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# PCR identification of *rpgip1* transgene in *Pisum sativum* L.

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## ABSTRACT

Recent efforts to increase Ascochyta blight resistance of pea have focused on the introduction of foreign genes by genetic engineering. The *rpgip1* gene from *Rubus idaeus* was introduced by *Agrobacterium*-mediated transformation into *Pisum sativum*, cv. Baroness with the aim to increase pea resistance to fungal diseases. Notwithstanding this success, practical applications have to be preceded by the development of analytical methods for screening. Singleplex and multiplex PCR assays were employed to test primer efficiency in identifying the *rpgip1* transgene in 11 pea genotypes. Five from ten primer combinations were effective in identifying transgene or insert sequences. PCR amplification using five other primer pairs revealed unspecific amplicons. According to *in silico* analyses, they arose from retrotransposons and pea genes including homologues of *rpgip1*. Two sets of primers were prepared with the aim of simultaneous amplification of different *rpgip1* fragments. Fingerprints were sums of bands observed from individual pairs so the utility of multiplex assays was demonstrated. An additional advantage of multiplex PCR was clear differentiation between the transgene and endogenous *pgip* genes present in the donor species, *R. idaeus*. Sequencing of two PCR products confirms that no substantial rearrangements at the *rpgip1* transgene arose during development of transgenic plants. However, a deletion occurred at 59 bp in the PGIP+VST line and a substitution at 392 bp in the PGIP line. The frequency of point mutations was not high (1.1 x $10^{-3}$) and comparable with the frequency expected for host genes based on the neutral theory of molecular evolution.

**Key words:** Transgenic pea, fungal diseases, *Rubus idaeus*, *pgip* homologues, multiplex PCR

## IZVLEČEK

### PCR IDENTIFIKACIJA TRANSGENA *rpgip1* PRI GRAHU (*Pisum sativum* L.)

Novejši dosežki pri povečanju odpornosti graha na Ascochyta so povezani z uvajanjem tujih genov s pomočjo genskega inženiringa. Gen *rpgip1* iz malinjaka (*Rubus idaeus*) je vključen v grah, cv. Baroness, s transformacijo z bakterijo *Agrobacterium*, da bi se povečalo odpornost graha na to glivično bolezen. Pred praktično uporabo te metode je potrebno razviti načine za spremljanje dedovanja tega transgena. Enojna in multipleks PCR sta bili uporabljeni za testiranje učinkovitosti začetnikov in za identificiranje transgena *rpgip1* pri 11 genotipih graha. Pet od desetih začetnikov je bilo uporabnih za identifikacijo transgenov ali za vključevanje sekvenc. PCR namnoževanje z drugimi petimi začetniki je dalo nespecifične namnožke. Glede na *in silico* analize so ti nastali zaradi retrotranspozonov in grahovih genov, ki vključujejo homologe *rpgip1*. Dva seta začetnikov sta bila pripravljena za istočasno namnoževanje različnih odlomkov *rpgip1*. Elektroferogrami so bili vsote črt individualnih parov, tako je prikazana uporabnost multipleksnega poskusa. Dodatna prednost multipleksnega PCR je razločna diferenciacija med transgenom in genom *pgip* prisotnim v donorski vrsti *R. idaeus*. Sekvenciranje dveh PCR produktov potrjuje, da ni pri *rpgip1* bistvenega prerazporejanja tekom razvoja transgenih rastlin. Toda pojavila se je delecija pri 59 bp v liniji PGIP+VST in substitucija pri 392 bp v liniji PGIP. Relativna pogostnost točkovnih mutacij ni bila visoka (1.1 x $10^{-3}$) in je bila primerljiva z pogostnostjo pri gostiteljivih genih, glede na nevtralno teorijo molekulske evolucije.

**Ključne besede:** Transgeni grah, glivične bolezni, *Rubus idaeus*, homologi *pgip*, multipleksna PCR

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## 1 INTRODUCTION

The pea (*Pisum sativum* L.) has been grown since the prehistoric ages providing forage (field peas), fresh vegetable (market peas) and the material for canning and freezing (vining peas). With approximately 26% - 33% proteins and a lower level of protein inhibitors than in soybean, peas are excellent protein supplements in human and animal diets (Cousin, 1997). Producing nearly two million tons of peas per year, Europe has recently provided one-fifth of world production. However, the yield is unstable with the average yield per hectare ranging from 1.3 tons to 4.8 tons (Eurostat, 2011). Consequently, many characters have to be improved and Ascochyta blight is the major factor limiting pea production up to 75% (McDonald and Peck, 2009). Symptoms include dark-brown lesions or flecks on all plant parts, seedling blight and foot rot. The disease is caused by related fungal pathogens of the Ascochyta complex (*Mycosphaerella pinodes, Ascochyta pisi, Phoma medicaginis*).

Pea genotypes differ in susceptibilities to Ascochyta pathogens but complete resistance to infection has not been observed (Timmerman-Vaughan *et al*., 2002). On the other hand, disease resistance is often encoded by major plus minor genes. Numerous genomic regions responsible for partial resistance have been located on linkage groups II, III, IV, V and VII (Timmerman-Vaughan *et al*., 2002; Prioul-Gervais *et al*., 2007). Unfortunately, the biological function of the underlying factors still remains unknown. The quantitative inheritance, the lack of information on the molecular mechanism underlying resistance and genotype-environment interaction emphasized by variation in disease development with both growing areas and climate conditions make the traditional resistant breeding difficult. Therefore, recent efforts to increase Ascochyta blight resistance of pea, directly correlated with the yield and quality of seeds, have focused on the introduction of foreign genes by genetic engineering.

A classic tactic for producing transgenic plants with increased resistance is to introduce *R* genes, products of which recognise pathogen *Avr* genes' determinants. When corresponding *R* and *Avr* genes are present, the disease resistance is brought in the classic gene-to gene manner. Good examples of this system are plant *pgip* genes encoding polygalacturonase inhibiting proteins (PGIPs) and fungal *PG* genes responsible for fungal endopolygalacturonases (PGs) - enzymes degrading polysaccharides of the cell wall. The PGIPs inhibit fungal endo-PGs, thus preventing the hydrolysis of the α-1,4 glycosidic bonds (Shanmugam, 2005; Di Matteo *et al*., 2006). A range of crops including tomato (Powell *et al*., 2000), apple (Szankowski *et al*., 2003), and wheat (Janni *et al*., 2008) were transformed with *pgip* genes to improve plant defence against fungal pathogens. In a case of pea, the *rpgip1* gene from red raspberry, *Rubus idaeus* (Accession N°AJ620336) was introduced by *Agrobacterium*-mediated transformation into cv. Baroness (Richter *et al*., 2006). Stable inheritance was confirmed and transgenic lines showed significant inhibitory effects on the polygalacturonases of *Colletotrichum lupini* and *Stenocarpella maydis* in a greenhouse experiment. Notwithstanding the usefulness of the transgenic approach to produce pea resistant to fungal diseases, practical applications have to be preceded by the development of analytical methods for screening. They are necessary to allow consumers a free choice between genetically modified (GM) and traditional crops. Controlling the structural integrity of the transgene during further manipulations is not less important. It is a norm that unexpected variation is found in transgenic lines during later experiments or commercial use. For instance, expression of *rpgip1* varied greatly among both transgenic pea individuals and subsequent generations (Richter *et al*., 2006). Among many reasons for this phenomenon, rearrangements at a transgene locus are frequently mentioned (Morino *et al*., 1999; Svitashev *et al*., 2002).

Direct sequence analysis is the most reliable way to identify transgenes and study their structure. However, it is too time and cost consuming when a large number of samples have to be tested. For this reason, various types of polymerase chain reaction (PCR) are adopted to enable reliable and rapid assessments of GMOs. Event-specific methods, which are preferred in EU, are based on a sequence unique to a certain GMO. Such assays have to be developed for each new GMO, so do for transgenic peas expressing antifungal genes (e.g., *rpgip1*). A critical point is that PCR should differentiate between a transgene and homologous, endogenous sequences. At least two pea sequences homologous to *rpgip1* are deposited in the GenBank maintained by the National Centre for Biotechnology Information (NCBI). Both genes can be a putative template for unintended amplification driven by primers specific to *rpgip1*. One possibility to overcome the problem is to design a set of primers to track different gene fragments. A simpler but yet a reliable method to differentiate between a transgene and homologues can employ several primers at once in so called multiplex PCR.

