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BIOTECH "*Zea* SYSTEM" APPLICATION IN WINTER WHEAT GENETICS AND BREEDING AT NARDI FUNDULEA

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ABSTRACT

The paper is a short review aiming to present results and specific protocols based on wheat x maize hybridization and immature embryo rescue techniques, used at the National Agricultural Research and Development Institute Fundulea, to develop completely homozygous genetic lines for performing genetics studies and to breed superior winter wheat cultivars.

Keywords: Biotechnology, *Zea* system, haploids, doubled haploids (DH), mapping populations, mutated and mutated - recombinant DH lines.

INTRODUCTION

Among biotechnological methods applicable to crop plants, those which make possible to attain homozygosity in only one generation fulfill ideally the old desiderata of shortening the duration of the breeding programs, increasing the selection pressure, accelerating the breeding cycles, obtaining stable and genetically uniform cultivars, and particularly saving resources. Moreover, by using such methods it becomes possible to develop in a shorter time homozygous genetic lines that can be further used to perform modern genetic analysis.

Two main procedures are now widely used to accomplish this essential demand: a) somatic embryogenesis (androgenesis, macrosporogenesis and gynogenesis) and b) zygotic embryogenesis (zygotes produced by inter-generic hybridization followed by chromosome elimination of the pollen source partner since the first division cycles of zygotes).

The advantage of these methods lies in the fact that they make possible to fix in a single generation the variability produced by recombination in F1, F2 etc., thus shortening the duration of the breeding program with 6-7 years and also generating an acceleration of the breeding cycles and increasing selection efficiency (correspondence phenotype/genotype).

METHODS

With previous experience acquired in inter-generic hybridization (wheat x alien related *Triticeae* species) we tested the possibilities to obtain wheat haploids following the hybridization wheat x maize. With the goal of establishing, if possible, an efficient working protocol for our conditions, with minimal logistic supports, the variant in which the hybridization work is carried out in greenhouse condition, in a single cycle per year with duration of about 40-50 days during the period of March-May, when certain environmental factors respectively daily and nocturnal temperatures and light brightness are more suitable for passing the pre- and flowering stages for both wheat and maize plants, was chosen.

The biotech system "*Zea*" based on zygotic embryogenesis following wheat - maize hybridization (Figure 1) apparently more laborious and costly has, however, certain advantages: all regenerants are haploid green plants while polyploids, aneuploids, albinotic plants and genotyping variability for regeneration capacity are common in somatic embryogenesis.

Among the genetic factors influencing haploid induction, the specific genotypic reaction of female form and pollen source could also be considered essential. Although,

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many researches revealed that the incompatibility *Kr* wheat genes were not strictly involved in wheat x maize crosses, as in other inter-generic hybridizations, we found some manifested differences using different maize pollinators (Giura, 1995; Mihăilescu and Giura, 1998).

However, as the maize hybrids are constantly replaced by new ones and the wheat F1 hybrids are genetically different each year, we are using now in wheat x maize crosses two sugary maize hybrids produced by ARDS Turda. These hybrids are characterized by a lower height, a richer branching of panicle and a longer duration of flowering that confers some advantages regarding the quantity of pollen per individual plant and the ease of collecting it.

