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Pollen Selection for Drought Tolerance in Sunflower

DOI 10.1515/helia-2015-0012
Received July 29, 2015; accepted August 13, 2015; previously published online August 26, 2015

Abstract: The drought resistance and genetic structure of F_2 sporophytic populations for some morphological marker traits after pollen selection for water stress tolerance in F_1 sunflower hybrids has been studied. The emasculated inflorescences were moistened with 10% and 20% of PEG 6000 solution in the experiments and with distilled water in the control. Freshly collected pollen was used for pollination after drying up the PEG solution on stigmas. F_2 resulted seeds were germinated in 20% solution of PEG 6000 for a period of 3 days, and then the percentage of seed germination was counted. Germinated and not germinated seeds were separately planted. Segregation ratios in F_2 populations for leaf venation and leaf chlorophyll deficiency marker traits were analyzed at early stage of development. Pollen treatment significantly increased the drought resistance of F_2 populations and changed the monogenic ratios for leaf chlorophyll deficiency marker trait.

Keywords: sunflower, F_1 hybrids, osmotic, pollen selection, water stress, drought tolerance, selective elimination, F_2 sporophytic generation

Introduction

Sunflower is a mesophyte, but it is very demanding to the presence of moisture. That is why the yield and efficiency of its growing is limited by moisture providing of the plants. At the same time owing to a strong root system, sunflower is relatively drought tolerant plant (Shpaar *et al*., 1999). Despite this fact, the yield losses may reach 50% because of drought in sunflower (Rauf, 2008). Therefore, increasing the drought resistance is an important direction of modern breeding of this crop.

In sunflower the evaluation of drought resistance may be carried out at different stages of plant development using different field and laboratory

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methods. Onemli and Gucer (2010) evaluated the drought tolerance at seedling stage applying the growing method. Nagarathna *et al.* (2012) analyzed the resistance to drought by some morphological traits and water use efficiency in adult plants. Biochemical parameters such as level of abscisic acid (Hoad, 1975), the content of aquaporins, dehydrins, fructose-1,6-bisphosphatase (Poormohammad *et al.*, 2007) as well as free proline content and peroxidase activity (Garcia *et al.*, 1987) are also used for determination of drought resistance. The exsiccator method, based on the evaluation of the cell surviving after dehydration, is known (Polevoy *et al.*, 2001). The ability of seed germination in the condition of osmotic stress is used for determining of drought resistance at the seed stage (Polevoy *et al.*, 2001).

Along with the traditional methods of selection at the level of sporophyte, the methods of gamete selection for drought tolerance are already developed for some crops. There are some data about the existence of high positive relationship between pollen resistance to high temperatures and the ability of seeds to germinate under high osmotic pressure in flax and castor. On this base the high temperature treatments of pollen were used to select the drought tolerant genotypes (Mishchenko, 1999; Odinets and Lyakh, 1997). In sorghum the gametophytic selection for drought tolerance was conducted using osmotically active substance, which served as a selective barrier for pollen (Patil *et al.*, 2006).

In sunflower the data on successful gametophytic selection for resistance to drought are not known, although the effectiveness of the pollen selection for resistance to diseases (Rani and Ravikumar, 2006), high (Lyakh and Totsky, 2014a) and low (Lyakh and Totsky, 2014b) temperatures is already shown.

The aim of this research was to study the influence of osmotic stress during pollen germination in F_1 sunflower hybrid on the drought tolerance of the F_2 resulting sporophytic offspring.

Materials and methods

F_1 "*virescent*" × "dichotomous venation" sunflower hybrid was used as the experimental material. The parental lines of this hybrid were obtained through induced mutagenesis (Lyakh *et al.*, 2005) and characterized by different tolerance to drought. "*Virescent*" mutant exceeded "dichotomous venation" line more than twice in the relative dry weight of seedling roots after seed germination in saccharose solution. That is why the "*virescent*" mutant was more resistant to drought than the "dichotomous venation" line (Totsky and Lyakh, 2014).

"Dichotomous venation" mutant has the marker trait of dense network of the fan-shaped veins. "*Virescent*" mutant has light yellow upper leaves during the first weeks of growth. Both mutant traits are easily identified at early stages of plant development.