In the present studies a set of primers was used to identify the *rpgip1* transgene in different genotypes of *P. sativum*. The primer efficiency in differentiating between the transgene and pea homologues was assessed as well as sets for multiplex PCR were proposed. We tried to understand why some primers preferentially amplified pea homologues instead of the *rpgip1* transgene. Results were discussed in the light of possible rearrangements of the transgene.

## 2 MATERIALS AND METHODS

### 2.1 Plant material

**Two transgenic lines:** PGIP – a homozygous line carrying two copies of the *rpgip1* gene from *Rubus idaeus*. PGIP+VST – a homozygous line with stacked antifungal genes, *rpgip1* and *Vst1* encoding stilbene synthase in *Vitis vinifera*. It was obtained by reciprocal crosses of the PGIP line expressing *rpgip1* and a line expressing the *Vst1* gene. Plants used in the analysis represented F$_8$ and further generations (Richter *et al.*, 2006). In total 15 plants per both lines were used in the analyses.

**Six *rpgip* hemizygotes:** F$_1$ hybrids derived from crosses between the PGIP or PGIP+VST lines and the parent cultivar Baroness (Bar x PGIP and Bar x PGIP+VST), the *arthritic* mutant from Paloma (*arth* x PGIP), the *ramosus* mutant from Parvus (PGIP x *ram* and PGIP+VST x *ram*) and the Polish cultivar Sokolik (PGIP+VST x Sok). All hybrids were expected to carry at least one copy of *rpgip1*.

**Negative controls:** Baroness – parent cultivar, from which transgenic lines were derived (15 plants in total); Bar x VST – the F$_1$ between Baroness and the transgenic line with *Vst1* gene; *ram* x Bar – the F$_1$ between the *ramosus* mutant from Parvus and Baroness.

**Positive controls:** pSCP1 plasmid – a binary vector using for pea transformation and carrying a *nos* promoter-driven *bar* gene and a double 35S promoter-driven the *rpgip1* gene from *R. idaeus* (Richter *et al.*, 2006). The *rpgip1* transgene sequence consists of 996 base pairs (bp) and corresponds to the 29 bp - 1024 bp of *rpgip1* in the NCBI file, accession N$^{o}$AJ6200336. Moreover, a wild ecotype of red raspberry (*R. idaeus*) was used for comparisons between transgenic and donor species fingerprints.

### 2.2 DNA isolation

Plant genomic DNA was extracted from about 1 g of young leaves by the modified CTAB procedure (Polok, 2007). The quality of DNA was verified on 1% agarose gels while the purity was assessed spectrophotometrically and it ranged between 93% and 99%. The DNA content of the samples ranged from 177 µg to 568 µg. The DNA of pSCP1 plasmid was provided by the Plant Biotech Unit, Leibniz Universität Hannover.

### 2.3 Primers

Eight primers distributed over the entire transgene were designed on the *rpgip1* sequence used in the pSCP1 transformation vector. Numbers in primers' abbreviations identify their 5'position on the *rpgip1* template. **Forward primers:** 1F: 5'atgatggacttcaagctctt3'; 6F: 5'ggacttcaagctcttctccc3'; 108F: 5'caagacagccttcaacaaccc3'; 421F: 5'cagctcaagaacctcacatt3'. **Reverse primers:** 366R: 5' cttgagatgtttaagcttgg3'; 733R: 5'ccacaatctgggtggtcttgt3'; 958R: 5'ggttatggaaatacgacgtg3', 971R: 5' gcaacttgggaggggagcac3'. Ten pairwise combinations of forward and reverse primers and two multiplex sets with four and three primers were applied (Table 1).

**Table 1.** Primer combinations and PCR conditions.

| | Primer combination | Annealing temperature [°C] | Number of cycles [n] | Predicted product length [bp] |
|---|---|---|---|---|
| **Singleplex** | 1F-366R | 58 | 30 | 366 |
| | 1F-733R | 58 | 30 | 733 |
| | 1F-958R | 58 | 30 | 958 |
| | 108F-366R | Touchdown:<br>48-0.8 then 40 | 10+20 | 259 |
| | 108F-733R | 48 | 30 | 626 |
| | 108F-958R | 58 | 30 | 851 |
| | 421F-366R | 40 | 30 | 942* |
| | 421F-733R | 50 | 30 | 313 |
| | 421F-958R | 58 | 35 | 538 |
| | 6F-971R | 41 | 35 | 958, 1700* |
| **Multiplex** | **Set 1:** | | | |
| | 1F-366R + 733R + 958R | 58 | 35 | 366, 733, 958 |
| | **Set 2:** | | | |
| | 108F + 421F-958R | 58 | 35 | 538, 851 |

*Product predicted for the plasmid and only when circular matrix is assumed.

### 2.4 PCR conditions

**Singleplex PCR:** PCR was performed in a 20 µl volume containing 20 mM $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 9.0 at 25°C, 1.5 mM $MgCl_2$, 2 µl of Enhancer with betaine (Epicentre Technology), 200 µM dNTPs, 1.0 µM primers, 0.75 U of *Tfl* polymerase (Epicentre Technology) and 80 ng of template DNA. The standard thermal conditions were: 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min. The final extension was at 72°C for 5 min. However, conditions for five pairs were optimized and finally a number of cycles were increased in two cases while annealing temperature was modified in three ones (Table 1). PCR products were loaded on 1.5% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide, separated in 1 x TBE buffer (Tris-Borate-EDTA) at 100 V constant power, visualized under UV light (312 nm), photographed with Olympus Camera and stored as .jpg files. **Multiplex PCR:** The same reagent concentrations and standard thermal conditions were applied as for singleplex PCR with the exception of a number of cycles that was increased to 35.

### 2.5 Sequencing

PCR products revealed by primers 1F-733R and 1F-958R in transgenic plants and the pSCP1 plasmid were sequenced. The PCR products were separated on 1.5% agarose gels, excised and purified using the gel-out system (AKOR Laboratories). The identities of the products were confirmed by nested PCR and restriction analyses. Sequencing was performed using the ABI3730 and BigDye Terminator Ready Cycle Sequencing Kit (Applied Biosystem) with the 1F primer by oligo.pl, Institute of Biochemistry and Biophisics PAS.

### 2.6 *In silico* and sequence analyses

The specificity of primers was tested by Primer-BLAST against *Pisum* sequences deposited in the non-redundant database at NCBI. Possibilities of unintended amplification were studied by performing *in silico* PCR on the *rpgip1* template and two pea homologues deposited at NCBI (AB0877839, AJ749705) using FastPCR software. Multiple alignment algorithms in CLUSTALX2 were applied for sequence comparisons. Sequences were viewed in Jalview 2.6.1 editor.

## 3 RESULTS

### 3.1 Amplification of the *rpgip1* transgene on the pSCP1 template

The majority of primer pairs amplified a product of a size corresponding to *rpgip1* while using the pSCP1 template (Fig. 1, 2). Thus, their usefulness in identification of the transgene was confirmed. Interestingly, only five pairs amplified a single, expected band (1F-366R, 1F-958R, 108F-366R, 108F-733R, 421F-733R), while the other five revealed two bands (1F-733R, 108F-958R, 421F-366R, 421F-958R and 6F-971R). The first of two products fell within the expected size range but the second was much shorter and faint. Surprisingly, primers 421F and 366R resulted in two, clearly visible but relatively short amplicons (350 bp, 490 bp) although the target sites' orientation rather excluded amplification (Table 1). *In silico* PCR used for quick primer analyzing on the *rpgip1* template demonstrated that some of additional, shorter products could result from unspecific amplification of the transgene. Likely, this explanation is valid for the 420 bp product revealed by 108F-958R but not for the other pairs of primers, for which even very weak searching criteria (7 mismatches, initial word size of 2) did not result in virtual, unspecific amplification. Therefore, it was assumed, that additional bands were probably derived from unintended amplification of other plasmid sequences. Pairs of primers amplifying additional plasmid sequences included 1F-733R (480 bp), 421F-958R (300 bp), 421F-366R (350 bp and 490 bp), and 6F-971R (740 bp).

