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INTERSPECIFIC HYBRIDIZATION BETWEEN DURUM WHEAT AND *AEGILOPS UMBELLULATA* (ZHUK.)

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Abstract

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The possibility for obtaining of interspecific hybrids between three durum wheat genotypes (*Triticum durum*, 2n = 4x = 28, AABB genomes) and one accession of *Aegilops umbellulata* (2n = 2x = 18, UU genome) during two different seasons has been studied. A low crossability rate of 3% average for all genotypes over two years was achieved. The observed variation of crossability is due to a greatest extent to the genotype of durum wheat parent with 82.91% from total variation. Hybrid plants were obtained only by means of embryo rescue method. The ability for *in vitro* regeneration was still independent of crossability of used durum wheat genotypes. All received F_1 hybrids plants were identical they exhibited good tillering ability and manifested traits from both parents. In spite of the observed partially fertility in F_1 hybrids between durum wheat and *Aegilops umbellulata* no germination of the hybrid seeds was ascertained. BC_1 seeds were obtained from F_1 hybrids of hybrid combination Beloslava × *Aegilops umbellulata* after backcrossing to the durum wheat parent. An increase of crossability with advancing of backcross progenies – from 1.6% in BC_1 hybrids to 26.2% in BC_2 hybrids has been found. Meiotic abnormalities including dyads and triads at the end of microsporogenesis as well as uninucleate microspores with a different shape and size were observed suggesting production of unreduced gametes in this cross.

Key words: crossability, embryo rescue, interspecific hybrids, meiotic abnormalities

Introduction

The wild and cultivated relatives of family *Gramineae* are source of useful alleles for wheat improvement. A promising breeding method for creation of new variability is the wild hybridization that became a more common practice after the advancement of hybridization techniques and embryo rescue method (Mujeeb-Kazi and Rajaram, 2002). Recently complex protocols associated with genetic transfers from the more distant alien species are established in results of impressive progress in molecular genetics, cytogenetics and genomics that allow simplification of the introgression of desired alien chromatin into wheat (Ceoloni and Jauhar, 2006). Regardless of the above mentioned advancement and the effort of many researchers to explore and include genetic plasma from wild and cultivated species of family *Gramineae*, this biodiversity is not yet sufficiently utilized in wheat breeding programs and only a limited number of the modern cultivars contain wild species in theirs pedigree (Chapman, 1989; Jauhar, 2006).

The *Aegilops* species are described as sources of desirable agronomic traits as biotic and abiotic resistance that could be introduced into cultivated wheat (Monneveux et al., 2000). It was found that the diploid *Aegilops* species as well as tetraploid species carrying the U-genome are very resistant to all foliar diseases (Dimov et al., 1993; Mamluk and Van Slageren, 1994). The diploid species *Aegilops umbellulata* Zhuk.(U*)* is

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of interest for wheat breeding program as source of genes for: resistance to stem rust (*Puccinia graminis tritici*), leaf rust (*Puccinia recondita*) (Özgen et al., 2004; Chhuneja et al., 2008) blotch pathogen *Septoria nodorum* Berk. (Maksimov et al., 2006), drought and salt tolerance (Uhr et al., 2007), high protein content (Karagoz et al., 2006).

Up to now, this wild relative was very rarely utilized for durum wheat improvement and there are very few reports for successful hybridization between the two species (Makino, 1981; Zaharieva et al., 2003; Özgen et al., 2004).

The objectives of this paper are to report the results obtained from interspecific hybridization between different Bulgarian durum wheat genotypes and wild species *Aegilops umbellulata* and as well the characterization of the produced hybrids.

Material and Methods

The plant materials used in this study include two durum wheat cultivars and one breeding line created in the Field Crops Institute (FCI) Chirpan, Bulgaria listed in Table 1 and one accession of *Aegilops umbellulata* (U) (kindly provided from the collection of Bulgarian Gene Bank in Institute of Plant Genetic Resources (IRGR) – Sadovo). The durum wheat genotypes were grown in the experimental field of FCI in two replications. Accession of *Aegilops umbellulata* was grown in greenhouse conditions so that its flowering time is synchronized with the wheat flowering time in the field. The hybridization has been realized in the field conditions during two different seasons - 2008/2009 years. Durum wheat genotypes were used as female parent. At least five spikes per replication were pollinated in each combination. Three days prior to anthesis durum wheat spikes were emasculated and bagged to avoid pollination with other wheat plants. Crosses were carried out using fresh pollen of the wild relative. The number of seeds set on durum wheat spikes was counted 16-20 days after pollination.