The technique of forced cross-pollination was used to obtain the seeds of F_1 hybrid in 2013. F_1 plants were grown in the field conditions of 2014. Before flowering, the inflorescences of F_1 plants were isolated. One part of these plants was emasculated within 1–5 days for artificial pollination. The other part was grown without emasculation for collecting fresh pollen. Fresh mature pollen derived from several not emasculated inflorescences of hybrid plants was used for pollination.

Viability test showed that pollen germination on standard artificial nutrient medium (Lyakh *et al.*, 2000), which contains 30% of polyethylene glycol (PEG) 6000, was more than 24%. At the same time, pollen germination on the artificial nutrient medium, which contained 40% and 50% of PEG 6000, was about 5.7% and 3% respectively. Thus, an increase of PEG 6000 content for 10% and 20% in artificial nutrient medium significantly decreased pollen germination.

The modified technique, proposed by Patil et al. for sorghum, was taken into account to carry out the gametophytic selection for drought tolerance in sunflower (Patil *et al.*, 2006). In the experiment the stigmas of emasculated inflorescences were moistened with 10% and 20% of PEG 6000 solution. In this case PEG 6000 solution was used as osmotic selective barrier for pollen. After drying up the PEG 6000 solution, treated inflorescences were pollinated with fresh pollen. The inflorescences moistened with distilled water were used for pollination in the control. One cm^3 of pollen was taken for pollination of each head.

Drought tolerance of F_2 sporophytic populations was evaluated by the seed germination in PEG solution. The seeds were placed in Petri dishes with 20% PEG solution and germinated at a temperature of 25 ± 2°C for a period of 3 days. After that, the percentage of seed germination was calculated (Polevoy *et al.*, 2001 with our modification where PEG solution was used instead of saccharose).

Germinated and not germinated in PEG solution seeds were separately planted in the phytotron under optimal conditions. At the stage of the second pair of true leaves the genetic structure of F_2 populations for "dichotomous venation" and "*virescent*" marker traits was analyzed. The experimental F_2 populations, obtained after treating the inflorescence with the PEG solution and composed of germinated and not germinated on PEG solution seeds in Petri dishes, were compared to control F_2 populations, obtained after treating the inflorescence with the distilled water and composed of germinated and not germinated on PEG solution seeds. The same comparison was carried out using only germinated in PEG solution seeds.

All experiments were performed in duplicate. The differences in drought tolerance were defined by the t-test at the 0.01 and 0.001 levels of probability. The χ^2 method was used to evaluate the differences in the segregation ratios for marker traits.

Results and discussion

The percentages of germination in 20% PEG 6000 solution of F_2 seeds, obtained with and without gametophytic selection for tolerance to water stress, were compared to determine the effect of pollen germination in the presence of osmotic on stigmas during pollination on drought tolerance of resulting sporophytic population (Table 1).

Table 1: Influence of pollen germination of F_1 hybrid in the presence of osmotic on drought resistance of F_2 resulting sporophytic offspring in sunflower.

Treatment	F_2 seeds		Germination, %
	total	germinated	
Control	156	30	19.2 ± 3.15
Pollen germination on the stigmas moistened with 10% solution of PEG 6000	154	106	68.8 ± 3.73***
Pollen germination on the stigmas moistened with 20% solution of PEG 6000	148	23	15.5 ± 2.97

Note: ***differences from the control are significant at the 0.001 level of probability.

As can be seen from the presented in Table 1 data, in "*virescent*" × "dichotomous venation" cross combination the percentage of seed germination in the experiment, where 20% solution of PEG 6000 was used, did not differ from the control. However, the percentage of F_2 seed germination under conditions of osmotic stress was significantly higher in the experimental F_2 population, which was obtained after pollination with fresh pollen of inflorescence moistened with 10% solution of PEG 6000. These results may indicate that such gametophytic selection was effective and resulted in drought resistance improvement of F_2 sporophytic population. Pollen germination on the stigmas, moistened with 20% solution of PEG 6000, was not effective in this respect, what can be explained, perhaps, by too severe selection that lost its selective effect.

Additionally, the study of the genetic structure of F_2 populations for "*virescent*" and "dichotomous venation" marker traits was conducted (Table 2).

Table 2: Influence of pollen germination of F_1 sunflower hybrid in the presence of osmotic on segregation ratio in F_2 generation for «dichotomous venation» and «*virescent*» marker traits.