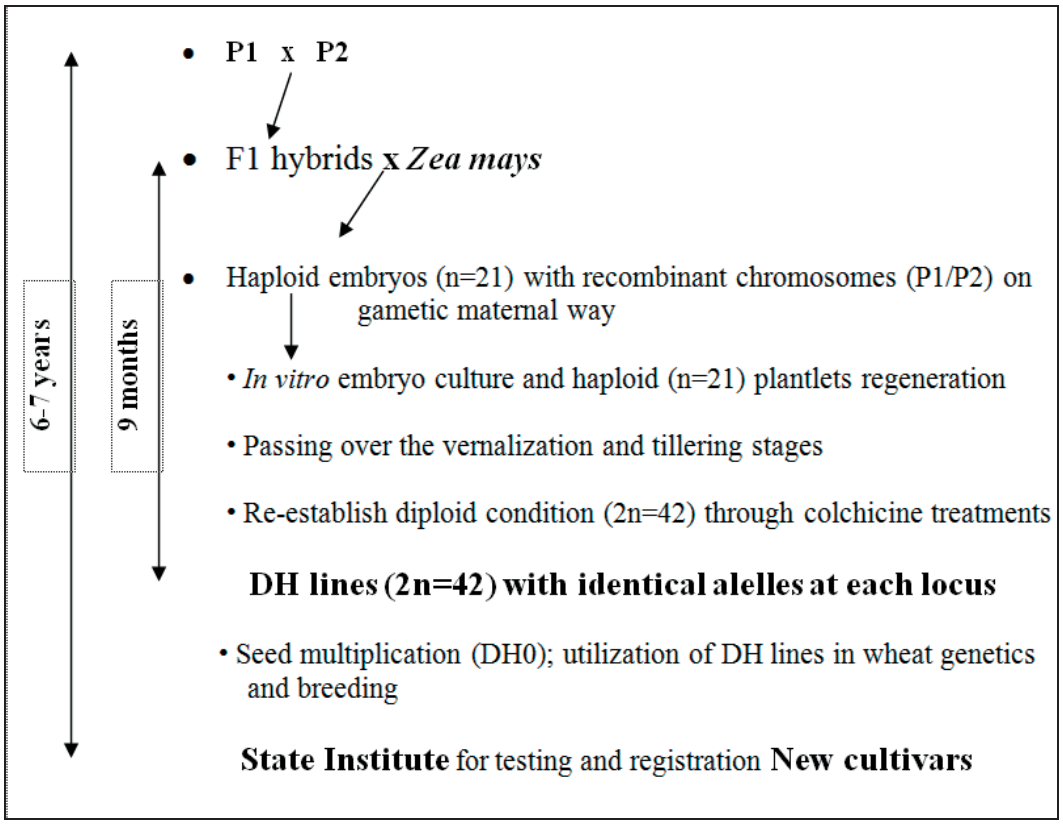

Figure 1. The main steps in the working protocol for obtaining winter wheat DH lines by using the "*Zea*" system

Between 1991 and 1995 several crossing procedures and 32 variants of plant growth regulators (PGR) and the mode of application *in vivo* were also tested and the superior PGR variants were cheked again in 1996.

The variant A2 of PGR combination consisting of 20 ppm 2-4D and 75 ppm GA3 spray treatment at 24 hours after pollination gave the best results: 21.4 haploid embryos/100 polinated flowers, 5.3 haploid embryos/spike and 2.4 haploid plantlets renenerated/spike after *in vitro* embryo culture on Gamborg B5 modified medium (Table 1).

The variants A2 and C1 were retested in 2012 on other F1`s wheat hybrids and the results were quite similar, variant A2 of *in vivo* PGR treatment by spraying the spike at 24 hours after pollination giving the best results.

Table 1. Plant growth regulator (PGR) combinations (1996)

Treatment variants	Pollinated flowers (no.)	Developed caryopses/100 pollinated flowers (%)	Haploid embryos/100 pollinated flowers (no.)	Haploid embryos/spike	Haploid plantlets regenerated/ spike
A: 20 ppm 2,4-D (**control**)	4,008	49.7	13.7	2.9	1.9
A1	3,920	49.7	13.8	3.2	1.8
A2: 20 ppm 2,4-D 75 ppm GA3	5,944	60.0*	**21.4***	**5.3***	**2.4***
A3	2,629	62.5*	12.1	3.2	1.1
B2	2,321	71.2*	13.4	3.0	0.4
C	2,528	73.9*	17.4	4.4	0.3
C1: 18 ppm 2,4-D, 9 ppm Dicamba, 2 ppm BA	2,914	68.6*	**21.4***	**5.4***	1.5
C2	2,494	74.3*	6.0°	1.4	0.9

*,°) significantly different from control for $p<0.05$.

Besides its applicability for haploid production in common wheat, the *Zea* system proved its efficiency in durum wheat and triticale too (Giura, 2004).

RESULTS

Since the introduction of this system in 1991 up to now, 13.695 DH (doubled haploid) lines have been produced for use in wheat genetics and breeding.

Development of genetic stocks of wheat DH lines:

Using *Zea* system it became possible to create DH-lines for special genetic studies such as mapping populations (that can be obtained from maize hybridization of F1`s between contrasting wheat genotypes for a certain important trait), recombinant substitution lines for individual pairs of chromosomes of real interest in modern genetic research at molecular level; selection for useful recessive or dominant mutants in the first generation after any mutagenic treatment and even genetic transformation at haploid level etc.

The following mapping populations have been developed by using such procedures:

a) **Mapping population Izvor / Jiana:** 62 DH lines to identify the associated markers for ***or*** gene (osmoregulation capacity) located on 7AL chromosome (Table 2).

