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THE CHOICE OF SUITABLE CONDITIONS FOR WHEAT GENETIC TRANSFORMATION

LENKA KLČOVÁ, KATARÍNA ONDREIČKOVÁ, DANIEL MIHÁLIK, MARCELA GUBIŠOVÁ

National Agricultural and Food Centre – Research Institute of Plant Production, Piešťany, Slovak Republic

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Wheat is one of three most important cereals worldwide. Its production rises every year. There is a possibility to improve quantitative and qualitative parameters by biolistic method of transformation. The process of introduction of desired gene into the wheat genome and plant regeneration is affected by many factors. To identify the suitable conditions, selection system, the influence of donor, plant environment and the regeneration capacity of wheat genotypes were tested. The scutella of immature embryos served as the initial explants. Plant regeneration was achieved by 6 out of 11 genotypes tested. The highest values were reached by the cultivar Ilias. The effect of donor, plant environment was evaluated by two genotypes. Plants from growth chamber appeared to be better source of explants compared to plants grown in the natural conditions. The selection system was optimized as follows: regeneration medium in the dark and subsequently in photoperiod conditions (DR) with 5 mg/l of phosphinotricin (PPT), shoot induction medium (DS) with 7 mg/l of PPT.

Key words: wheat (*Triticum aestivum* L.), genotype, selection, biolistic transformation, donor plant

Wheat belongs to the most important cereals. As a leading breadmaking crop, it is the primary source of human nutrition. To improve yield and nutritional value, there is a possibility of quantitative or qualitative trait modification. The methods of genetic property modification are presented by the classical and mutation breeding or genetic transformation. Since the possibilities of classical breeding methods are limited and the process is time consuming, genetic transformations offer an interesting and used alternative for the agricultural crops. Despite its agronomical importance, wheat belongs to the last successfully transformed cereals (Vasil *et al.* 1992). The current successes with the wheat genetic modification include the improvement of breadmaking quality, production traits, photosynthesis efficiency, resistance to abiotic or biotic stress (Kasirajan *et al*. 2014; Abouseadaa *et al*. 2015; Bruce *et al.* 2015). In spite of many results were reached, the genetic modification of wheat is not a routine method. The progress still lags behind the other cereals due to the difficulties associated with gene delivery and transgenic plant recovery (He *et al*. 2015).

In wheat, microprojectile bombardment is more effective transformation method compared to DNA transfer via *Agrobacterium tumefaciens* (Li *et al*. 2012). The advantages of *Agrobacterium*-mediated transformation are the efficiency, cost-effectivness and the incorporation of low-copy number of the transgene, but some species are recalcitrant to this method. Microprojectile bombardment is relatively genotype-independent and is applicable to a large number of target tissues. The principle is the utilization of large acceleration of the microparticles coa-

Mgr. Lenka Klčová, PhD. (*Corresponding author), Mgr. Katarína Ondreičková, PhD., Mgr. Daniel Mihálik, PhD., Mgr. Marcela Gubišová, PhD., National Agricultural and Food Centre – Research Institute of Plant Production, Bratislavská cesta 122, Piešťany, SK- 921 68, Slovak Republic. E-mail: l.klcova@vurv.sk

ted with DNA to transfer it to the target tissue.

Genetic modification is dependent on the regeneration of fertile plants of given species in *in vitro* culture. Wheat belongs to the plant species rather recalcitrant to *in vitro* regeneration. Genotype has the main impact on the regeneration ability (Varshney & Altpeter 2001; Shah *et al*. 2009). Although the model genotypes, without any prospect of practical application, are used for transformation prevailingly, there is a tendency to introduce the transgene into agronomically acceptable cultivars (Greer *et al*. 2009; Zhang *et al*. 2015). Wheat regenerants were obtained from various types of the explants: immature inflorescences, coleoptiles, apical meristems, leaf bases, microspores, anthers, embryos. The best regeneration capacity is proved by immature embryos and they are the most frequently used type of explant (Tamás *et al.* 2004). Also, a strong dependence on the growth conditions of the explant donor plants was described (Pastori *et al*. 2001; Wang *et al*. 2014). In an effort to ensure efficient regeneration, different culture media, individual components and growth regulators were tested.

One of the critical factors of an efficient transformation is to provide the selection system that allows the division of only transformed cells and the generation of regenerative structures. The type, concentration and the length of selection agent treatment are questionable depending on the species/genotype transformed. In wheat, the introduction of *bar* gene is commonly used for the selection of transformed tissues.