Pollen treatment	F_2 phenotypes			Segregation ratio	χ^2
	normal plants	plants with marker trait	total		
				«*Virescent*» marker trait	
Control	198	42	240	4.7:1	–
Pollen germination on the stigmas moistened with 10% solution of PEG 6000	216	68	284	3.2:1**	8.04
Pollen germination on the stigmas moistened with 20% solution of PEG 6000	150	52	202	2.9:1**	9.39
				«Dichotomous venation» marker trait	
Control	186	53	239	3.5:1	–
Pollen germination on the stigmas moistened with 10% solution of PEG 6000	213	71	284	3:1	1.27
Pollen germination on the stigmas moistened with 20% solution of PEG 6000	156	44	200	3.5:1	0.01

Note: $\chi^2_{0.01}(df = 1) = 6.64$.
**differences from the control are significant at the 0.01 level of probability.

Change in the segregation for marker traits may show that the genes, which determine some marker traits, directly affect the drought tolerance of plants or are linked to the genes, which determine the plant resistance to osmotic stress and dry environmental conditions.

The results of marker analysis for "*virescent*" trait showed the modification in segregation for both experimental F_2 populations, which were obtained after gametophytic selection, compared to the control. In all cases, there was a significant increase of the number of plants with the "*virescent*" trait. For the "dichotomous venation" marker trait the differences in segregation ratios between control and experimental F_2 populations were not observed. Thus, we can suppose that the genes, which control this marker trait, are not linked to the genes that determine the drought tolerance.

Therefore, we can state the fact of the changing the genetic structure of F_2 populations after carrying out gametophytic selection for drought tolerance because it favored the survival of gametes with the "*virescent*" marker trait.

The data on the effect of pollen germination on stigmas moistened with the osmotic on the genetic structure of F_2 populations, which were composed of germinated on PEG 6000 solution seeds, are presented in the Table 3. It should be noted that these plants are more drought-resistant part of the plant population.

Table 3: Genetic structure of F_2 sunflower populations composed of germinated in PEG solution seeds.

Pollen treatment	F_2 phenotypes			Segregation ratio	χ^2
	normal plants	plants with marker trait	total		
				«*Virescent*» marker trait	
Control	26	1	27	26:1	–
Pollen germination on the stigmas moistened with 10% solution of PEG 6000	85	16	101	5.3:1***	41.72
Pollen germination on the stigmas moistened with 20% solution of PEG 6000	14	7	21	2:1***	51.69
				«Dichotomous venation» marker trait	
Control	21	6	27	3.5:1	–
Pollen germination on the stigmas moistened with 10% solution of PEG 6000	76	25	101	3:1	0.37
Pollen germination on the stigmas moistened with 20% solution of PEG 6000	15	6	21	2.5:1	0.49

Note: $\chi^2_{0.001}(df = 1) = 10.83$.
***differences from the control are significant at the 0.001 level of probability.

The results of marker analysis, which are presented in Table 3, show that the differences in the segregation ratio between the control and experimental populations for "dichotomous venation" marker trait were not observed. However, pollen germination of F_1 hybrids on stigmas moistened with 10% and 20% solution of PEG 6000 modified the genetic structure of drought resistant part of F_2 population for "*virescent*" marker trait. In both cases, the number of plants with the "*virescent*" marker trait significantly increased. It should be noted that the plants with "*virescent*" marker trait were almost absent (26:1) in the drought resistant part of control F_2 population, whereas in the drought resistant part of

experimental F_2 populations, where stigmas were moistened with the 10% and 20% solution of PEG 6000, the number of such plants raised more than 5 and 10 times respectively. Thus, we can say that increasing the number of plants with the "*virescent*" marker trait in whole population was mainly caused due to enlarging the number of these plants in drought tolerant part of experimental F_2 populations.

In summary, it must be said that pollination with fresh pollen of stigmas moistened with the osmotically active substance (PEG 6000 solution) can increase the drought resistance of F_2 sporophytic populations and modify their genetic structure for some marker traits. Also, it should be noted, that simultaneous enhancing the drought resistance of F_2 population and the number of plants with the "*virescent*" marker trait in whole F_2 population, and number of these plants in the most drought resistant part of F_2 population after gametophytic selection, indicates that the gene or genes, which determine the "*virescent*" trait, directly influenced the drought resistance or were linked to the genes responsible for drought tolerance of pollen and plants. At the same time, the absence of changes in the segregation ratio for the "dichotomous venation" marker trait indicates that genes, which determine this trait, were not linked to the genes that affected the drought tolerance in sunflower.