The hybrid seeds were removed from the spikelets 16-20 days after pollination, washed in 70% C_2H_5OH and sterilized in 10% sodium hypochlorite solution for 10 minute. After several rinsing in sterile water, embryos were isolated under aseptic conditions and cultivated in Petri dishes on modified medium, containing macro- and micro salts of medium MS (Murashiege and Skoog, 1962), vitamins according Chu et al. (1990), asparagines – 200 mg/l, casein hydrolysate - 200 mg/l, kinetin – 0.1 mg/l, indole-3-acetic acid (IAA) – 0.1 mg/l, abscisic acid (ABA) – 0.1 mg/l. The cultures were incubated in darkness at 26±2°C for the first two weeks and then, when the first regeneration indications appeared, were transferred to a culture room under a 16-hours light period and light intensity of 3000 Lux. The hybrid embryos were transferred to fresh media every three weeks. In the second true leaf stage, the regenerated plants were transferred into pot with soil mixture and watered with nutrition solution. The preliminary adaptations of regenerated plants were carried out in covered containers and then were cultivated in open-air.

For cytological investigation of meiotic irregularities spikes from hybrids plants with anthers containing pollen mother cells and microspores at different stage of microsporogenesis were fixed for three hours in a fresh Clarke's solution (ethanol:acetic acid, 3:1 v/v). Then they were transferred to 70% ethanol and stored at 4ºC. Anthers were squashed and pollen mother cells and microspores were stained with aceto-carmine.

The following traits: plant height, spike length, general tillering, leaf length, leaf width, leaf hairiness, angled stem, resistance to leaf diseases and plant shape were studied in parents and F_1 hybrid plants.

The results of crossability were processed via two-factor analysis of variance to prove genotype differences and influence of studied factors on the detected variability using the program package STATISTICA 7.0 (2004).

The F_1 hybrid plants were used as female parent and were backcrossed to durum wheat and the new generation obtained was marked as BC_1.

Results

Three durum wheat genotypes – Bulgarian cultivars Victoria and Beloslava and breeding line D-7383 were hybridized to one accession of wild relative *Aegilops umbellulata*. The crossability between them expressed

as a percent of pollinated florets produced caryopsis with embryo is presented in Table 1. From 1432 pollinated flowers were obtained 43 seeds for two years, i.e. the main crossability for all hybrid combinations was 3.0 %. It varied from 2.7 % in the first year to 3.6 % in the second year. Variation in percentage of seed set was also found among the durum wheat genotypes as female parents – the lowest at breeding line D-7383 – 0 % in the first year to 1.9 % in the second year and the highest at cultivar Victoria – 4.6 % to 6.8 % respectively. As a whole very low - less than 7% crossability was achieved. The presented results of analyses of variance (Table 2) confirm that the differences between durum wheat genotypes are statistically significant. The variation of crossability at the hybridization between durum wheat and *Aegilops umbellulata* in our experiment is to the greatest extent, due to the genotype of cultivated parent with 82.91 % from total variation. The influence of years and interaction between year and genotypes on the variation of crossability is vastly less, although the variances of both factors are statistically significant.

In our experiment hybrid, plants were obtained only by means of the embryo rescue method (Table 3). The most hybrid caryopsis had small-differentiated embryos with normal endosperm. From 43 isolated embryos, 13 plants were regenerated and four were successfully adapted. The percent of regenerated plants - 30.2 % and especially the percent of adapted plants – 9.8 % are comparatively low. The dying of big part of the regenerated plants in different development stages was due to weak vitality of hybrid plants and not controlled conditions during acclimatization and further cultivation. The highest percent of regenerated and adapted plants was achieved for hybrid combination D-7383 x

Table 1
Crossability between durum wheat genotypes and wild relative *Aegilops umbellulata*