The aim of work was to test the regeneration capacity of selected wheat genotypes, to evaluate the effect of donor plant environment and to optimize the selection system to identify the suitable conditions for wheat transformation.

Wheat plants of the model genotype Bobwhite, responsible cultivar CY-45 and Slovak cultivars Ilias, Ilona, Torysa, Venistar, Madejka, Viglanka, PS Pintta, IS Karpatia, Astella were grown in field or controlled (growth chamber) conditions. Immature caryopses were surface-sterilized, scutella were aseptically isolated and preconditioned on the callus induction medium (DC): MS salts + myo-inositol 100 mg/l + thiamin 1 mg/l + sucrose 30 g/l + gerlite 2.8 g/l supplemented with 2 mg/l of 2,4-dichlorophenoxyacetic acid (Gubišová *et al.* 2011) in the dark, at 25°C, for 2 weeks. Subsequently, the immature scutella were plated onto the regenera-

T a b l e 1

The regeneration of 11 wheat cultivars (various letters indicate the statistically significant difference detected by the *LSD* test, $\alpha = 0.05$)

Cultivar	Callogenesis [%]	Regeneration [%]	Efficiency [R/ep*]
Ilias	99.1^{a}	78.3^{a}	7.09^{a}
Ilona	19.2^{d}	0^{d}	0^{c}
IS Karpatia	25.0^{d}	0^{d}	0^{c}
Madejka	98.3^{a}	15.8^{b,c}	0.64^{c}
PS Pintta	64.1^{b}	0^{d}	0^{c}
Torysa	36.6^{c,d}	0^{d}	0^{c}
Venistar	100^{a}	0^{d}	0^{c}
Viglanka	99.0^{a}	16.0^{b,c}	0.61^{c}
Astella	73.3^{b}	0.8^{d}	0.01^{c}
Bobwhite	54.1^{b,c}	14.1^{c}	1.63^{b}
CY-45	100^{a}	20.8^{b}	1.49^{b}

*R/ep: number of regenerants per explant plated

tion medium (DR): DC medium supplemented with 2.5 mg/l of $CuSO_4.5\ H_2O$ and cultivated 2 weeks in the dark. To promote regeneration, the calli were exposed to 16/8 h photoperiod with light intensity 50 µmol/m²/s and temperature regime 25/20°C. After 2 weeks explants were transferred onto the shoot induction medium (DS): DC medium without 2,4-D. To optimize the selection system, various concentrations of phosphinothricin (PPT) were added to the DR and DS culture media (5, 7 or 10 mg/l). The regeneration frequency was evaluated after 2, 4 and 6 weeks of cultivation on the selection media.

The frequency of callogenesis, regeneration [%] and efficiency of regeneration (represented by the number of regenerants per embryo plated) were evaluated. Data were processed by one-way analysis of variance (ANOVA) followed by *LSD* test ($\alpha = 0.05$).

Immature scutella of eleven wheat cultivars were screened for their regeneration ability. All genotypes tested were able to produce callus, but with a significantly different frequency (Table 1). The lowest production was recorded by genotype Ilona (19.2%), the high values by Ilias, Madejka, Viglanka and 100% by Venistar and CY-45. The number of wheat genotypes used for genetic transformation is limited, with most of it having inappropriate agronomic properties. For this reason, many cultivated genotypes are tested. The wide range of the ability of callogenesis (6.2–100%) by wheat genotypes was described by Murín *et al*. (2012). Similarly, Shah *et al*. (2009) recorded the range 36.3–86.3% in a ten-genotype group. In European cultivars, callogenesis varied from 56.1 to 98.7% (Varshney & Altpeter 2001). Zhang *et al*. (2015) tested 18 Chi-

