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In vitro evaluation of boron tolerance in wheat (*Triticum aestivum* L.) genotypes

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Summary: Wheat tolerance to high boron concentrations was determined using mature embryo culture. The testing was performed on a modified MS nutrient medium to which boric acid was added in two concentrations: 15 mM and 30 mM. The control medium contained no excess boric acid. The experiment involved 14 Serbian varieties. After one month of cultivation callus fresh weight (CFW) was measured and reductions of fresh callus weight (RFCW) at boron (B) concentration of 15 mM in relation to the control were calculated. The genotypes differed significantly according to their reaction to different B concentrations. Six genotypes had RFCWs below 50.0 %, while eight genotypes had RFCWs above 50.1 %. Cultivars Nevesinjka and Pesma were considered the most tolerant genotypes, while cultivars Balada and Vila were the most sensitive. The results confirmed that differences in genotype reactions to excess B are visible at the cellular level and that they may serve as a selection criterion.
Key words: excess boron, mature embryo, tolerance, wheat

Introduction

Boron is one of the seven microelements essential for plant growth and development. Its deficit in higher plants causes morphological and physiological changes, which may reduce yields of crop plants (Lazarević et al. 2003, Ziaeyan & Rajaie 2009). The range between optimal and excess B concentrations in plants is small. Long-term fertilizer application or anthropogenic pollution bring about B excess relatively quickly, even in plants that require increased B amounts, such as sugar beet and spinach (Nable et al. 1997).

Growing tolerant plant species and identification of highly tolerant genotypes are the best ways to overcome the problems caused by high B concentrations in the soil (Torun et al. 2006). Incorporation of B tolerance into existing high-yielding cultivars and development of new special-purpose cultivars could significantly increase production potentials of regions in which boron-rich soils occupy large areas.

Conventional methods used in breeding programs for plant evaluation for B tolerance are labor intensive and prone to experimental error. In spite of these limitations, notable success has been achieved in wheat breeding for tolerance to excess B (Eastwood et al. 2004). However, it is often considered desirable to have a controlled-environment screening system, in which plant reaction could be evaluated uniformly and rapidly. Methods of *in vitro* culture are frequently used as supplement to classical breeding methods for tolerance to abiotic stresses (Huang & Graham 1990, Stoddard et al. 2006).

In an attempt to find a rapid method for B tolerance evaluation in a large number of wheat genotypes, *in vitro* zygotic embryo culture was used in this study. The objectives

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of the study were: (1) to determine the effect of different B concentrations on callus growth, (2) to check if it is possible to determine different levels of B tolerance at the cellular level in wheat genotypes and (3) to assess the applicability of the method in wheat breeding for tolerance to high B concentrations.

Materials and methods

In vitro test

In this study 14 wheat (*Triticum aestivum* L.) cultivars (Pobeda, Nevesinjka, Venera, Renesansa, Balada, Ljiljana, Kantata, Sonata, Simfonija, Rapsodija, Pesma, Cipovka, Vila and Balerina) were analyzed. The material for embryo isolation was biologically mature wheat seeds, which had been soaked in distilled water for four hours. Surface sterilization was done with Clorox (5.25 % solution of NaClO) diluted with four parts of sterile distilled water for 30 minutes. The embryos were isolated together with the scutellum and rinsed (3x) in Clorox diluted with nine parts of sterile distilled water. After rinsing, the embryos were immersed in a solution of streptomycin (250 mg l^{-1}) and penicillin (600 mg l^{-1}) for 30 minutes.

After sterilization and isolation, the embryos were placed onto a modified MS (Murashige & Skoog 1962) nutrient medium. The medium contained the standard MS mineral solution, 0.6 % agar, 3 % sucrose, and it was supplemented with the following growth regulators: 2.4-dichlorophenoxyacetic acid (2.4-D) - 1.5 mg l^{-1}; naphthylacetic acid (NAA) - 0.5 mg l^{-1}, thiamin - 0.5 mg l^{-1} and glutamine - 150 mg l^{-1}. Boric acid was added to the medium in two concentrations, 15 mM and 30 mM, while the medium for control group contained no excess boric acid. The experiment was conducted in five replications. Each replication consisted of one tube (10x100 mm) containing two embryos. Fresh callus weight (FCW) was measured after two months of cultivation. The entire material was cultivated in a growth chamber, at constant temperature (25-27 °C), 1.500 lx and a 16/8h light/dark photoperiod.

Data processing

All results were processed in the statistical program Statistica 7 (StatSoft, Inc. Corporation, Tulsa, OK, USA). Before applying the analysis of variance, homogeneity was verified by Hartley, Cochran and Bartlett tests. If the tests indicated as necessary, appropriate data transformations and adequate variance analysis models were applied.