### 3.2 Efficiency of primer combinations in identification of the *rpgip1* transgene in *P. sativum*

Amplification patterns of the pSCP1 vector demonstrated that all primer pairs could theoretically detect the transgene and eventually other sequences within an insert. Surprisingly, among ten pairs of primers checked, only 50% of combinations gave the plasmid banding pattern in transgenic PGIP and PGIP+VST plants and no amplification in the parent cultivar, Baroness (Fig. 1). This first group included 1F-366R, 1F-733R, 1F-958R, 421F-733R, 421F-958R pairs. They can further be used in identifying the transgene in various backgrounds as confirmed by fingerprints of *rpgip1* hemizygotes similar to these of transgenic lines and no amplification in negative controls (Table 2).

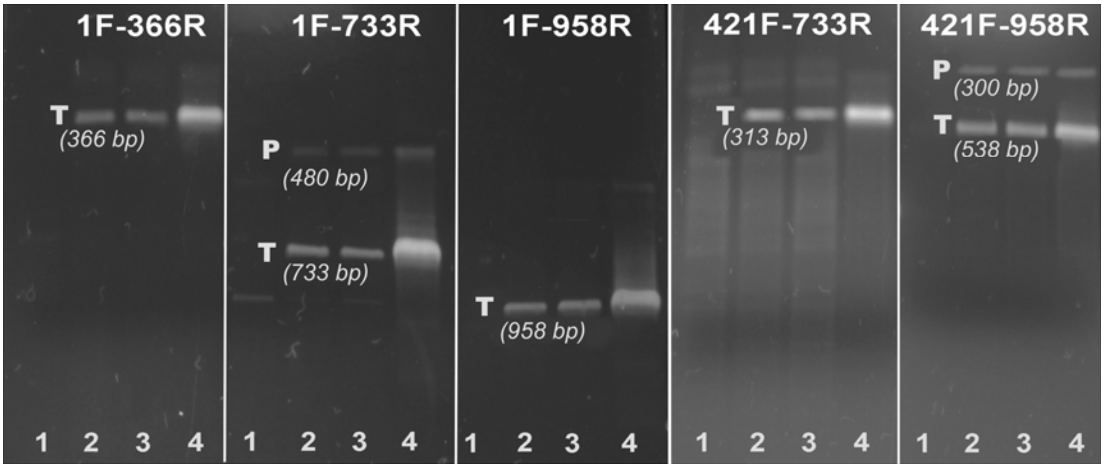

**Fig 1**. Identification of the *rpgip1* transgene and plasmid sequences in *P. sativum*. 1 - Baroness – negative control, 2 - PGIP, 3 - PGIP+VST, 4 - pSCP1 plasmid. T - products corresponding to the transgene, P – products corresponding to other insert sequences.

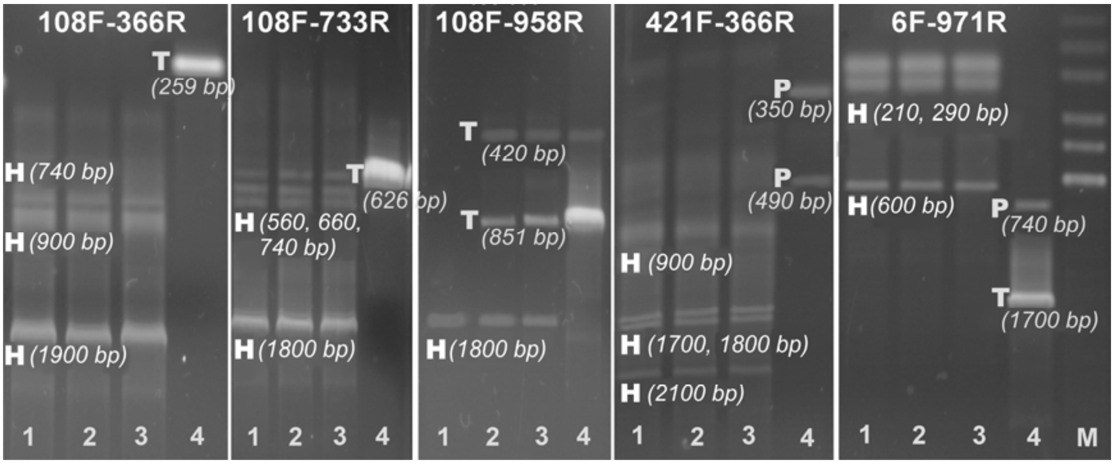

**Fig 2.** Unspecific amplification of *P. sativum* genomic DNA revealed by primers complementary to the *rpgip1* transgene. 1 - Baroness – negative control, 2 - PGIP, 3 - PGIP+VST, 4 - pSCP1 plasmid. T - products corresponding to the transgene, P – products corresponding to other insert sequences, H – products corresponding to endogenous pea sequences homologous to the transgene (transposons or *rpgip1* homologues), M – DNA marker.

**Table 2.** Validation of primer combinations in identifying the *rpgip1* transgene in *P. sativum*.

| **Primer combination** | **Estimated product length [bp]** | **Negative controls** | | | **Transgenic lines** | | ***rpgip1* hemizygotes** | | | | | | **Donor species** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Baroness** | **Bar x VST** | **ram x Bar** | **PGIP** | **PGIP+VST** | **Bar x PGIP** | **arth x PGIP** | **PGIP x ram** | **Bar x PGIP+VST** | **PGIP+VST x ram** | **PGIP+VST x Sok** | ***Rubus idaeus*** |
| **1 Primer pairs identifying the *rpgip1* transgene or/and plasmid sequences** | | | | | | | | | | | | | |
| 1F-366R | 366 | 0 | 0 | 0 | T | T | T | T | T | T | T | T | 366 |
| 1F-733R | 480 | 0 | 0 | 0 | P | P | 0 | 0 | P | P | P | P | 1000 |
| | 733 | 0 | 0 | 0 | T | T | T | T | T | T | T | T | |
| 1F-958R | 958 | 0 | 0 | 0 | T | T | T | T | T | T | T | T | 1100 |
| 421F-733R | 313 | 0 | 0 | 0 | T | T | T | T | T | 0 | T | T | 450 |
| 421F-958R | 300 | 0 | 0 | 0 | P | P | P | P | P | 0 | P | P | 538, 600 |
| | 538 | 0 | 0 | 0 | T | T | T | T | T | 0 | T | T | |
| **2 Primer pairs revealed unintended amplification on pea homologous sequences** | | | | | | | | | | | | | |
| 108F-366R | 740, 900, 1900 | H | H | H | H | H | H | H | H | H | H | H | 0 |
| 108F-733R | 560, 660, 740, 1800 | H | H | H | H | H | H | H | H | H | H | H | 500 |
| 108F-958R | 420 | 0 | 0 | 0 | T | T | T | T | T | T | T | T | 1000, 1500 |
| | 851 | 0 | 0 | 0 | T | T | T | T | T | T | T | T | |
| | 1800 | H | H | H | H | H | H | H | H | H | H | H | |
| 421F-366R | 900, 1700, 1800, 2100 | H | H | H | H | H | H | H | H | H | H | H | 1000, 1800, 2100 |
| 6F-971R | 210, 290, 600 | H | H | H | H | H | H | H | H | H | H | H | 580 |
| **3 Multiplex PCR** | | | | | | | | | | | | | |
| **Set1:** 1F, 366R, 733R 958F | 366 | 0 | 0 | 0 | T | T | T | T | T | T | T | T | 366, 1000, 1100 |
| | 480 | 0 | 0 | 0 | P | P | P | P | P | P | P | P | |
| | 733 | 0 | 0 | 0 | T | T | T | T | T | T | T | T | |
| | 958 | 0 | 0 | 0 | T | T | T | T | T | T | T | T | |
| **Set2:** 108F, 421F, 958R | 300 | 0 | 0 | 0 | P | P | P | P | P | 0 | 0 | 0 | 538, 1000, 1500 |
| | 420 | 0 | 0 | 0 | P | P | P | P | P | 0 | 0 | 0 | |
| | 538 | 0 | 0 | 0 | T | T | T | T | T | 0 | T | T | |
| | 851 | 0 | 0 | 0 | T | T | T | T | T | T | T | T | |
| | 1800 | H | H | H | H | H | H | H | H | H | H | H | |

0 – lack of amplification. T – products corresponding to the *rpgip1* transgene, P – products corresponding to plasmid/insert sequences, H – products resulted from unintended amplification of homologous pea sequences, for *R. idaeus*, an approximate size of observed products is given.