It should be noted that the laboratory method, which may not always correspond to the field methods, was only used to evaluate the drought tolerance of F_2 seeds. However, this approach was quite sufficient to reveal the effectiveness of pollen selection for drought tolerance in sunflower.

In our previous studies it was shown that not only stigmas moistening with the osmotically active substance before pollination but also pollen heating was effective to improve the drought resistance of sunflower (Lyakh and Totsky, 2014a). Pollen heating was performed in air bath oven where the low humidity of air in addition to high temperature influenced pollen as well.

To separate the effect of water stress on pollen from the effect of high temperature the method, proposed by Patil *et al.* (2006) for sorghum, where PEG 6000 at the concentration of 36% was applied as osmotically active substance. To adapt this technique for sunflower a series of experiments that included the pollen germination on artificial medium with different content of PEG 6000 was conducted. The results of these studies resulted in the conclusion that the treatment of sunflower inflorescences with 10% and 20% solutions of PEG 6000 *in vivo* can serve as an effective selective barrier for pollen.

As it was shown in our paper, gametophytic selection in F_1 can modify not only drought resistance of sunflower, but also the genetic structure of F_2 populations for the "*virescent*" marker trait. Other authors' studies also suggest changing the genetic structure of populations as a result of gametophytic

selection. Thus, pollen heating in F_1 hybrid increased in their offspring the number of plants with the marker traits, which were characteristic for drought tolerant genotypes, in castor (Odinets and Lyakh, 1997). After pollen storage of F_1 hybrid at low temperature, the modification of BC_1 population structure for corolla color and some quantitative traits was found in linseed (Lyakh *et al.*, 2000; Mishchenko, 1999). The structure of F_2 populations for flower color after gametophytic selection for cold resistance was changed in chickpea as well (Clarke *et al.*, 2004). In sorghum the gametophytic selection for drought resistance modified the structure of F_2 populations for such quantitative traits as the duration of the "emergence-flowering" period, plant height, panicle length, panicle width, panicle weight, 1000 seed weight and seed yield (Patil *et al.*, 2006).

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Sélection de pollen pour la tolérance à la sécheresse chez le tournesol

Résumé

La résistance à la sécheresse et la structure génétique des populations F_2 pour certains traits marqueurs morphologiques après sélection de pollen pour la tolérance aux stress hydrique dans les hybrides F_1 de tournesol ont été étudiés. Les inflorescences émasculés ont été humidifiées avec 10% et 20% de solution de PEG 6000 dans les expériences et avec de l'eau distillée dans le contrôle. Pollen fraîchement ont été utilisées pour la pollinisation après le tarissement des stigmates. Le graines F_2 ont germer en solution à 20% de PEG 6000 pour une durée de 3 jours et ensuite le pourcentage de germination des graines a été compté. Graines germées et non germées ont été plantés séparément. Ratios de ségrégation des populations F_2 pour traits de nervation des feuilles et insuffisance de chlorophylle dans la feuille ont été analysés au stade précoce de développement. Traitement de pollen a augmenté significativement la résistance à la sécheresse des populations F_2 et a modifié les ratios monogéniques pour trait de insuffisance de chlorophylle dans la feuille.

La selección de polen por tolerancia a la sequía en girasol

Resumen

La resistencia a la sequía y de la estructura genética de las populaciones esporophyticas F_2 para algunos morfológicos marcadores rasgos después de la selección de polen para la tolerancia a estrés hídrico en los híbridos F_1 de girasol se ha estudiado. Las inflorescencias castradas se humedecieron con 10% y 20% de soluciónes de PEG 6000 en los experimentos y con agua destilada en el control. Polen fresco se utilizó para la polinización después del secado de los estigmas. Semillas F_2 fueron germinadas en solución de 20% de PEG 6000 por un período de 3 días y luego se contó el porcentaje de germinación de las semillas. Semillas germinadas y no germinadas fueron plantadas por separado. Relaciones de segregación en populaciones F_2 para rasgos marcadores de hoja venación y insuficiencia de clorofila de la hoja fueron analizadas en la etapa temprana de desarrollo. Tratamiento de polen aumentó significativamente la resistencia a la sequía de las populaciones F_2 y cambió las relaciones monogénicas para rasgo marcador de insuficiencia de clorofila de la hoja.