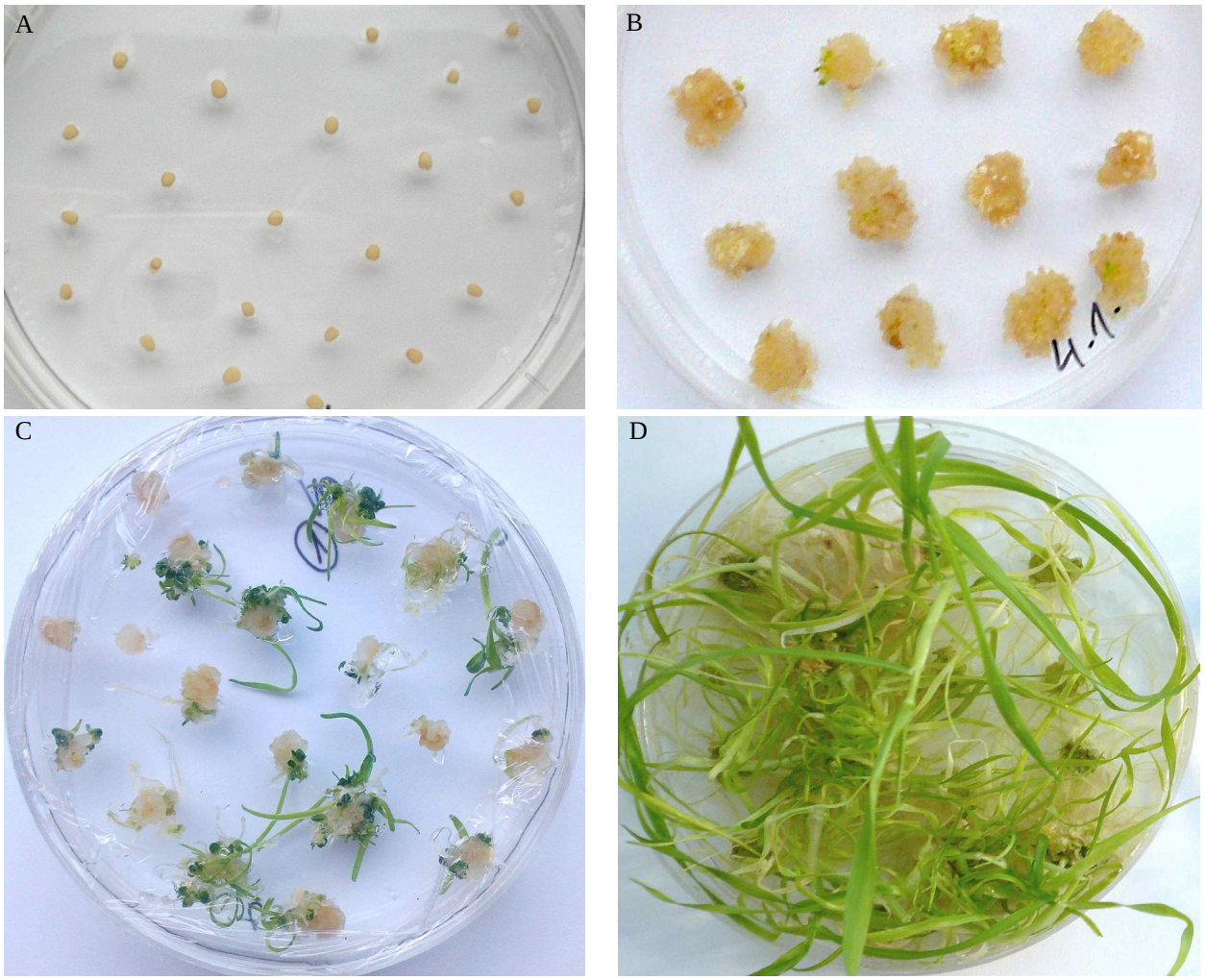

Figure 1. *In vitro* wheat regeneration from immature scutella, cultivar Ilias (A – isolated scutella, B – callus formation, C – regeneration, D – regenerated shoots)

nese genotypes with the result 10–100% of callus formation.

The production of shoots from induced callus was achieved by six out of 11 genotypes tested. Differences in both frequency and effectivity were statistically significant (Table 1). The cultivars Ilona, IS Karpatia, PS Pintta, Torysa and Venistar did not generate the shoots. From regenerating genotypes, Astella has the lowest regeneration efficiency. The highest percentage of regenerating explants was given by the Slovak variety Ilias (Figure 1), with regeneration efficiency 4.3 times exceeding model genotype Bobwhite.

Wheat *in vitro* regeneration is markedly genotype dependent. In European genotypes, Rasco-Gaunt *et al.* (2001) recorded a range of regeneration frequency 0–80%, which is in accord with our results (0–78.3%). Varshney and Altpeter (2001) tested 38 genotypes, with regeneration varied from 51.9% to maximum and efficiency between 1 and 16.8 regenerants. Wide differences (0–61%) of regeneration frequency of Hungarian genotypes were

T a b l e 2

The regeneration of two wheat genotypes in respect to donor plant growth conditions (various letters indicate the statistically significant difference detected by the *LSD* test, $\alpha = 0.05$)