PCR amplification from pea genomic DNA using other five pairs of primers revealed completely different fingerprints than observed in the plasmid (Fig. 2). This second group comprised pairs: 108F-366R, 108F-733R, 108F-958R, 421F-366R and 6F-971R. Unexpected products were found both in transgenic and non-transgenic plants, hence providing an evidence for unspecific amplification from homologous sequences in pea genome (Table 2). All pairs but 108F-958R failed to detect either the transgene or other insert sequences. When PCR was performed with 108F-958R primers, the combination of a plasmid pattern and additional bands was noticed (Fig. 2). Hence, this pair may be in favour when a goal is to distinguish between the transgene and its endogenous homologues.

Based on the results for each pair of primers, two sets consisting of four (set 1: 1F, 366R, 733R, 958R) and three primers (set 2: 108F, 421F, 958R) were prepared with the aim of simultaneous amplification of different *rpgip1* fragments. Fingerprints obtained from these two multiplex assays were sums of bands observed from individual pairs (Fig. 3) so the utility of using several primers at once was demonstrated. Alike individual pairs, the first set amplified only sequences of a transgenic origin (Table 2). The second one gave products of both transgene/insert and endogenous origins. Likely, this pattern was caused by the 108F primer, combinations of which amplified mainly homologues. An additional advantage of multiplex PCR over singleplex is clear differentiation between the transgene and *pgip* genes present in the donor species, *R. idaeus* (Fig. 3). For example, bands revealed by 1F-366R and 421F-958R pairs in red raspberry were indistinguishable from the *rpgip1* amplicons in transgenic pea. Simultaneous amplification by 108F, 421F and 958R (set 2) gave a three-band pattern in *R. idaeus* that was clearly distinct from the transgene fingerprint.

## 3.3 Structure of the *rpgip1* locus in transgenic plants

The *rpgip1* transgene authenticity was confirmed by sequencing six PCR products, each two from the PGIP, PGIP+VST lines and the pSCP1 plasmid. The sequences derived from 1F-733R primers had 678 bp in transgenic plants and 691 bp in the pSCP1. Expectedly, sequences obtained from 1F-958R primers were longer and had 903 bp and 889 bp, respectively. Irrespective of the template (plant or plasmid DNA), shorter and longer nucleotide sequences were identical on the length of ca. 690 bp, what confirmed that both primer pairs identified the *rpgip1* transgene. This fact also enabled to use only longer sequences in further comparisons. Generally, significant rearrangements were not observed in the transgene (Fig. 4). However, all sequences were shorter than the original *rpgip1* construct. They started from the 47 bp position instead of 1 bp despite the 1F primer was used in sequencing reactions. The deletion of cytosine at 59 in relation to the *rpgip1* template occurred in the PGIP+VST line and thymine was substituted by adenine at 392 in the PGIP line. In total 1806 unique base pairs were sequenced in two transgenic lines, so that the frequency of point mutations was 1.1 x $10^{-3}$ or one mutation per 909 nucleotides.

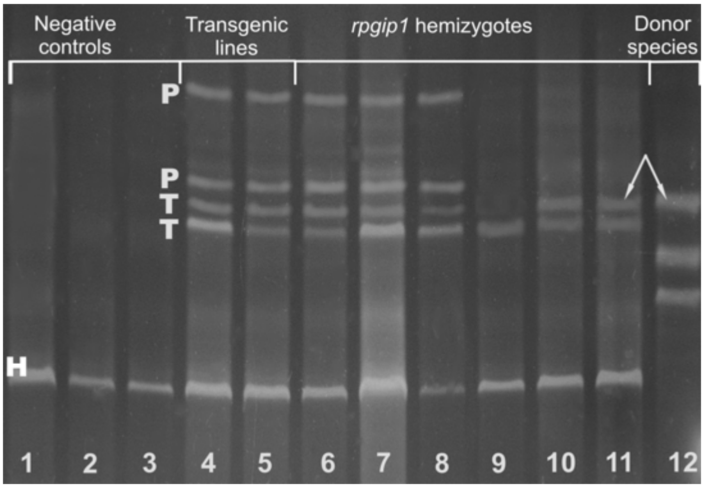

**Fig. 3.** Multiplex PCR profiles revealed by primers 108F, 421F and 958R (set 2). 1 - Baroness, 2 – Bar x Vst, 3 – *ram* x Bar, 4 – PGIP, 5 – PGIP+VST, 6 – Bar x PGIP, 7 – *arth* x PGIP, 8 – PGIP x *ram*, 9 – Bar x PGIP+VST, 10 – PGIP+VST x *ram*, 11 – PGIP+VST x Sok, 12 – *Rubus idaeus*. T - products corresponding to the transgene, P – products corresponding to other insert sequences, H – products corresponding to endogenous pea sequences homologous to the transgene (transposons or *rpgip1* homologues). Arrows indicate amplicons of transgenic and *R. idaeus* origin that have similar mobility.

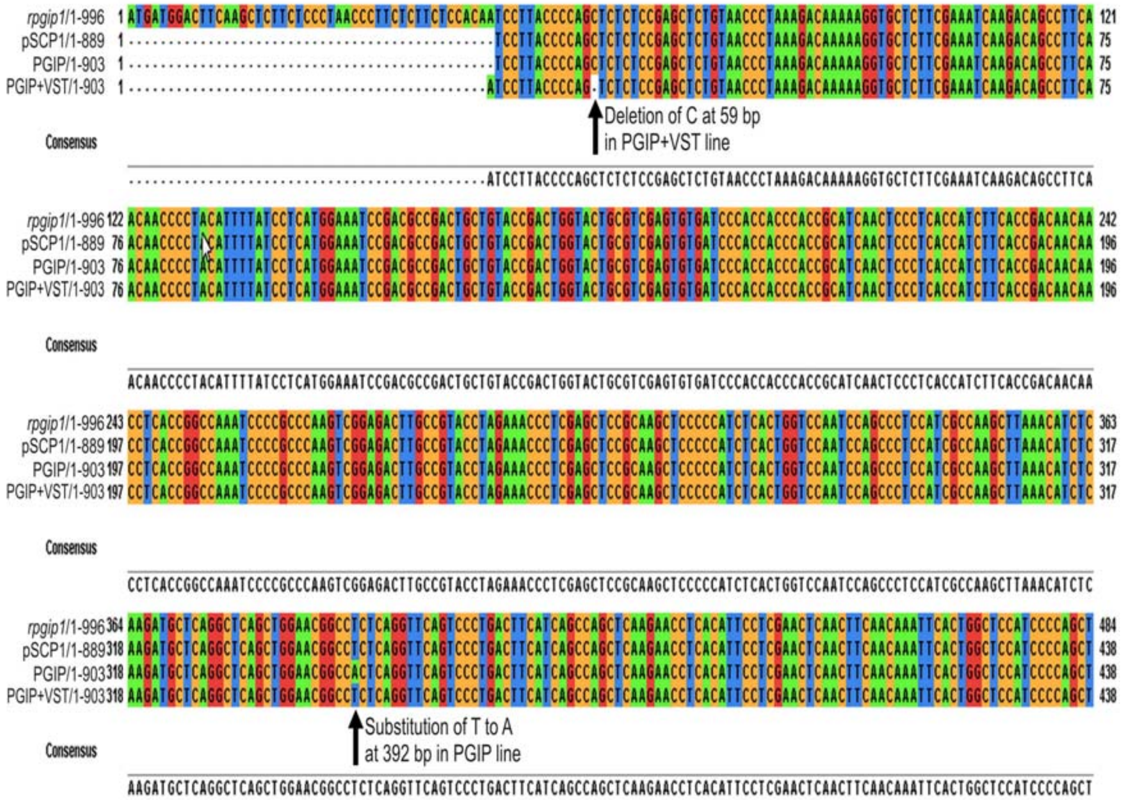

**Fig. 4**. Alignment of the *rpgip1* sequences obtained from the PCR product revealed by primers 1F-958R. *rpgip1*/1-996 – a sequence used for transformation, pSCP1/1-889 – a sequence from the pSCP1 plasmid, PGIP/1-903 – a sequence from the PGIP line, PGIP+VST/1-903 – a sequence from the PGIP+VST line. Arrows indicate point mutations.