Reduction of fresh callus weight (RFCW) on the medium with 15 mM boric acid, compared against the control, served as an indicator of genotype tolerance to excess B. Reduction was calculated by the formula:

$$RFCW = 100 - \frac{FCW(15\ mM) \times 100}{FCW(control)}$$

where:

RFCW - reduction in fresh callus weight

FCW(15 mM) - fresh callus weight at the medium with 15 mM boric acid

FCW(control) - fresh callus weight at the control medium

Results and discussion

Table 1 shows the results of measurement of fresh callus weight of the 14 wheat genotypes from the *in vitro* test. After the transformation the homogeneity of the variances was achieved and the transformed data were used in the analysis of variance (Factorial ANOVA). The growth of callus tissue, expressed by FCW, was found to be under highly significant effects of the genotype, the media with the different B contents, and their interactions (Tab. 2).

The presence of excess B in the nutrient medium inhibited the growth of wheat calli. When average values for the 14 genotypes are considered (Fig. 1), a significant reduction of FCW in relation to the control group can be noted already in the first treatment, i.e., when 15 mM boric acid was added to the medium. The average FCW of 13.7 mg, in the medium with15 mM boric acid, was a reduction by 61.1 % in relation to the control. Further increase of boric acid concentration to 30 mM, caused an even higher inhibition of FCW. The average FCW of 6.7 mg, at this B concentration, was a reduction by 81.0 % in relation to the control.

Tab. 1. Fresh callus weight (FCW) of 14 wheat genotypes at different treatments: 1 - control medium without excess B, 2 - with 15 mM boric acid and 3 - with 30 mM boric acid; and reduction of fresh callus weight (RFCW) at the concentration of 15 mM boric acid

Tab. 1. Sveža masa kalusa (SMK) kod 14 genotipova pšenice na različitim tretmanima: 1 - kontrolna podloga bez suviška B, 2 - sa 15 mM borne kiseline i 3 - sa 30 mM borne kiseline; kao i redukcija sveže mase kalusa (RSMK) na koncentraciji od 15 mM borne kiseline

No. *Br.*	Genotype *Genotip*	FCW / *SMK* (mg)			RFCW / *RSMK* (%)
		1	2	3	
1	Pobeda	72.7	20.0	9.3	72.5
2	Nevesinjka	82.4	45.5	8.8	44.7
3	Venera	13.1	4.8	3.9	63.0
4	Renesansa	20.9	11.3	3.9	45.6
5	Balada	44.5	5.7	15.6	87.1
6	Ljiljana	20.2	6.6	4.4	67.4
7	Kantata	29.1	7.3	5.7	74.8
8	Sonata	16.7	9.4	3.4	43.7
9	Simfonija	20.7	17.9	5.5	13.5
10	Rapsodija	13.4	8.0	2.6	39.9
11	Pesma	29.6	16.7	7.2	43.5
12	Cipovka	31.0	13.5	6.2	56.4
13	Vila	59.8	12.5	10.6	79.1
14	Balerina	38.5	12.7	6.4	66.9
	Mean / *Prosek*	35.2	13.7	6.7	57.0

*Tab. 2. Factorial ANOVA for effects of genotype ("Var. 1"), boron concentration ("Var. 2") and their interaction ("Var. 1" * "Var. 2") on fresh callus weight of 14 wheat genotypes*

*Tab. 2. Fartorijalna ANOVA za efekat genotipa ("Var. 1"), koncentracije bora ("Var. 2") i njihove interakcije ("Var. 1" * "Var. 2") na svežu masu kalusa kod 14 genotipova pšenice*

Effect / *Efekat*	SS	Df	MS	F	P
Intercept	28873.83	1	28873.83	1877.125	0.000**
"Var. 1"	9161.23	13	704.71	45.814	0.000**
"Var. 2"	12343.52	2	6171.76	401.234	0.000**
"Var. 1" * "Var. 2"	6647.10	26	255.66	16.621	0.000**
Error / *Greška*	646.04	42	15.38		

Most of the tested genotypes reacted in accordance with the average reaction. However, five genotypes (Venera, Renesansa, Sonata, Simfonija, Rapsodija) showed no significant difference in FCW between the control and the medium with 15 mM boric acid (Fig. 1), mainly because of their poor callusing ability already on the control medium (Tab. 1).

A comparison of genotype reactions to the applied B concentrations showed that high callus growth inhibition occurred in all genotypes with the B concentration of 30 mM (Figure 1). Obviously, this concentration was not suitable for genotype ranking based on their tolerance to excess B. The average reaction of the genotypes to both B concentrations also showed not to be sufficiently discriminating. Therefore, the genotype reaction to 15 mM boric acid was used as an acceptable indicator of tolerance to B excess (Tab. 1). Since the data on RFCW were expressed in percents, transformation was performed prior to statistical analysis. The One-Way ANOVA indicated that there is highly significant genotypic effect on RFCW (Tab. 3).