Cross combination	Florets Pollinated *No.*	Seeds *No.*	Cross-ability *%*
2007			
Victoria × *Aegilops umbellulata*	392	18	4.6
Beloslava × *Aegilops umbellulata*	414	6	1.5
D-7383 × *Aegilops umbellulata*	94	0	0.0
Total	900	24	2.7
2008			
Victoria × *Aegilops umbellulata*	176	12	6.8
Beloslava × *Aegilops umbellulata*	212	5	2.4
D-7383 ×*Aegilops umbellulata*	144	2	1.9
Total	532	19	3.6
Total for 2 years	1432	43	3.0
Backcross progenies			
(Beloslava × *Aegilops umbellulata*) BC_1F_1	392	7	1.8
(Beloslava × *Aegilops umbellulata*) BC_2F_1	164	43	26.2

Table 2
Analysis of variance for crossability between durum wheat genotypes and *Aegilops umbellulata*

Source of variation	Degrees of freedom *d.f.*	Sum of squares *SS*	Variance *MS*	η^2 *%*
Total	17	90.76		
Genotypes	2	75.25	37.62***	82.91
Years	1	12.50	12.50***	13.77
Interaction (G ×Y)	2	1.39	0.70*	1.53
Error	12	1.62	0.13	

η2 – eta-squared - effect size of the studied factors from the total variation

Table 3
Plants regeneration of interspecific hybrids between durum wheat and *Aegilops umbellulata* after embryo rescue

Cross combinations	Cultivated embryos	Regenerated plants		Adapted plants	
	No.	*No.*	*%*	*No.*	*%*
Victoria × *Aegilops umbellulata*	30	7	23.3	0	0.0
Beloslava × *Aegilops umbellulata*	11	4	36.4	2	18.2
D-7383 × *Aegilops umbellulata*	2	2	100.0	2	100.0
Total	43	13	30.2	4	9.3

Aegilpops umbellulata, while no plants were regenerated from hybrid combination Victoria x *Aegilops umbellulata* possessing the highest crossability.

All F_1 hybrid plants obtained were identical, exhibited good tillering ability and manifested traits from both parents (Figure 1). The mean values of different morphological and agronomical traits of F_1 hybrid plants from the cross Beloslava x *Aegilops umbellulata* and their parents were presented in Table 4. The shape of plants and spikes was as erect as durum wheat. The F_1 hybrid spikelets and seeds had a morphology similar to wheat. The leaf length, leaf width and number of tillers were intermediate between the two parents. By other traits such as plant height, spike length, highly leaf hairiness and resistance to leaf diseases hybrid plants resembled the wild parent *Aegilops umbellulata.*

A part of the spikes of regenerated F_1 hybrid plants were selfed and the rest were backcrossed with pollen from durum wheat. F_1 hybrids showed a partial fertility – 13.8 % and formed few seeds. The hybrid seeds did not germinated because of hybrid inviability and no F_2 plants were produced.

Fig. 1. Spikes of F_1 hybrid Beloslava x *Aegilops umbellulata* (center), durum wheat parent cultivar Beloslava (right) and wild parent (left)

BC_1 seeds were obtained after backcrossing of F_1 hybrids (Beloslava × *Aegilops umbellulata*) to the durum wheat parent. The crossability rate was no higher than that accounted for the F_1 hybrids. An increase in crossability with advancing of backcross progenies – from 1.6 % in BC_1 hybrids to 26.2 % in BC_2 hybrids was found (Table 1). The fertility of hybrid plants rose after each subsequent backcrossing. The germination rate in backcrossed seeds was better compared to selfed F_1 hybrids.

Cytological investigations of F_1 and BC_1 hybrid plants during the anaphase II and uninucleate microspores stage of microspores revealed irregular passing of meiosis. During the late anaphase II stage multiple bridges, fragments (Figure 2a), irregular cytokinesis and laggard chromosomes (Figure 2b) in low frequency were found. Abnormal tetrads, manifested by frequent formation of dyads, triads and polyads (Figure 2d-g) as well as irregular position of microspores in the tetrads (Figure 2h) were observed. Formation of micronuclei and microcytes was detected, too (Figure 2c, e). These

Table 4
Comparison between F_1 hybrid *Triticum durum* (cultivar Beloslava) × *Aegilops umbelullata* and his parents