### 3.4 Endogenous homologous sequences responsible for unintended amplification on pea genomic DNA

To find out the nature of unintended amplification by several primer pairs, the site specificity of primers was checked by performing a sequence homology search through all known template sequences for *Pisum sativum* at NCBI. Four pairs that otherwise were effective in *rpgip1* identification could also amplify other sequences from pea genome. Pairs 1F-733R, 1F-958 could identify the *Ogre* retrotransposon (AY299397.1) while 421F-733R and 421F-958R demonstrated affinity to an unknown sequence (CU655881.1) and the *Tfl* gene responsible for late flowering (AY430579.1). Among primers unsuccessful in transgene identification, two pairs, 108F-733R and 108F-958R could identify the *Psmar-2 – Mariner* retrotransposon (AY833551.1).

Because the *pgip* genes are ubiquitous in plants, unintended amplification of endogenous homologues can not be excluded. Two putative pea homologues of *rpgip1* were identified by NCBI searching, i.e., AB0877839 and AJ749705. Both sequences were derived from mRNA and they share of 65% and 60% of similarity to the *rpgip1* transgene. Alignment of both sequences and the *rpgip1* transgene at a protein level revealed several conserved regions between 164 and 331 amino acids corresponded to conserved residues at the nucleotide level. On the other hand, *in silico* PCR conducted on both pea homologues using all primer pairs complementary to *rpgip1* from *R. idaeus* produced none amplicons even though weak criteria were applied (4 - 7 mismatches allowed at the 3’ end of primers).

## 4 DISCUSSION

The classical approach to GMO screening involves amplification of sequences common to many transgenic plants, such as the cauliflower mosaic virus 35S promoter or the *Agrobacterium tumefaciens nos* terminator (Bonfini *et al.*, 2001). Transgenic peas expressing *rpgip1*, like most GM plants, contain these sequences what enables to use numerous commercial kits for their routine screening. It should be stressed however, that the detection of common GMO markers only indicates that a sample contains DNA from any GM plant and provides neither trait nor transgene confirmation. An alternative is to focus on target sequences but then any PCR-based strategy depends on a transgene and organism. Besides the majority of studied primer pairs identified the *rpgip1* transgene, some of them revealed additional bands corresponded to pea homologous sequences. Thus, the choice will depend very much on the objective of the PCR analysis. For routine screening purposes five pairs of primers (1F-366R, 1F-733R, 1F-958R, 421F-733R, 421F-958R) identifying unequivocally the *rpgip1* transgene can be recommended to detect as many *rpgip1* fragments as possible. If only a single pair is used, a small rearrangement within the transgene may prevent amplification and a GMO material may pass unnoticed. Notwithstanding several primer pairs can overcome this problem, the method is troublesome if many singleplex PCRs have to be used to test hundreds of samples as it is typical of food testing or GMO spread in the environment. Each pair needs a separate PCR reaction and sizing by agarose gel electrophoresis. Two proposed multiplex PCR assays allowing one-step identification of up to five GM-derived products provide promising simplification in detecting transgenic pea expressing antifungal genes. This procedure is in agreement with the current trends as emphasized by multiplex assays developed for the simultaneous detection of GM maize and soybean lines (Forte *et al*., 2005; Yoke-Kqueen *et al*., 2011). Rapidity and cost-efficiency are in favour of multiplex assays.

As more and more traits are introduced into plants, a challenge is how genetic variation of both donors and recipients influences the identification of GMOs. Risks rely on the false-positive results arising from wild ecotypes of a donor species. Primers 1F-366R gave a product of the same size in transgenic peas and wild raspberry. Similar fingerprints were observed for 421F-958R primers. This means that samples containing raspberry products may be misidentified as GM pea derivatives. It is therefore necessary to employ multi-primers assays that often entail clearer differentiation as the present studies demonstrated. Surprisingly, such considerations have been scarce, presumably because the majority of so far marketed GM plants carry bacterial or viral genes that are avoided in detection procedures (Bonfini *et al*., 2001).

A major aspect of the *rpgip1* detection in transgenic pea is the distinguishing between transgenes and endogenous homologues as shown for transgenic peas. This problem is rarely raised because most transgenic plants harbour insect or herbicide resistance genes that have not counterparts in plant genomes. Recently, genes from more or less related plant taxa are employed in plant transformation. A favourite example involves the wheat HMG gene, *Dy10* encoding glutenin subunit and introduced into several wheat cultivars (Abdalla, 2007). But even more distant relationships do not prevent a transgene from interfering with endogenous homologues. The *rpgip1* donor species, *R. idaeus* belongs to the Rosaceae family whereas the recipient, *P. sativum* is a member of Fabaceae. Nevertheless, five primer pairs amplified pea homologous sequences instead of the transgene. False-positive results originated from other genome homologues are critical in breeding transgenic crops. A common practice involves transformation of well responding genotypes and then, the transgene is introduced to other breeding lines by ordinary genetic crosses. Different allelic variants of homologues can have different affinity to primers designed on transgenes as well as a transgene may be rearranged during breeding. At this point, one can imagine employing one multiplex for preliminary analyses of the *rpgip1* transgene structure (e.g., set 1) and another to reveal the *rpgip1* transgene, insert sequences and homologues at once (e.g., set 2).

Important outcomes from the present studies is that some primers can identify homologues instead of the *rpgip1* transgene. Expected patterns of the pSCP1 template in addition to primer specificity check and lack of unspecific products by *in silico* PCR enable to exclude the well-known points of consideration for unspecific amplification. What remains contentious is single-primer binding to the DNA template (Ma *et al*., 2011) However, each primer was used in different combinations and only some of them resulted in unspecific amplification. On the other hand, only sequences with few mismatches (1 or 2) at the 3'end of primers can be used for effective PCR. A single mismatch at the last 10 bases can reduce the primer binding and can cause unintended amplification. Although positions of point mutations recognized at the *rpgip1* transgene are apart from primer binding sites, they demonstrate such possibilities. Of course, the prerequisite for unspecific products is the presence of sequences complementary to *rpgip1* primers in the pea genome. Otherwise, the amplification fails giving false-negative results. For plant genomes, highly copied short or long direct repeats can become the target for non-specific amplification. Indeed, four primers showed partial homology to pea retrotransposons. Just as transposons, endogenous *pgip*s and especially their conserved regions are good templates for primers directed towards the transgene. Unexpectedly, *in silico* PCR on two known pea homologues did not confirm this thesis. This simulation inevitably entails the presence of so far unknown *pgip* genes in pea. Plants have evolved many PGIPs differing in inhibition profiles and recognition specificity to counterpart many PGs secreted by pathogens. Obviously, whether observed amplicons represent so far unknown pea *pgip*s or other genomic sequences has to be clarified by sequencing and phylogenetic analyses.