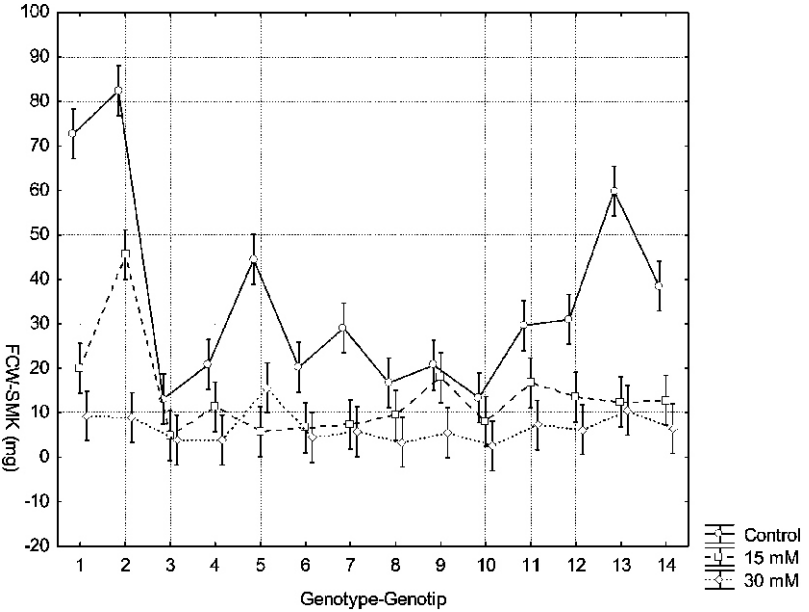

Fig. 1. Effect of different boron treatments (1 - control medium without excess B, 2 - with 15 mM boric acid and 3 - with 30 mM boric acid) on fresh callus weight (FCW) of 14 wheat genotypes. Vertical bars denote 0.95 confidence intervals.
Graf. 1. Uticaj različitih tretmana borom (1 - kontrolna podloga bez suviška B, 2 - sa15 mM borne kiseline i 3 - sa 30 mM borne kiseline) na svežu masu kalusa (SMK) kod 14 genotipova pšenice. Vertikalne linije označavaju 0,95 interval poverenja.

Tab. 3. One-Way ANOVA for effect of genotype on reduction of fresh callus weight (RFCW) of 14 wheat genotypes
Tab. 3. Jednofaktorijalna ANOVA za efekat genotipa na redukciju sveže mase kalusa kod 14 genotipova pšenice

Effect / *Efekat*	SS	Df	MS	F	P
Intercept	67846.83	1	67846.83	9216.528	0.000**
Genotype/ *Genotip*	3904.80	13	300.37	40.803	0.000**
Error / *Greška*	103.06	14	7.36		

Formation of FCW was affected by genotypes tolerance to excess B as well as by its callusing ability in *in vitro* culture, expressed at the control medium. Because of that, the classification of genotype into the certain tolerance level was corrected depending on its callusing ability on the control medium. Thus, out of seven genotypes which should be classified as the tolerant, according to the RFCW values below 50 % (Fig. 2), only two genotypes (Nevesinjka and Pesma) expressed sufficient level of B tolerance. The other genotypes from the tolerant group (Renesansa, Sonata, Simfonija, Rapsodija, Cipovka) had low callusing ability on the control medium and their level of B tolerance should be checked by other methodologies.

The analyzed genotypes were found to exhibit different callusing rates on the control medium and different reactions to the applied concentrations of boric acid. In the majority of genotypes fresh callus weight decreased significantly with increase in the boric acid concentration, both with respect to the control and between groups. The obtained results are in agreement with the results that Huang and Graham (1990) obtained for wheat, Ismail (2003) for corn and peas, Molassiotis et al. (2006) for apple and Rajaie et al. (2009) for lemon.