- Cultivar Izvor has a good osmotic adjustment capability (maintenance of leaf turgor through osmotic adjustment);
- Jiana (breeding line) exhibited a low osmotic adjustment:

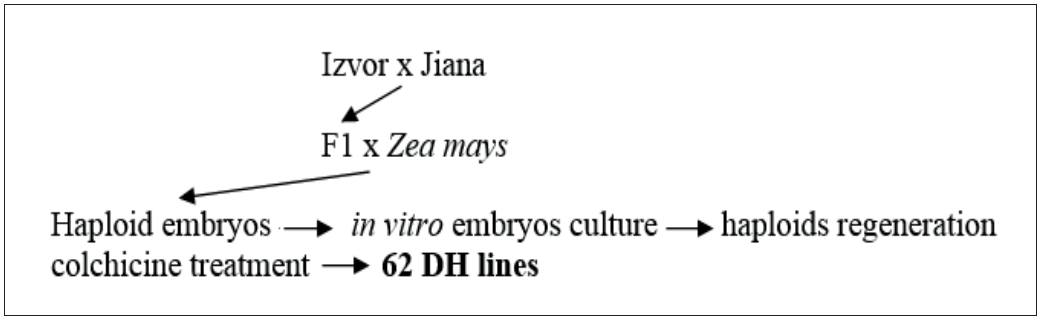

Table 2. Genetic distance between several markers and the "*or*" gene estimated by JoinMap®4 software (Ciucă et al., 2010)

Marker	Estimated distance from the *or* gene	Recombination frequency	LOD
Xwmc9	9.1	0.0806	11.44
Xwmc596	9.1	0.0806	11.44
Xwmc603	9.1	0.0806	11.44
Xbarc108	9.3	0.0833	9.46
Xgwm260	13.0	0.1321	7.61
Xbarc121	29.1	0.2182	4.12

The molecular analysis proved that gene *or* is located on 7A chromosome (7AL) in centromeric region, in the proximity of Xwmc9, Xwmc596 and Xwmc603 primers.

b) **Mapping population MV3-1/F132:** 151 DH-lines for pyramiding the crossability recessive alleles and selection of DH lines for the highest intergeneric crossability.

It is well known that alien useful gene introgression in cultivated wheat gene pool by wide hybridization can greatly contribute to genetic progress in wheat breeding. However, the success of wide hybridization is difficult and depends upon cross compatibility (crossability) between wheat and alien species. Unfortunately, the modern wheat genotypes carry dominant restrictive alleles at *Kr* loci thereby reducing the chances of their use as recipient parents. New crossable DH lines with acceptable agronomic performances were obtained by crossing two sub-lines of Martonvasary 9 genotype (*kr1kr1*) with a modern Romanian genotype F.132 (1-30), carrying recessive allelic variant at *Kr2* and probably at *Kr*3 loci. The resulted F1 was crossed with maize and 151 DH lines were generated. Testcrosses with rye (cv. Harkovskaya) in different years facilitated identification of some lines with high levels (60-70%) of inter-generic crossability (Giura, 2016). These DH lines carry *kr1kr1kr2kr2* recessive alleles and probably even *kr3kr3*.

c) **Mapping population G.603/F.132:** 87 DH-lines were produced in order to identify the associated markers/QTL`s for grain size trait in wheat.

Genetic analyses for grain size on F3 disomics selected from monosomics F2 populations (Favorit monosomics - 21 lines x G.603) revealed a complex genetic control of this trait by genes located on several chromosomes (Giura and Săulescu, 1996). In order to advance the genetic analysis, we proceeded to develop a mapping population using as hybridization partner the line F.132 with smaller grains. F1`s were then crossed with maize and 87 DH lines were obtained which are now under field evaluation to identify DH-lines with higher thousand kernel weight (TKW) values regardless of annual environmental conditions and carrying also other useful genes.

Mutated and mutated/recombinant DH lines

In the case of experimental mutagenesis, the implementation of DH technology makes possible to fix in a single generation any modification induced in the DNA structure by using physical or chemical mutagenic agents. If the direct and reciprocal hybridization of the M1`s generation from two parents are intercrossed, it is possible to fix under homozygous condition, any recessive mutation and mutation/recombination respectively, without resorting to the classical cycles of selection in heterozygous populations (Figure 2).