One final point to consider is point mutations that may arise at transgenic loci through successive reproductive generations. Data from *Arabidopsis thaliana* (Papazova *et al*., 2008) and maize MON810 (La Paz *et al*., 2010) suggest the high stability of transgenes and flanking sequences from one side but from the other, an example of oat lines has demonstrated multiple transgene rearrangements, truncated sequences and complex transgene loci (Makarevitch *et al*., 2003). These contrary results can simply mean that transgene behaviour depends on a gene and organism. The *rpgip1* transgene sequenced from the GM peas did not show any large rearrangements apart from a truncated fragment of 46 bp at 5'end. However, this may result from a sequencing procedure and explaining this lack needs further identification of transgene junctions. Noteworthy, two point mutations, one deletion and one substitution were recognized at the *rpgip1* locus in transgenic peas. Likewise, 34 point mutations such as small deletions and base pair substitutions have occurred in the transgene coding region of Roundup Ready soybean transgenic plants during 10 years since their release (Ogasawara *et al*., 2005). In both cases the rate of mutations is comparable, 1.1 x $10^{-3}$ at the *rpgip1* transgene and 0.87 x $10^{-3}$ at a transgene in soybean. Remarkably, the same research on Roundup Ready soybean has demonstrated similar mutation rates (0.9 x $10^{-3}$) at *Cong* gene, the host locus encoding conglycinin storage proteins. According to the neutral theory of molecular evolution, the frequency of mutations at the transgene should be comparable with that for host genes but whether or not this hypothesis is true for the *rpgip1* transgene corroborates further comparisons.

To conclude, using several pairs of primers targeted towards different fragments of the *rpgip1* transgene is an advantage over a single pair because at once they identify the transgene, distinguish between the transgene and endogenous homologues as well as enable to avoid false positive results due to contamination from donor species genes. Mutliplex assays provide further cost effective simplification of the procedure. Mutations at transgenic loci may complicate GMO identification but they do not seem to be more frequent than at host genes.

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# Mutagenic treatment induces high transposon variation in barley (*Hordeum vulgare* L.)

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## ABSTRACT

With more than 2700 mutant-derived cultivars, mutation techniques belong to the most efficient breeding methods. Despite a relatively good understanding of mutagenesis there is no agreement about the range of genome changes in mutants. Visualizing the gain and the loss of transposon insertion sites in SSAP profiles, the present studies aimed to answer whether or not mutants can be regarded as near isogenic lines with respect to their parent cultivar. Activities of *BARE-1* retrotransposon and *Tpo1*-like DNA transposon from the CACTA superfamily were analysed in ten barley mutants derived by mutagenic treatment of two cultivars, Brenda and Scarlett. A large number and proportion of mutations (on average 21.4 mutations encompassing 19.7% loci) confirm high efficiency of applied mutagens. However, differences exist among both cultivars and transposons. The lack of clear correlation between transposon activities and morphology reflects different mechanisms shaping the mutant architecture. With respect to a transposon type, *BARE-1* and *Tpo1*-like were equally active in inducing mutations. However, their activity differs in that the former was mainly responsible for new insertions while the latter equally for insertions and deletions. An excess of new insertions over recombinational loss suggests a transposon burst as a response to stress caused by chemical mutagens. Low estimations of Nei's similarities, well within the range of semispecies demonstrate the role of mutagenic factors in diversification of populations. This way mutagenic treatment not only provides rough breeding materials but also can be used as a model in evolutionary studies.

**Key words:** Induced mutants, SSAP, *BARE-1* retrotransposon, CACTA transposon, genetic similarity

## IZVLEČEK

### MUTAGENO TRETIRANJE POVZROČA VISOKO VARIABILNOST TRANSPOZONOV PRI JEČMENU (*Hordeum vulgare* L.)

Pri več kot 2700 kultivarjih gojenih rastlin so bili mutanti izhodišče za požlahtnitev, torej je induciranje mutacij ena od učinkovitih metod žlahtnjenja rastlin. Kljub razmeroma dobremu razumevanju procesa mutageneze ni soglasja o tem, kakšen je pri mutantih obseg sprememb genoma. S prikazom pridobitve ali izgube na mestih insercije transpozonov pri SSAP profilih poskuša ta raziskava pojasniti, če so mutanti skoro izogene linije v primerjavi z izhodiščnimi kultivarji. Aktivnost retrotranspozona *BARE-1* in transpozonu *Tpo1* podobnega iz superdružine CACTA je bila raziskana pri desetih mutantih ječmena, dobljenih z mutagenim tretiranjem dveh kultivarjev, Brenda in Scarlett. Veliko število mutacij in njihov delež (v povprečju 21,4 mutacij na 19,7% lokusih) potrjuje učinkovitost uporabljenih mutagenov. Toda med obema kultivarjema in transpozoni so razlike. Ugotovljeno je, da ni jasne povezave med aktivnostjo transpozonov in morfologijo, kar se odraža v razlikah v mehanizmu formiranja oblik pri mutantih. Glede na vrsto transpozona sta *BARE-1* in *Tpo1*-u sličen enako aktivna pri induciranju mutacij. Toda so razlike v aktivnosti glede na to, da prvo navedeni povzroča predvsem nove insercije, medtem ko drugi povzroča tako insercije kot delecije. Višek novih insercij v primerjavi z rekombinacijsko izgubo nakazuje, da je nastanek transpozonov povzročen s stresom, ki ga povzročijo kemični mutageni. Nizka ocena podobnosti po Nei-u, ki je v okviru podobne kot pri podvrstah, kaže na vlogo mutagenih dejavnikov pri diverzifikaciji populacij. Na ta način mutageno obravnavanje ne daje samo izhodiščnega materiala za žlahtnjenje rastlin, ampak je lahko uporabno tudi kot model v evolucijskih raziskavah.

**Key words:** inducirane mutacije, SSAP, *BARE-1* retrotranspozon, CACTA transpozon, genetska podobnost

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## 1 INTRODUCTION

Mutation techniques employing chemical or physical agents belong to the most cost effective, simple and uncontroversial methods for changing plant traits. Today more than 2700 mutant-derived cultivars are registered in the FAO/IAEA Mutant Germplasm Repository (2006). Increased yields and enhanced quality of mutation-derived cultivars have made a considerable impact on world food security and economy. Outstanding examples involve Zhefu 802 in rice, Jauhar 78 and Soghat 90 in wheat, NIAB-78 in cotton. It is also worth to mention barley cultivars, Diamant, Golden Promise and their 150 derivatives that have added billion of dollars to the value of the European brewing industry (Ahloowalia *et al*., 2004). In addition, induced mutations offer the unique features in gene discovery and functional genomic programmes. For instance, a key gene responsible for domestication of maize, *teosinte branched1* (*tb1*) was identified in a recessive mutant of maize resembling the nearest close relatives, annual teosinte (Hubbard *et al.,* 2002).

For research purposes, chemical mutagens such EMS (ethylmethane sulphonate), MNH (N-methyl-N-nitrosourea) and $NaN_3$ (sodium azide) are the most widely used because they induce highly required mutations at single nucleotide pairs. Despite a relatively good understanding of this process there is no agreement about the range of genome changes in mutants. Mutations are induced with the frequency $10^{-5}$-$10^{-3}$ per locus, what implies that a mutant at a given locus can be found in a population of at least one thousand $M_1$ plants. This probability is much lower when two or more mutations are searched. Hence, a commonly held view is that mutants and their parent cultivar are similar to near isogenic lines, differing in a limited number of mutated loci (Maluszynski *et al*., 2001). Even though initial variation is greater, selection is believed to fixate mutated alleles as exemplified by semi-dwarfism alleles such as *sd1* in rice (Rutger, 2009) or *Rht* in wheat (Konzak, 1988). Additional yield changes of these mutants have often been explained as pleiotropic effects of mutated alleles. Recent studies showing that both the rice *sd1* and wheat *Rht* genes encode defective enzymes in the GA-biosynthetic pathway (Hedden, 2003) seem to provide strong evidence for this view.