Traits	*Triticum durum* Parent	*Aegilops umbellulata* Parent	F_1 hybrid
	Average	*Average*	*Average*
Plant height, cm	90.3	52.3	50.0
Number of tillers	15	29	21
Spike length, cm	8.5	4.0	5.5
Leaf length, cm	40.0	11.5	20.2
Leaf width, cm	2.2	0.6	1.2
Leaf hairiness	light hair	high/ heavy hair	high/ heavy hair
Angled stem	no	yes	no
Resistance to leaf diseases	S	R	R
Plant shape	dressed	rosette	dressed

R - resisting
S - sensitive

meiotic anomalies resulted in the formation of irregular microspores during the next stage of the microsporogenesis - microspores with irregular shape, size and number (Figure 2i) and degenerating microspores (Figure 2k). In BC_1 progeny decreased number of types and frequency of meiotic abnormalities was observed.

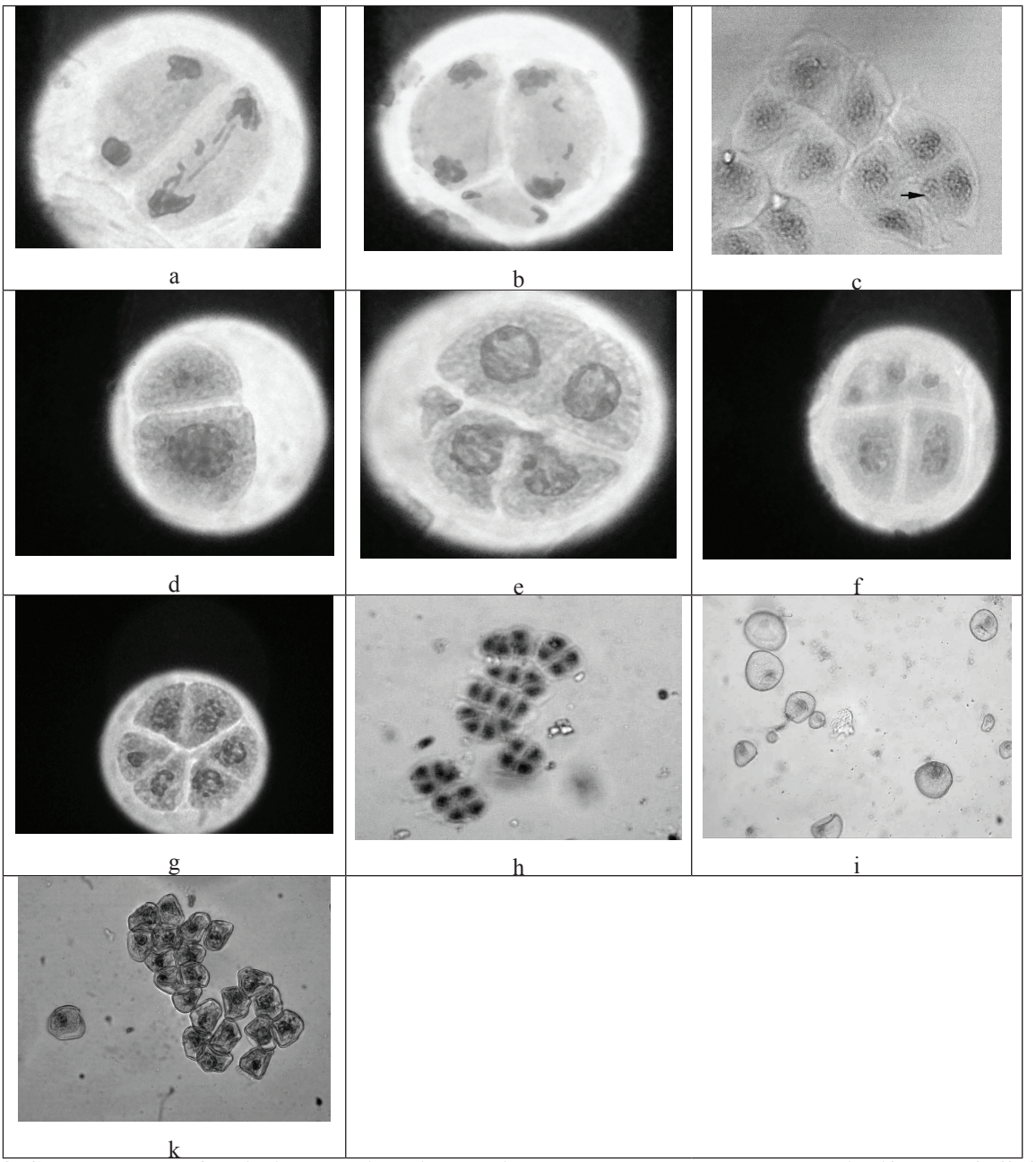

Fig. 2. Some aspects of meiotic behaviour in hybrid between durum wheat and *Aegilops umbellulata:* a – late anaphase II with bridges and segments, b - late anaphase II with irregular cytokinesis and laggards chromosomes, c – tetrads with microspores with micronuclei, d - diad with microspores of different sizes and micronuclei, e – triad with microcite, f – triad with one trinucliated microspore, g - hexsad, h - irregular position of microspores in the tetrads, i - microspores with irregular shape and size, k – degenerating microspores

Discussion

A prerequisite for using wild species as germplasm is a successful hybridization and backcrossing. Unfortunately, the most of the well adapted wheat cultivars are not crossable with the alien species. Thereby the numbers of breeding lines that can be used for alien introgression are restricted. Very few genotypes of cultivated wheat (so-called genetic models) possess the recessive *kr*-alleles of the genes responsible for high crossability (Manickavelu et al., 2009). Hitherto the most intra and interspecific hybrids have been produced with these genotypes (Mujeeb-Kazi and Rajaram, 2002). The results presented here reveal the possibility interspecific hybrids between commercial durum wheat cultivars possessing good agronomic characteristics and diploid wild species *Aegilops umbellulata* to be produced even at low crossability rate – 3% average for all genotypes over two years. The observed low crossability is in conformity with the law of genetic distance between the both species and their genome homeology. In the few publications appeared so far on hybridization between cultivated wheat as female parent and