	Donor plant	Bobwhite	CY-45
Callogenesis	*field*	62.8^{b}	96.0^{a}
[%]	*growth chamber*	98.6^{a}	100.0^{a}
Regeneration	*field*	13.8^{b}	$32.9^{a,b}$
[%]	*growth chamber*	61.4^{a}	$40.2^{a,b}$

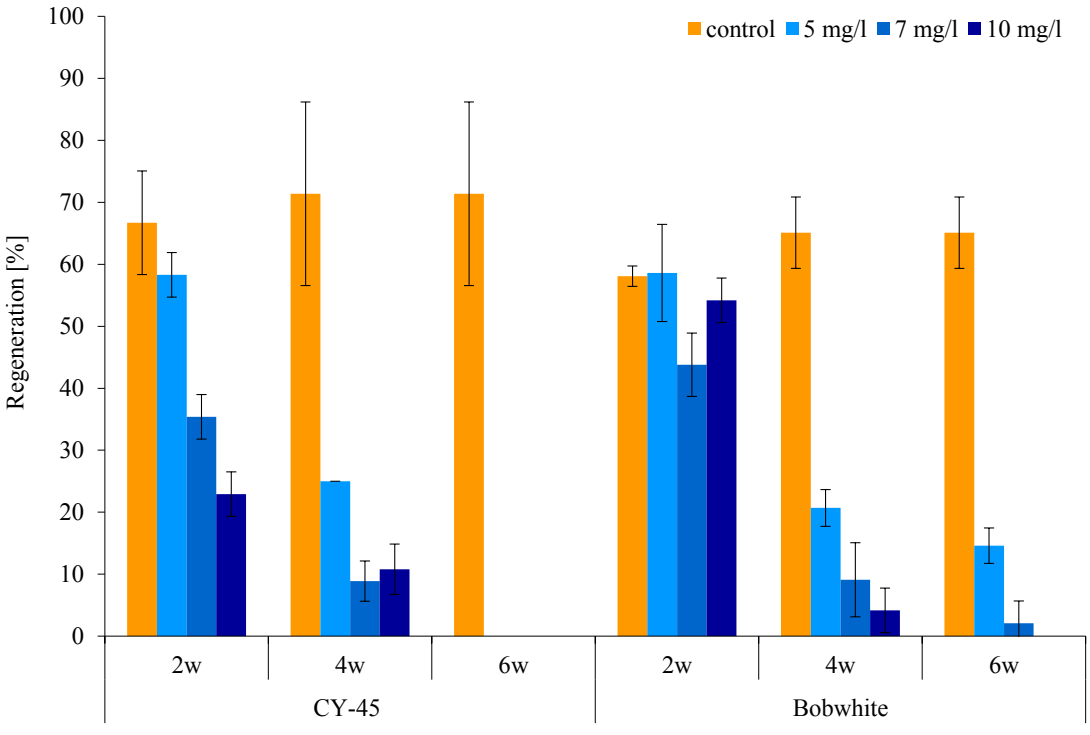

Figure 2. The effect of different concentration of the selection agent (0, 5, 7, 10 mg/l PPT) on the regeneration frequency of two wheat genotypes throughout 6 weeks (the average +/- standard deviation)

described by Tamás *et al.* (2004). The regeneration ranged from 3.5 to 31% in Chinese cultivars (Zhang *et al*. 2015). Murín *et al*. (2012) evaluated 23 cultivars with the result from none to 20% recovery, The upper limit was shifted to 37.5% by Dicamba supplementation. The efficiency varied up to 0.9 shoots. Nevertheless, 2,4-D predominates in wheat cultures, namely at the concentration 2 mg/l. We applied copper addition to the induction medium, similarly to He *et al*. (2015).

To evaluate the effect of donor plant environment, scutella culture derived from field grown or controlled conditions grown plants of genotypes Bobwhite and CY-45 were initiated. Higher callogenesis was reached by CY-45 genotype, where no differences with the starting material used were noticed. The callus production of genotype Bobwhite was significantly superior when using plants grown in the growth chamber. The regeneration frequency differences were documented among the donor plant growth conditions only by Bobwhite (Table 2). The explants from growth chamber conditions regenerated better. The dependence between wheat regeneration and the growth conditions of the explant donor plants was described also by Pastori *et al*. (2001). In addition, a variety of regeneration rates are shown by the same genotype under the same culture conditions at different times (Wang *et al.* 2014).