A counterproposal frequently expressed is that during induced mutagenesis a lot of changes are generated in one nucleus and even though the selection is directed towards a certain trait, finally mutants differ by hundreds of mutated genes. Because these mutations are hardly visible in ordinary morphological screening, they have been described as background mutations. Great variation of quantitative traits commonly observed in induced mutants (Kulkarni *et al*., 1999; Barshile and Apparao, 2009; Sial *et al*., 2010) in addition to heterosis effects in crosses involving mutants from the same parent cultivar (or mutants and its parent cultivar) weight in favour of the high mutant diversity (Polok *et al*., 1997). Why then, can not these mutations be revealed by molecular techniques? In fact, microarray analyses have demonstrated that ionizing radiation may change expressions of more than 11 thousand genes (Batista *et al*., 2008). Unfortunately, there is a lack of systematic studies on the frequency of mutations induced by different mutagens at the DNA level. Presumably, breeding goals underlie behind a common drive for approaches detecting mutations in genes of particular economic interest.

Mutations are induced randomly in plant genomes, in which genes are only a tiny portion. In cereals, up to 80% of genomic DNA consists of repetitive DNA, mainly LTR retrotransposons (Devos, 2010). Being highly abundant and evenly distributed in plant genomes, transposons are good targets for studying mutation frequency in induced mutants. Furthermore, many mutants appear to result from transposon insertions such as albino rice plants (Fujino *et al.,* 2005) or barley forms differing in susceptibility to powdery mildew (Wei *et al*., 2002). Mutations originated from transposon movements are easy to follow using Sequence Specific Amplification Polymorphism (SSAP). In the SSAP, products are derived from a DNA fragment between transposon sequences and restriction sites in the flanking sequences (Polok, 2007). When a transposon has integrated into a new site, it will appear as a polymorphic band present only in plants, in which the insertion has taken place. Barley (*Hordeum vulg*are L.), with a genome of ca. 5000 Mb comprising mainly transposon sequences (e.g., *BARE-1*, *BAGY*, *MITE*, *Nikita Sukkula*, *Sabrina*) is a suitable model for studying effects of mutagenic treatments. Among retrotransposons, *BARE-1* elements occupy approximately 9.6% of the barley genome (Soleimani *et al*., 2006). There is no estimations of the DNA transposons' content in barley, nevertheless they can make up from 4.8% to 13.7% of cereal genomes (Devos, 2010).

In the present studies changes in a number of transposon insertion sites in barley mutants were estimated in order to answer whether mutants can be regarded as near isogenic lines with respect to their parent cultivar or perhaps they carry a lot of mutations and hence, they are highly diverged. Moreover, the behaviour of retrotransposons and DNA transposons in response to mutagenic treatment was compared. Diversity of induced mutants was also compared with inter- and intraspecific diversities of natural populations.

## 2 MATERIALS AND METHODS

### 2.1 Plant Material

Ten mutants of *Hordeum vulgare* (L.) from the collection of the Department of Genetics, University of Warmia and Mazury were used in assessing transposon variation. These mutants were selected on the basis of their large morphological changes. Mutants were derived from two spring cultivars (parent cultivars), Brenda and Scarlett by double treatment of seeds with MNH and $NaN_3$. All mutants represented stable, advanced generations, at least $M_8$. **Mutants from Brenda:** 011B – stiff straw, dark leaves, shorter spikes, roots shorter and yield lower than in Brenda; 033B – semi-dwarf, stiff straw, spotted leaves (yellow and purple), short leaves, shorter roots and lower yield than in Brenda; 051B – taller than Brenda, purple leaf tips, short, thick leaves, yield higher than in Brenda; 052B – light green leaves, yield comparable with Brenda; 054B – *brachytic* type; 070B – semi-dwarf, shorter roots and lower yield than in Brenda. **Mutants from Scarlett:** 016S – dwarf, stiff and very short leaves, low yield; 026S – semi-dwarf, prostrate, twisted awns, short roots, very low yield, late; 029S – *tigrina*, low yield; 038S – dwarf, spots on leaves resembling disease symptoms, very low yield.

Plant genomic DNA was extracted from 1 g of 4-week old leaves using modified CTAB method (Polok, 2007). For each genotype 10 plants were used. The purity of DNA samples ranged between 89% - 93% and the quantity between 61 - 237 µg.

### 2.2 Transposon analysis by SSAP

The SSAP methodology combines the general principle of AFLP with the sequence-specific PCR. This approach is similar to AFLP in that genomic DNA is digested with restriction enzymes, enzyme-specific adapters are ligated to the restriction products and the resultant fragments are pre-amplified. The final step, selective amplification uses a single adapter-specific primer together with a transposon-specific primer. The SSAP method as described by Polok (2007) was employed with the following modifications. Briefly, 360 ng DNA was digested with *Mse*I (3 U) and *Pst*I (3 U) and ligated to enzyme specific adapters. Ligation mixtures were diluted 10-fold to obtain final DNA concentration of 1 ng/µl. Pre-amplifications were carried out in a 15 µl final volume containing 20 mM $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 9.0 at 25°C, 2.0 mM $MgCl_2$, 1.5 µl of Enhancer with betaine (Epicentre Technology), 200 µM dNTPs, 0.5 µM primers without selective nucleotides (Mse0, Pst0), 0.5 U of *Tfl* polymerase (Epicentre Technology) and 5 µl of diluted ligation mixtures (equal to 5 ng of DNA). Thermal conditions were: 94°C for 3 min, followed by 30 cycles of 94°C for 60 s, 60°C for 60 s, 72°C for 60 s. The final extension was at 72°C for 7 min. Pre-amplifications were diluted 5-fold and used for selective amplifications using an extended *Pst*I primer (Pst-AT) in combinations with a transposon based primer. Selective amplifications were performed in a 10 µl final volume containing 20 mM $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 9.0 at 25°C, 1.5 mM $MgCl_2$, 1 µl of Enhancer with betaine (Epicentre Technology), 250 µM dNTPs, 0.5 µM Pst-AT primer, 0.5 µM transposon specific primer, 0.5 U of *Tfl* polymerase (Epicentre Technology) and 2.5 µl of diluted preamplified DNA. Thermal conditions were: 12 cycles of 94°C for 30 s, a touchdown phase starting at 65°C for 30 s with decrements of 0.7°C per cycle, and 72°C for 60 s; then 28 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s. Products of selective amplifications were denatured with 8 µl of 98% formamide at 94°C for 7 min, loaded on 6% denaturing polyacrylamide gels and separated at 45 W of constant power up to Bromophenol Blue run out of the gel. The gels were silver stained at 4°C, dried, scanned using a flatbed scanner and pictures were stored as .jpg files.

Following primers were used in the SSAP:
**SSAP standard primers. Mse adapters:** MseF: 5'GACGATGAGTCCTGAG3', MseR: 5'TACTCAGGACTCAT3'; **Pst adapters:** PstF: 5'CTCGTAGACTGCGTACATGCA3', PstR: 5'TGTACGCAGTCTAC3'; **pre-amplification primers:** Mse0: 5'GATGAGTCCTGAGTAA3', Pst0: 5'GACTGCGTACATGCA G3'; **primers with selective 3' bases:** Pst-AT - 5'GACTGC GTACATGCAG**AT**3'.
**Transposon based primers. *BARE-1*:** 5'CTAGGGCATAATTCCAACAA3' - primer specific to the family of *BARE-1* retrotransposons belonging to Ty1-*copia* like elements; ***Tpo1*-like:** 5'GATTGCCTTTTTCCTAGTAGTG-3' – primer produced from a terminal sequence of the *L. perenne* DNA transposon, *Tpo1* belonging to the CACTA superfamily and highly abundant in grass genomes.

### 2.3 Data analysis

All bands that could be reliably read were scored either presence of a transposon insertion (1) or a deletion of a transposon site (0) and defined as follow. **An insertional locus:** a locus, in which a transposon insertion (band) was present in at least one genotype from a given combination (cultivar plus its mutants). **Number of insertions:** in a given genotype, total number of insertional loci, in which a transposon (band) was present. **New insertion**: a band was present in a mutant but no in its parent cultivar. **Deletion:** a band was present in a parent cultivar and lacking in its mutant. Total number of mutations was calculated by summarizing new insertions and deletions. The relative frequency of mutations was estimated as the percentage of mutations in relation to a total number of insertional loci observed in a given combination.