Thus, by using an original protocol including two genotypes, two gamma-ray (Gy) irradiation cycles and DH technology, it was possible to generate 143 mutant DH lines of the wheat parents after the first irradiation cycle and 415 mutant/recombinant DH lines after second irradiation cycle (Giura, 2013).

Under field conditions both types of mutants showed an extensive range of genetic variability for disease resistance (Table 3), morphological traits (Figure 3), productivity, glaucousness etc.

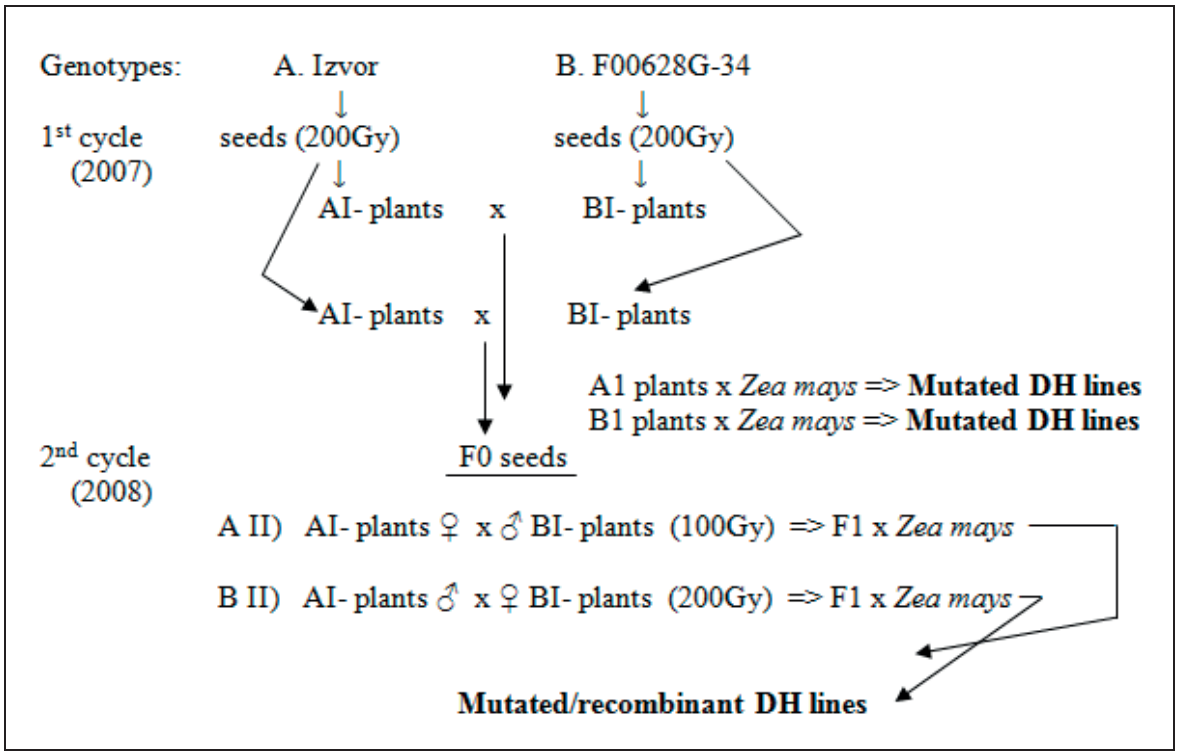

Figure 2. Mutagenic protocol with two irradiation cycles- gamma rays (Gy) and DH technology using *Zea* system

Extremely interesting is the grain size trait, respectively the thousand kernel weight (TKW) which, in the case of several lines exceeded 50 grams on average of 5 years. As recently studies (Sharma et al., 2014) argue that up to now, the progress of selection for a superior yielding capacity was directly related to the gradual increase of the grain size and degree of grain filling, the mutated and mutated/recombinant DH lines with higher TKW values may represent valuable donors for breeding programs.