diploid grass *Aegilops umbellulata* has been reported higher crossability than that obtained in our experiment – 17.9% for cross with *Triticum durum* (Zaharieva et al., 2003) and from 12.5 to 68.8% for cross with *Triticum aestivum* (Bochev, 1993). The above discrepancy and the observed significant differences in crossability among used in our experiment durum wheat lines confirmed that success of alien hybridization is highly genotype dependent.

The embryo rescue was necessary to obtain interspecific hybrids between durum wheat and *Aegilops umbellulata* both in our and previous studies (Zaharieva et al., 2003). The used durum wheat genotypes differed in *in vitro* response of immature embryos and further plant regeneration. Genotypic differences in success rate of hybrid embryo rescue were already reported in wild hybridization of wheat (Kapila and Sethi, 1993; Saïdi et al., 1998). The ability for in vitro regeneration was still independent from crossability of used durum wheat genotypes. Other researchers have reached the same conclusion in investigations of various crosses between distant species of family *Gramineae* (Fujigaki and Tozu, 1993; Wojciechowska and Pudelska, 2002). The small number of regenerated plants resulting from the rescue of immature hybrid embryos is to be expected and has been reported in a number of previous studies, too. The failure of many of the rescued hybrid embryos to germinate and reduced viability of the hybrid seedlings is common in interspecific crosses, possibly due to activation of post-zygotic incompatibility mechanisms (Bajaj, 1990).

The presented results underscore that the effectiveness of hybridization between durum wheat and *Aegilops umbellulata* depends not only on crossability, but also on ability of *in vitro* cultivated embryos to develop into plants.

In spite of the partially fertility observed in F_1 hybrids between durum wheat and *Aegilops umbellulata* no germination of the hybrid seeds was ascertained. The lack of germination of the seeds obtained from the regenerated *in vitro* plants is again an expression of the above mentioned effect of post-zygotic incompatibility occurred after wild hybridization. This occurrence has been revealed in different intra - and interspecific crosses (Sears, 1943; Tikhenko et al., 2008). Zaharieva and Monneveux (2006) emphasized on lower hybrid seed viability in crosses involving the diploid *Aegilops* species that is confirmed in our study.