The selection was optimized by two non-transformed genotypes (Bobwhite, CY-45) with phosphinothricin as a selection agent. After 2 weeks on the selection medium, regeneration of all variants were observed (22.9–58.6%). Four-week cultivation on the selection medium resulted in the statistically significant differences between the control and the selection media. In the case of Bobwhite, the shoots were emerged (although minimal number) even after 6-week culture at the concentration 5 mg/l of PPT (Figure 2, 3). Based on the results and the length of cultivation during transformation (6–10 weeks), the selection protocols were optimized. The regeneration medium (DR) is supplemented with 5 mg/l of PPT, already during the cultivation in the dark. In the subsequent cultivation on DR medium under

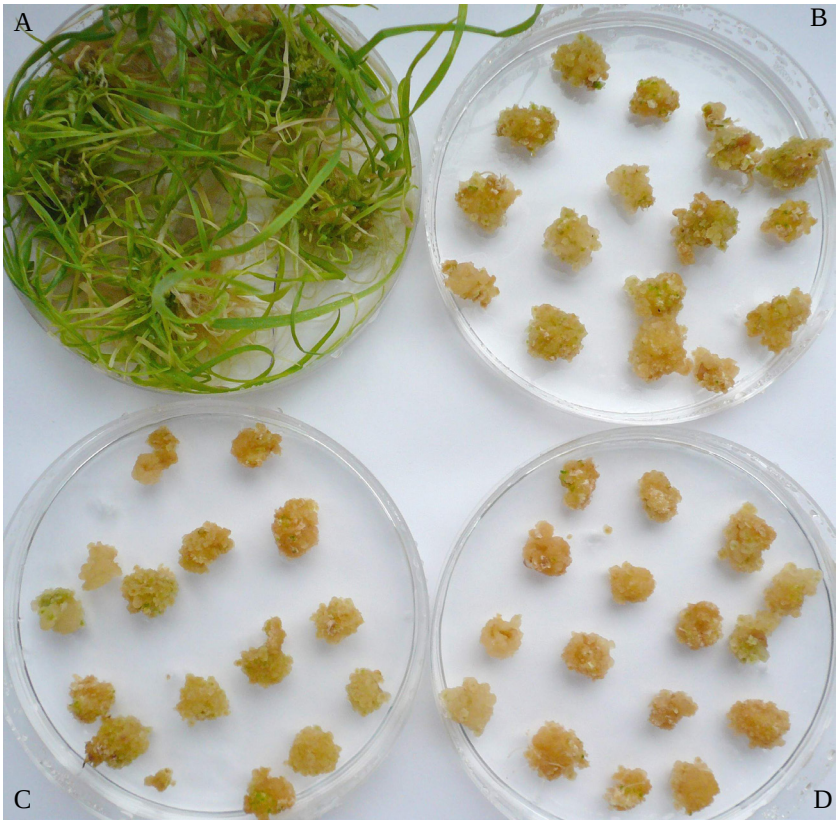

Figure 3. The regeneration of wheat immature embryos of CY-45 after 6 weeks on the selection media with different concentrations of PPT (A – control, B – 5 mg/l PPT, C – 7 mg/l PPT, D – 10 mg/l PPT)

the photoperiod conditions the PPT concentration is maintained (5 mg/l). The shoot induction medium (DS) is supplemented with 7 mg/l of PPT.

Most of authors introduce the selection immediately after explant bombardment at a concentration 2–5 mg/l of PPT (Li *et al*. 2012; Zhang *et al*. 2015). Rana *et al*. (2014) selected after two weeks of cultivation (3 mg/l of BASTA). Higher levels of BASTA were used by Kasirajan *et al*. (2013), in spite of they recorded more than 50% of the escape plants. Similar amounts are reported for bialafos. In our experiments, the concentration of 5 mg/l was sufficient for genotype CY-45, but non-transformed shoots were recovered by Bobwhite in later phases even under these conditions. Therefore, the PPT level in the last culture interval was increased. Successful genetic transformation is dependent on the regeneration of given species in *in vitro* culture. Some factors affecting the process of wheat regeneration were evaluated. The regeneration ability of eleven wheat genotypes was screened. All genotypes were capable to form callus. The regeneration of shoots was achieved by six of 11 genotypes tested. The highest frequency and effectivity of regeneration was provided by the Slovak cultivar Ilias. The effect of donor plant environment was tested in scutella culture derived from field or controlled conditions grown plants. Plants from growth chamber appeared to be better source of explants for wheat *in vitro* cultivation. The selection system was optimized by supplementation of media with increasing concentrations of phosphinothricin. After two weeks the regeneration was observed in all variants, after 4 weeks the differences between the control and the selection media were evident. Based on the results, the selection protocol was proposed.

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