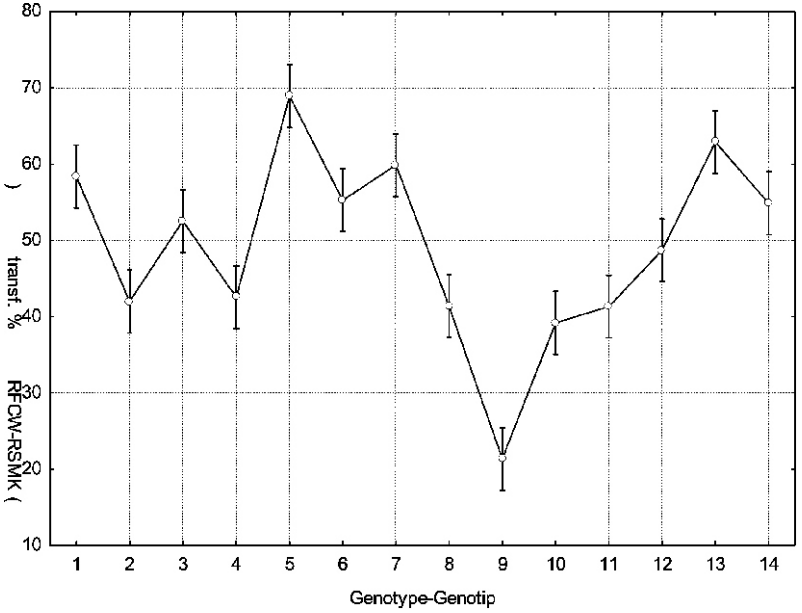

Fig. 2. Reduction of fresh callus weight (RFCW) of 14 wheat genotypes at the concentration of 15 mM boric acid. Vertical bars denote 0.95 confidence intervals
Graf. 2. Redukcija sveže mase kalusa (RSMK) kod 14 genotipova pšenice pri koncentraciji od 15 mM borne kiseline. Vertikalne linije označavaju 0,95 interval poverenja

The *in vitro* culture test showed significant differences among the genotypes regarding their tolerance to increased B concentrations. This confirmed earlier findings that differences in genotype reaction to the toxic effect of B are visible at the cellular level and that they may serve as a selection criterion (Mouhtaridou et al. 2004, Kondic-Spika et al. 2006, Kondic-Spika et al. 2007, Martinez-Ballesta et al. 2008).

The obtained results on tolerance levels of individual genotypes are in agreement with the study of Kraljevic-Balalic et al. (2002) who determined B concentration in leaves of 30 wheat genotypes at the heading stage. They concluded that cultivars Nevesinjka, Kalyan Sona and Peking 11 may serve as adequate sources of tolerance to excess B as well as parent components in breeding programs. Our findings about the cultivar Nevesinjka are in full agreement with their results, as well as with the results of Brdar et al. (2008).

Cultivar Pobeda was characterized as sensitive genotype in our study, as well as in the study of Brdar et al. (2007). Authors Kraljevic-Balalic et al. (2002) classified this cultivar in the group of medium tolerant genotypes. Different conclusions about the level of tolerance of some genotypes could be explained by different methodologies, physiological stages, B concentrations applied and the time of analysis, different selection criteria used in the studies, or by the experimental error obtained in one of the studies.

There are many advantages of using *in vitro* culture as a method for B tolerance screening. It is rapid and produces an objective metric value that can be statistically analyzed. The method is also reproducible and, as it is conducted under controlled environment, it is not subject to seasonal variations which influence glasshouse and field experiments. The method of *in vitro* embryo culture enables a uniform and rapid assessment of plant reaction to stress and it could be rec-

ommended for screening a large number of genotypes for breeding purposes. Just a small number of potentially tolerant genotypes, selected in *in vitro* test, should be checked in a filed experiment, in order to confirm or reject the obtained results.

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In vitro evaluacija tolerantnosti genotipova pšenice (*Triticum aestivum* L.) prema boru

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Izvod: Tolerantnost pšenice prema visokim koncentracijama bora ispitana je primenom kulture zrelog embriona. Testiranje je vršeno na modifikovanoj MS hranljivoj podlozi, kojoj je borna kiselina dodata u dve koncentracije: 15 mM i 30 mM. Kontrolna podloga nije sadržala bornu kiselinu u suvišku. Eksperiment je izveden na 14 sorti pšenice proizvedenih u Srbiji. Posle mesec dana gajenja na podlogama izmerena je sveža masa kalusa (SMK) i izračunata redukcija sveže mase kalusa (RSMK) na podlozi sa 15 mM borne kiseline, u odnosu na kontrolu. Genotipovi su se značajno razlikovali u pogledu njihove reakcije na različite koncentracije bora. Šest genotipova imalo je RSMK ispod 50,0 %, dok je osam genotipova imalo RSMK iznad 50,1 %. Sorte Nevesinjka i Pesma ocenjene su kao najtolerantnije, dok su sorte Balada i Vila bile najosetljivije na prisustvo bora u podlozi. Rezultati su potvrdili da su

reakcije genotipova pšenice na suvišak bora vidljive i na ćelijskom nivou, te da se ova reakcija može koristiti kao selekcioni kriterijum za procenu tolerantnosti na bor.
Ključne reči: pšenica, suvišak bora, tolerantnost, zreli embrioni

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