Due to partial sterility of these interspecific hybrids we were able to develop them further by direct back-crossing with the durum wheat parent as previously experienced by other authors (Zaharieva et al., 2003). A restoration of fertility in backcross progenies was achieved which is prerequisite to utilize backcross strategy for introgression of desirable characters from *Aegilops umbellulata* into durum wheat in the future generation.

The meiotic behavior observed during the anaphase II and uninucleate microspores stage of the F_1 and BC_1 hybrid plants elucidates the disturbances of the fertility in the hybrid progenies. The very small microspores most likely result from detected micronuclei and turned then to sterile pollen grains. Many arguments have been published that frequency of micronuclei in tetrads is correlated with frequency of univalent at metaphase I and that elimination of a micronuclei from the microspore is a kind of chromosomes elimination (Davies, 1974, Baptista-Giacomelli et al., 2000). Chromosomes elimination in interspecific hybrids

is one prerequisite for production of addition and substitution lines, that can be used in our further efforts to involvement of *Aegilops umbellulata* for durum wheat improvement.

The partial fertility of F_1 hybrids and backcross progeny in our study could be explained with the presence of unreduced gametes (2n) in small frequency. Unreduced gametes (2n) transmit the whole chromosome complement of parents to their offspring and are mostly involved in the fertility restoration of interspecific hybrids (Bretagnolle and Thompson, 1995). Meiotic abnormalities including dyads and triads as well as uninucleate microspores with a different shape and size were observed suggesting production of unreduced gametes in cross between durum wheat cultivar Beloslava and *Aegilops umbellulata.* The formation of unreduced gametes is well documented in triploid F_1 hybrids between different tetraploid wheat species and some *Aegilops* species (Fukuda and Sakamoto, 1992; Li and Liu, 1993; David et al., 2004; Zhang et al., 2010). Our cytological evidences for occurrence of 2n-gametes in the hybridization between durum wheat and *Aegilops umbellulata* supplement this finding.

Genetic recombination between chromosomes of homeological genomes is an important pre-requisite for transferring of desired traits from distantly related plant species into the cultivated wheat (Ceoloni and Jauhar, 2006). One of the feasible paths of spontaneous arising of recombinant chromosome is the homoeologous pairing in hybrids plants during the origin of 2n-gametes. Recently by genomic *in situ* hybridization (GISH) was revealed that intergenomic recombination occurs during formation of 2n-gametes in distantly related F_1 hybrids through homoeologous crossing-over as well as through the assortment of homoeologous chromosomes (Lim et al., 2003**;** David et al., 2004; Barba-Gonzalez et al., 2005). The observed in our study bridges and segments during the anaphase II could be an evidence of occurred recombination between homoeologous chromosomes of the involved in hybridization parents.

Conclusions

At interspecific hybridization between durum wheat genotypes as female parent and one accession of wild relative *Aegilops umbellulata* was achieved low crossability rate of 3 % average for all genotypes over two years. The observed variation of crossability is to the greatest extent, due to the genotype of cultivated parent with 82.91 % from the total variation.

Hybrid plants were obtained only by means of embryo rescue method. From 43 isolated embryos, 13 plants were regenerated and four were successfully adapted. The ability for *in vitro* regeneration is still independent of crossability of used durum wheat genotypes.

All received F_1 hybrid plants from the cross Beloslava x *Aegilops umbellulata* were identical, exhibited good tillering ability and manifested traits from both parents. An increase in crossability with advancing of backcross progenies – from 1.6 % in BC_1 hybrids to 26.2 % in BC_2 hybrids was found. Meiotic abnormalities including dyads and triads as well as uninucleate microspores with a different shape and size were observed suggesting production of unreduced gametes in this cross. The observed bridges and segments during the anaphase II could be an evidence of occurred recombination between homoeologous chromosomes of the involved in hybridization parents.

Here presented results are only initial step of the involvement of wild species *Aegilops umbellulata* in long process of production of durum wheat breeding lines with introgressed alien genes. Successive progenies are going to be screened at morphological, physiological, cytological and molecular level for hybrid identification and enhancing of genetic variation for biotic - and abiotic stress resistance traits and its incorporation into Bulgarian durum wheat.

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