ECOLOGICAL ASPECTS OF *IN VITRO* WHEAT HERBICIDE TOLERANCE TESTING

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ABSTRACT

To avoid soil pollution caused by filed trials for herbicide tolerance of wheat genotypes, an alternative *in vitro* test was created and analyzed in this study. For validation of *in vitro* results, a comparative *in situ* test was conducted in a greenhouse. Four winter wheat cultivars (Renesansa, Stepa, Proteinka and Pesma) and three herbicide (tribenuron) concentrations (I-18.75 mgl^{-1}, II-37.50 mgl^{-1}, III-56.25 mgl^{-1}) were used in both tests. Zygotic embryo culture was used as the method for *in vitro* testing. Since different parameters were analyzed in the *in vitro* and *in situ* experiments, only the final conclusions about herbicide tolerance of different genotypes were compared.
Tribenuron-methyl had no significant effect on callus fresh weight only in cv. Proteinka. Similarly, the *in situ* experiment showed that tribenuron-methyl had no significant effect on plant height and number of kernels per spike in the same cultivar (cv. Proteinka), while the highest concentration of tribenuron-methyl caused significant decreases of these traits in the other cultivars. This compatibility of results from different tests can be considered as a proof of validity of *in vitro* test, which is more ecologically friendly than field trials.
Key words: herbicide tolerance, in vitro, wheat.

INTRODUCTION

Pesticides consist of a broad group of heterogeneous chemicals. They are of significant public benefit by increasing agricultural productivity and by decreasing the prevalence of diseases. However, they also cause public concern due to their potential adverse effects on human health, which is most obvious in the developing fetus and young children. In addition, they have consequences for animal life (1).
Weed infestation of crops causes extensive losses in the production of food. Estimates range from 10 to 30% of the annual crop yield (2). Despite a multitude of herbicides available to farmers, there are considerable limitations to their effectiveness.
Sulfonyl ureas (SUs) are a family of environmentally compatible herbicides that were discovered by DuPont Crop Protection in 1975 and first commercialized for wheat and barley crops in 1982. Sulfonyl urea herbicides are extraordinarily potent, requiring application doses one hundred times smaller then the herbicides they were intended to replace. The use of such potent chemicals also carries some obvious drawbacks. When any pesticide is applied from an airplane or helicopter, there is always some drift of the chemical from the target crop into adjacent fields and further away. Residues of drifting pesticides have been found as far as a mile away from target fields (3). In field trials for testing herbicide tolerance of different crops or weed species, the risk of drifting

herbicides is much stronger, because of multiple field doses used (doubled, tripled, etc.). In case of such strong preparations as SU herbicides these risks are more obvious.
The objective of this study was to create an alternative *in vitro* test for herbicide tolerance, to avoid these environmental risks. Also, in order to prove validity of the *in vitro* results, they were compared with results of standard *in situ* test for herbicide tolerance.

MATERIAL AND METHODS

Tolerance of four winter wheat cultivars (Renesansa, Stepa, Proteinka and Pesma) to different concentrations of tribenuron-methyl was investigated in comparative *in vitro* (a) and *in situ* (b) experiments. Tribenuron-methyl (Granstar 75-WG preparation) is selective herbicide from the group of sulfonyl urea preparations, which is used to control broadleaf weeds in wheat fields.

a) *In vitro* experiment
Air-dry mature grains of four randomly selected wheat genotypes were immersed in distilled water for four hours and surface sterilized in a 5.25% solution of NaOCl. Isolated embryos were sterilized using an earlier described procedure (4) and inoculated onto a modified MS (5) medium. Tribenuron-methyl was added to the medium in three different concentrations (I-18.75 mgl^{-1}, II-37.50 mgl^{-1}, III-56.25 mgl^{-1}). Since herbicide formulation contains 750g/kg of the active substance (tribenuron-methyl), the above concentrations of tribenuron-methyl are equivalent to the following doses of the preparation: I – 25g/ha (typical field dose), II – 50 g/ha (double field dose) and III – 75 g/ha (triple field dose). The control groups of embryos were cultivated on a herbicide-free medium.
During the two months of cultivation, the callus tissues were subcultured every 3 weeks on fresh medium with the same herbicide concentrations. Survival rates of calluses and plants were visually observed during the cultivation. Callus fresh weight was determined at the end of cultivation.

b) *In situ* experiment
The *in situ* experiment was performed in a greenhouse using a complete randomized block design with 10 replicates and the same cultivars as in the *in vitro* experiment. Plants in tillering phase were treated with the same herbicide concentrations as in the *in vitro* experiment.
Plant height and number of kernels per spike were analyzed in this part of the experiment.

Data analysis
Results were assessed by standard analysis of variance for a randomized complete block design. Least significant difference (LSD) values were calculated when the F value was found to be significant.
Since different parameters were analyzed in the *in vitro* and the *in situ* experiments, it was not possible to compare them directly. Only the final conclusions about herbicide tolerance of different genotypes were compared.

RESULTS AND DISCUSSION

a) *In vitro* experiment

Increases in herbicide concentration led to a gradual decrease in callus survival rate in all genotypes. However, the genotypes differed in the callus survival rate when compared at same levels of concentration (Tab. 1). Cv. Pesma had the highest survival rates with all tribenuron-methyl concentrations. The remaining three cultivars differed only at the highest concentration (III-56.25 mgl^{-1}), where cv. Proteinka had 71.3%, Stepa 67.9% and Renesansa-55.3% of survived calluses.