Table 3. Dispersion for leaf rust adult plant resistance/susceptibility in 338 DH lines population after two irradiation cycles (2011)

Specification	No DH lines	%
Very susceptible	32	9.47
Susceptible	95	28.11
Middle susceptible	18	5.33
Middle resistant	6	1.78
Middle susceptible (Hy*)	2	0.59
Resistant	86	25.44
Resistant (Hy*)	97	28.70
Very resistant	2	0.59
*) hyper sensibility trough necrosis	338	100.00

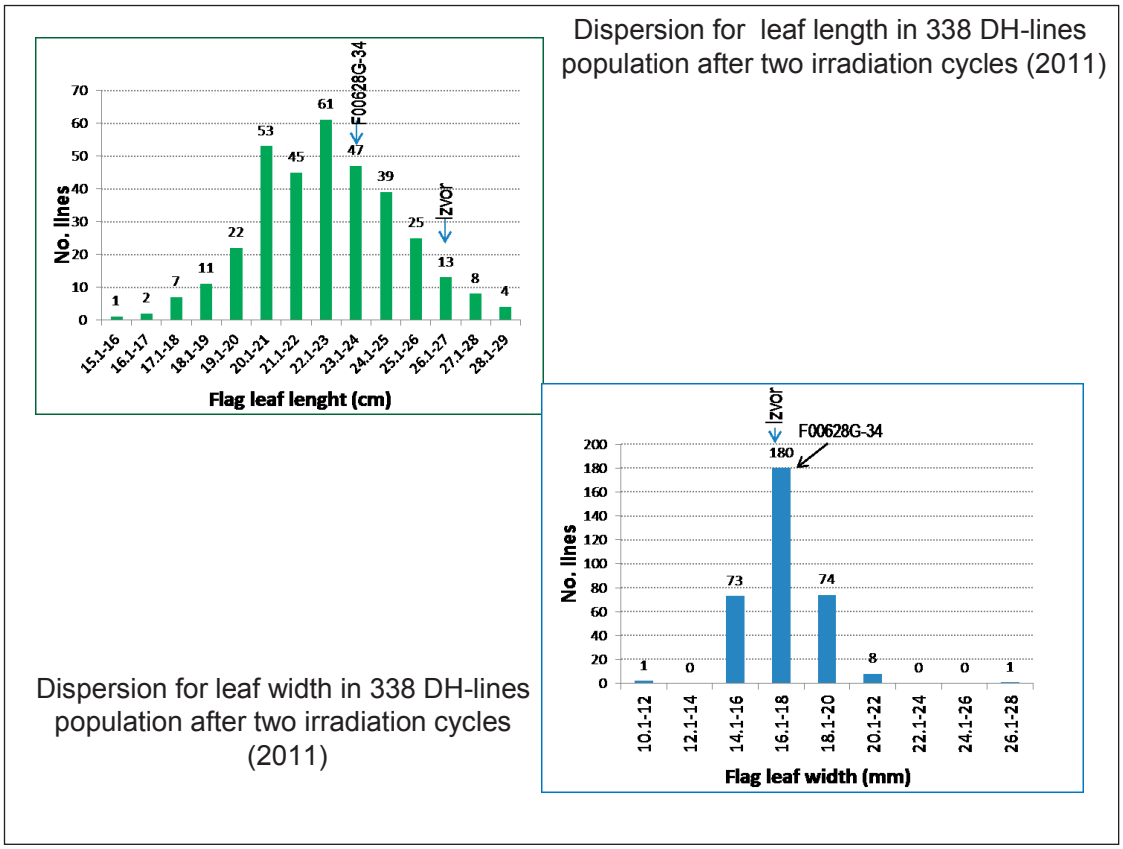

Figure 3. Dispersion for leaf morphological traits

Single chromosome recombinant substitution lines (SCRS) for 7B chromosome

Previous genetic analysis for protein content based on substitution lines Favorit/F.26-70 revealed the involvement of chromosome 7B in controlling the protein content and rheological properties of the dough (high gluten tenacity) and 3 days earlier flowering compared to Favorit parent (Giura and Ittu, 1986). The main difference between 7B substitution line and recipient parent Favorit for some traits are presented in Table 4.

Table 4. Differences between substitution line Favorit/F.26-70(7B) and the recipient parent Favorit for several traits

Specification	Donor parent F26-70	Recipient parent Favorit	Substitution line Favorit/F26-70 (7B)	Difference
Date of heading (days after 1 May)	26	29	26	-3 days
TKW	48.83	43.94	43.70	-0.24
Protein (%N x 5.7)	16.26	14.41	15.19	+0.78*
Protein (mg/grain)	5.89	5.46	5.77	+0.31**
Protein yield (q/ha)	5.64	5.61	5.90	+0.29*
Yield (q/ha)	40.06	45.43	45.58	+0.15
Farinograph development time (min)	2.5	4.0	5.5	+1.5**
Farinograph mixing (min)	1.0	2.5	4.5	+2.0*
Extensogram action (135`)	0.75	1.04	2.11	+1.7**
Loaf volume (cm^3)	591	544	548	+4.0

*) p 0.05-0.01; **) p< 0.01.

