanisms in breeding for osmotic tolerance. However, studies carried out using a larger collection of wheat showed no correlation between the mechanisms of intrinsic and induced OA (David, 2012), creating reasonable optimism for their integration by breeding for drought tolerance.
Thus, Bezenchukskaya 210, Omskaya stepnaya, and Solnechnaya 573 successfully resist osmotic stress through the intrinsic adjustment mechanism. Oazis, Pamyati Yanchenko, and Zhemchuzhina Sibirii are likely to have both intrinsic and induced OA. It can be assumed that these accessions contain the or gene, which determines one of the OA mechanisms. In 12S2-24 line, only the mechanism of induced adjustment acts, but it does not compensate for the negative changes in the cell cytoplasm that occur under induced osmotic stress. Line 12S1-14 does not withstand osmotic stress and its pollen grains are most exposed to dehydration and, as a result, lose turgor.

According to Morgan (1999), the expression of the or gene, responsible for osmotic adjustment in wheat pollen, affects the transport of K+ cations sucking up from the environment. However, in our experiment, some genotypes coped with osmotic stress by intrinsic OA during the incubation of pollen in a culture medium that does not contain potassium ions (K+). It should be noted that this fact does not exclude the participation of K+ as an osmolyte, which can be mobilized from internal cell reserves. Similar results were obtained by David (2012) when screening an extensive collection of bread and durum wheat. Also, it is important to take into account that in many plant species, individual solutes do not contribute much to OA. This mechanism is realized by the accumulation of a variety of substances (Sanders & Arndt, 2012).

The agronomic criterion of drought tolerance of crops is the yield in drought conditions. Blum (2017) analyzed the data of various researchers on the relationship of OA with the yield of plants exposed to drought. He concluded that in most studies a positive association between OA and yield under stress was found. Such an effect was shown to be in wheat, barley, sorghum, maize, sunflowers, chickpeas, pea, pigeon-pea and peanuts (Blum, 2017). There is also an alternative point of view, according to which the value of OA as a component contributing to higher yields under drought conditions is questioned. On the contrary, OA is believed to be associated with “costs” in terms of yield. Probably, it just supports survival (Munns, 1988; Serraj & Sinclair, 2002; Turner, 2017). Challenging skepticism about the role of OA, Blum (2011, 2017) views this discussion as very important, as it stimulated further research in this direction and contributed to the emergence of new ideas. With regard to wheat, the agronomic value of OA in yield stabilization during drought is confirmed by the breeding a drought-resistant variety Mulgara, which is characterized by a high capacity for OA (Richards, 2006). Ciucă et al. (2010) believe that osmotic adjustment plays an important role in the formation of the wheat yield in dry years in Romania, as confirmed by the Izvor variety, which shows an excellent performance under drought and at the same time has a high capacity for OA (Bâncică et al., 2008).

In the south of Western Siberia, the conditions for vegetation of spring crops develop in different ways. They can be both very favorable moistened and sharply arid. The probability of droughts is about 30%. Both early summer droughts of varying duration and intensity and drought throughout the growing season are possible (Rozova & Ziborov, 2016). We compared the capability of pollen grains to OA under in vitro induced osmotic stress with field drought tolerance of durum wheat genotypes. The field drought tolerance index (TI) was calculated for the genotypes as a ratio between the mean yield obtained under drought stress and the corresponding trait value in favorable conditions. In 2012 there was a severe drought throughout the entire vegetation (with a short-term discharge of the moisture deficit at the end of the first decade of July), accompanied by high average daily temperatures, significantly exceeding the mean annual values, especially in the first half of the vegetation. Weather conditions in 2009, on the contrary, were characterized as very favorable for the growth and development of plants throughout the growing season.

The total OA slightly correlated with field drought tolerance. The determination index was 20% (Fig. 5A). However, a high significant correlation between TI and intrinsic adjustment was established (Fig. 5B). More than 40% of the variation was determined by the influence of the intrinsic OA mechanism. The relationship between induced OA and field drought tolerance of durum wheat genotypes has not been found. This type of osmotic adaptation, based on the expression of the gene regulating the transport of external osmolytes, is poorly involved in the material studied and could serve as a reserve for increasing the drought resistance of local varieties. The search for sources of induced osmotic adjustment and their involvement in the breeding program can be considered one of the ways to create drought resistance genotypes.
Figure 5. Relationship between total OA (A), intrinsic OA (B) and grain yield of spring durum wheat genotypes cultivated under field drought conditions

It should be noted that a low correlation (or lack of correlation) between the index of field drought tolerance and total OA in the presence of a significant genetic variation of the trait is quite possible because other mechanisms of resistance to stress may act as dominant ones under certain drought scenarios. Such examples are known in the scientific literature (Blum, 2017). So, OA in chickpea at the stage of grain filling did not correlate with the yield under drought, while this mechanism showed such a link at an earlier growth stage. Probably, under the water deficit, the stem reserve utilization for grain filling might have had a greater contribution to the yield (Leport et al., 1999). In our experiment, the mechanism of intrinsic adjustment in genotypes prevailed during the adaptation of pollen under induced osmotic stress, which probably caused a close correlation of this trait with yield under drought. There was no correspondence between TI and the induced OA, despite the fact that some genotypes had this mechanism.

Conclusions

Thus, we have established significant differences among varieties of spring durum wheat relative to OA mechanisms responsible for dehydration avoidance. The response of pollen grains to in vitro induced osmotic stress reflected various strategies for the behavior of genotypes involving or not transporting K+ cations from the cultivation medium. Most of the accessions tested are capable of supporting the cellular turgor, using only intrinsic adjustment mechanism, or combining it with osmolyte induced OA. One line has been shown to exhibit the induced osmotic adaptation, which caused some restoration of the projected cytoplasm area. Finally, one line did not resist osmotic stress, reducing the pollen turgor both under stress and in the presence of osmolyte. We succeeded in revealing a good correspondence between the pollen reactions under induced in vitro osmotic stress and the field drought tolerance index of genotypes. This made it possible to test the diversity of the accessions for tolerance to drought by scanning the pollen surface. Assessment of the behavior of the male gametophyte population of wheat under conditions of induced stress allows screening and determining drought-tolerant genotypes.

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References


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