Table 1. Effect of different tribenuron-methyl concentrations on wheat calluses and regenerants

Genotype	Concentration (mgl^{-1})	Survival of calli (%)	Survival of plants (%)	Callus fresh weight (mg)
	Control	100	100	186.5
PESMA	I-18.75	87.0	70.2	186.2
	II-37.50	85.5	48.4	154.7*
	III-56.25	81.0	47.2	151.5*
	Control	100	100	73.4
STEPA	I-18.75	82.6	68.2	64.3
	II-37.50	74.4	50	47.7
	III-56.25	67.9	50	40.2*
	Control	100	100	118.2
RENESANSA	I-18.75	82.2	59.6	109.5
	II-37.50	84.5	50.6	82.0*
	III-56.25	55.3	44.4	77.8**
	Control	100	100	74.7
PROTEINKA	I-18.75	75.4	80.0	61.7
	II-37.50	78.7	40.0	74.3
	III-56.25	71.3	34.3	70.2
LSD	0.005			28.74
	0.001			38.37

b) *In situ* experiment

Regenerants were more susceptible to the presence of tribenuron-methyl in the nutrient medium than calluses. At the lowest concentration, all genotypes had more that 50% of survived regenerants. However, in treatments II and III only cv. Stepa had 50% of survived plants while the other cultivars had lower survival rates (Table 1).

The results of callus fresh weight indicated that the presence of tribenuron-methyl in the medium had an inhibitory effect on callus growth in all genotypes, except in cv.

Proteinka. In cultivars Pesma and Renesansa, tribenuron-methyl concentrations II and III caused significant decreases in callus fresh weigh, while in cv. Stepa, only the highest concentration (III) significantly decreased callus fresh weight (Table 1).

Table 2. Effect of tribenuron-methyl on plant height and number of kernels per wheat spike

Genotype	Concentration (mgl^{-1})	Plant height (cm)	No of kernels per spike
	Control	67.6	23.0
PESMA	I-18.75	69.7	25.0
	II-37.50	66.3	23.6
	III-56.25	60.2**	23.1
	Control	59.6	28.1
STEPA	I-18.75	58.4	26.1
	II-37.50	58.2	26.8
	III-56.25	54.1**	23.7
	Control	69.8	30.6
RENESANSA	I-18.75	67.4	29.8
	II-37.50	66.4*	27.8
	III-56.25	62.1**	24.8*
	Control	61.0	25.5
PROTEINKA	I-18.75	60.5	26.3
	II-37.50	60.4	24.9
	III-56.25	60.5	26.9
LSD	0.005	2.835	4.757
	0.001	4.073	6.834

The results of the *in situ* experiment showed that high concentrations of tribenuron-methyl have an inhibitory effect on plant growth. Treatment I had no effect on plant height in either genotype, while in treatment II only cv. Renesansa had a significantly decreased plant height. The highest concentration (III) of the herbicide caused significant decreases in plant height in all genotypes, except cv. Proteinka (Table 2).

According to the results for the number of kernels per spike, all genotypes exhibited a high level of herbicide tolerance, except cv. Renesansa. In this cultivar the highest concentration of tribenuron-methyl caused a significant decrease in the number of kernels per spike (Table 2).

The results showed that tribenuron-methyl, at all the concentrations, caused necroses of callus tisses and regenerants in all the cultivars. However, the *in vitro* results for the three examined parameters gave different information on genotype susceptibility to the herbicide. According to the callus survival rate, the most tolerant genotype was cv.

Pesma, while cv. Stepa had the highest level of tolerance in regenerated plants. Cultivar Proteinka was the most tolerant genotype, according to the callus fresh weight results. On the other hand, the results of standard *in situ* test have shown that cv. Proteinka had the highest level of tribenuron-methyl tolerance. It can be concluded that among the *in vitro* parameters, callus fresh weight is the best one for separating tolerant from sensitive genotypes. It could be due to some necroses of calluses and regenerants that may be induced by other factors in the *in vitro* culture and that may occur independently of herbicide exposure. It is not possible to separate necroses caused by herbicide from necroses caused by other factors. However, in the case of callus growth, as indicated by callus fresh weight, it is possible to contend that growth inhibition of healthy calluses was caused by herbicide activity. Here we should have in mind a unique mode of action of the sulfonyl urea herbicides, which inhibit acetolactate synthase (ALS), a key enzyme required for plant cell growth (6).

According to our resuts, however, the callus tissues and regenerants were much more susceptible to the herbicide then the whole *in situ* plants. This is in agreement with results of other authors (6; 7; 8). These authors suggested that less sensitive reaction of sprayed plants could be due to a partial degradation of the herbicide during its uptake and translocation to the target tissues. On the other hand, callus tissues cultured on a selective medium are directly exposed to the herbicide and should be more susceptible to lower herbicide concentrations.

When tested *in vivo*, the wheat cultivar most resistant to tribenuron-methyl (cv. Proteinka) was also the most resistant at callus level *in vitro*, i.e., there was a correlation between tolerance to tribenuron-methyl in the intact wheat plants and tolerance to tribenuron-methyl of wheat *in vitro*. Similar results for other herbicides and other crop species have been reported (7; 9; 10).

CONCLUSION

In conclusion, the results of the present study demonstrate that, at least for the investigated cultivars, the response of wheat against tribenuron-methyl *in vitro*, indicated by callus fresh weight is directly related to the tolerance expressed at the whole-plant level (*in vivo*). *In vitro* screening tests for herbicide tolerance can be advantageous over conventional tests, on account of lower environmental risk, less time required for the determination of tolerance level, as well as considerably less space required for screening a large number of different genotypes.

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