A more detailed genetic analysis was attained using substitution recombinant lines for chromosome 7B [Favorit/F.26-70(7B)] produced by using both classic procedure (development of recombinant disomics) and *Zea* system (development of recombinant DHL`s via recombinant haploids 7B). The later procedure is quicker, surer and more reliable, requiring only the hybridization of the substitution line with the recurrent parent and then the F1`s crossed with maize (Figure 4). It is worth to mention that the development of recombinant substitution lines for individual pairs of chromosomes by classical procedure requires more stages, two of them being based on cytological analyses to select firstly recombinant 7B monosomic progenies (2n=41) from Favorit/Favorit (F26-70 7B) F1 hybrids and then, to select from self-pollinated F1`s, recombinant (SCRLs) disomics (2n=42).

The study was carried out within EWAC cooperation (Germany, Russia and Romania).

The SCRLs were grown both with and without vernalization at IPK-Gatersleben (Germany) in a greenhouse under short day (10 h day/14 h night) and long day (14 h day/10 h night) regimes. Vernalization was achieved by holding seedlings at 4°C under a 12/12 day/night regime for 8 weeks.

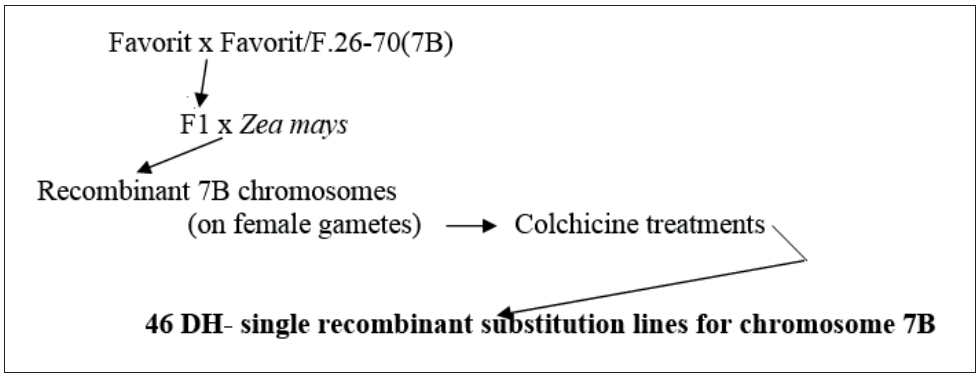

Figure 4. Development of SCRLs DH-lines for chromosome 7B using the *Zea* system

Earlier flowering under long days was controlled by a newly identified gene *Bpd-B2*, located on 7BS at 8.8 cM distal and 20.7 cM proximal to microsatellite markers Xgwm0537 and Xgwm0255, respectively. The earlier flowering induced by *Ppd-B2* gene was significantly correlated with higher protein content and a major gene for this character *Gpc-B2* (Grain protein content-B2) was mapped at 4.4 cM proximal to *Ppd-B2*) (Klestkina et al., 2009). It was noticed that this gene did not affect grain size and no negative correlation between yield and protein content was noticed (Giura et al., 2008). Therefore, this gene is probably involved in nitrogen uptake and/or translocation and the carriers need to be further analyzed.

Use of DH system in wheat breeding

In practical breeding, application of the *Zea* system can significantly shorten the time to reach required homozygous condition to test for yielding capacity. Moreover, a substantially acceleration of genetic progress can be obtained using as parents for the next cycle of crossing the best yielding DH lines in course of evaluation (Săulescu et al., 2012). Also, DH cultivars completely meet the requirements for uniformity and stability in accordance with compulsory standards of EU.

Six out of the ten cultivars registered between 2004 and 2018 are doubled haploids (DH): Faur (2004), Glosa (2005), Litera (2010), FDL Miranda (2011), Pitar (2016), Semnal (2017). They were grown in 2018 on about 45% of the wheat area in Romania.

CONCLUSIONS

The implementation of the Biotech *Zea* system made it possible to obtain winter wheat genetic lines (mapping populations) with the help of which genes of interest were

identified and positioned on chromosomes, thereby advancing also genetic studies at molecular level. At the same time, the use of *Zea* system in the wheat breeding program has led to the creation of new and superior homozygous cultivars, in a shorter time.

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