Factor ANOVA was used to check for cultivar, transposon type and interaction effects. The LSD test (Least Significant Differences) was used to examine differences between all possible pairs of means. All data were standardized and used in PCA (Principal Component Analysis). Genetic identities and distances were determined with the Nei and Li formula (Nei and Li, 1979). STATISTICA 9.0 software with own macros was used in calculations.

## 3 RESULTS

### 3.1 Insertional loci in Brenda and Scarlett genotypes revealed by SSAP

Two transposons were used in SSAP reactions, namely *BARE-1* belonging to the Ty1-*copia*-like retrotransposons and *Tpo1*, a member of the CACTA superfamily of DNA transposons. The first element is the best characterized retrotransposon in barley, thus it was not surprising that it produced high quality fingerprints. The *Tpo1*-like specific primer was designed on the *L. perenne Tpo1* template (Polok, 2007). This primer produced a great number of bands in SSAP fingerprints confirming high abundance of *Tpo1*-like transposons in barley. Both transposons were very effective in revealing insertional loci as confirmed by 110 loci observed in Brenda genotypes and 107 insertional loci in a case of Scarlett genotypes. Intra-cultivar variation was not observed what confirmed their uniformity. When cultivars were taken together with their mutants, high polymorphism was observed (Fig. 1). In total 41 insertional loci were polymorphic in Brenda, which was 37%. In Scarlett, respective values were 46 loci and 43%.

### 3.2 Mutation frequency in induced barley mutants

Due to the uniformity of parent cultivars, a significant degree of polymorphism was assumed to result from mutation events. Two types of changes in SSAP profiles were observed in mutants i.e., new bands not present in a respective parent cultivar and the loss of bands (Fig. 1). The large average number of mutations (21.4), encompassing 19.7% indicated high efficiency of applied mutagens in inducing point mutations (Table 1). However, differences existed among both cultivars and transposons. Brenda seemed to be more resistant than Scarlett as emphasized by the smaller average number of SSAP changes (17.7 and 26.5, respectively) and the lower overall proportion of mutated loci (16% and 24%). This observation is in agreement with well known dependence of mutagen efficiency on genetic background.

**Table 1.** Transposon-derived mutations in barley mutants.

| | Total | | | *BARE-1* | | | *Tpo1*-like | | |
|---|---|---|---|---|---|---|---|---|---|
| Mutant line | N° of loci | Mutated loci | | N° of loci | Mutated loci | | N° of loci | Mutated loci | |
| | | [n] | [%] | | [n] | [%] | | [n] | [%] |
| Brenda | | | | | | | | | |
| 011B | 110 | 15 | 13.6 | 60 | 3 | 4.9 | 50 | 12 | 24.0 |
| 033B | | 12 | 10.9 | | 6 | 9.8 | | 6 | 12.0 |
| 051B | | 16 | 14.5 | | 10 | 16.4 | | 6 | 12.0 |
| 052B | | 16 | 14.5 | | 8 | 13.1 | | 8 | 16.0 |
| 054B | | 20 | 18.2 | | 10 | 16.4 | | 10 | 20.0 |
| 070B | | 27 | 24.5 | | 13 | 21.3 | | 14 | 28.0 |
| Mean$_{Brenda}$<br>±SD | | 17.7a<br>±1.8 | 15.9a<br>±1.6 | | 8.3a<br>±1.3 | 13.7a<br>±2.3 | | 9.3<br>±1.3 | 18.5<br>±2.3 |
| Scarlett | | | | | | | | | |
| 016S | 107 | 29 | 27.1 | 66 | 24 | 36.4 | 41 | 5 | 12.2 |
| 026S | | 26 | 24.3 | | 18 | 27.3 | | 8 | 19.5 |
| 029S | | 28 | 26.2 | | 19 | 28.8 | | 9 | 21.9 |
| 038S | | 25 | 23.3 | | 16 | 24.2 | | 9 | 21.9 |
| Mean$_{Scarlett}$<br>±SD | | 26.5b<br>±2.2 | 24.4b<br>±1.9 | | **19.3**b [B]<br>±1.6 | **29.1**b [B]<br>±2.8 | | **7.8** [A]<br>±1.6 | 18.8 [A]<br>±2.8 |
| Mean$_{All}$<br>±SD | | 21.4<br>±6.3 | 19.7<br>±6.0 | | 12.7<br>±6.5 | 19.8<br>±9.5 | | 8.7±<br>2.8 | 18.7<br>±5.4 |

Different letters mean significant differences between means of Brenda and Scarlett mutants at P = 0.05. Different capitals in brackets mean significant differences between means of *BARE-1* and *Tpo1*-like at P=0.05, Mean$_{All}$ – an average of all ten mutants.

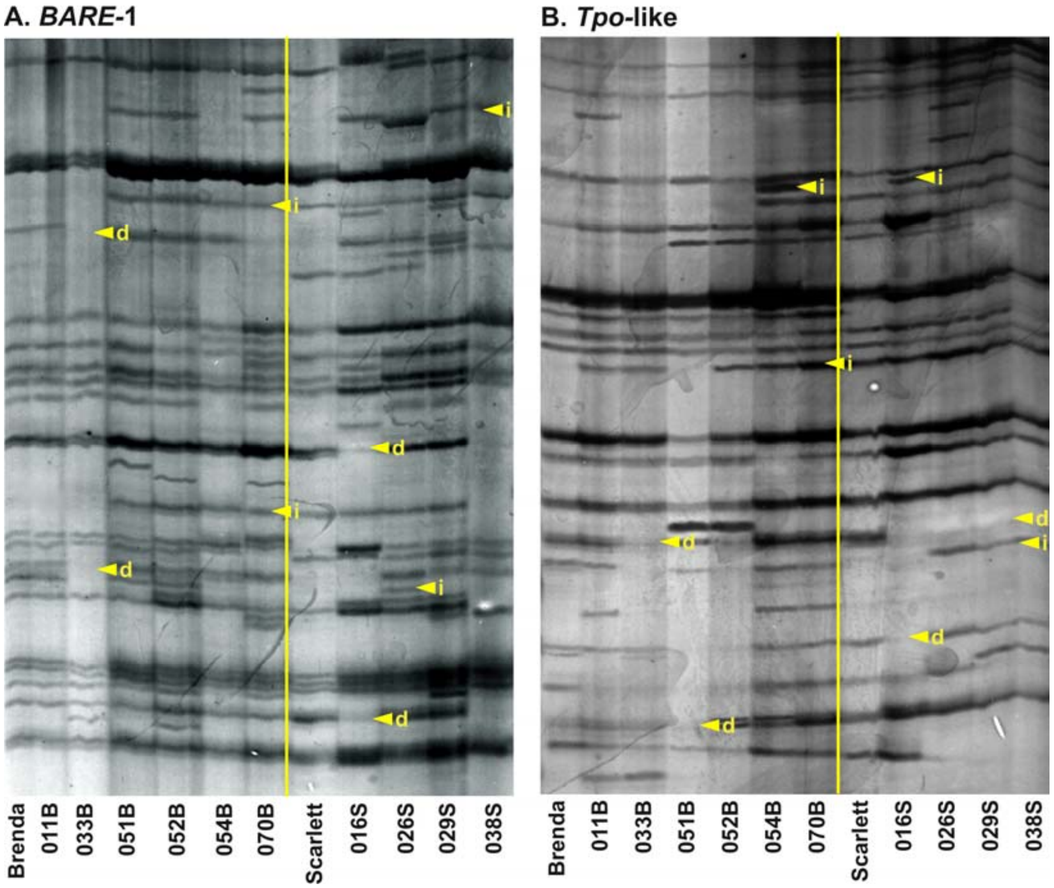

**Fig. 1.** SSAP fingerprints demonstrating insertion sites of *BARE-1* (A) and *Tpo1*-like (B) transposons in barley mutants. Arrows indicate examples of new insertions (i) and deletions (d).

With respect to a transposon type, *BARE-1* and *Tpo1*-like were equally active in inducing mutations. Profiles of both transposons were characterized by similar number of mutated loci and their proportion (Table 1). However, *BARE-1* fingerprints of Scarlett mutants were unusual in possessing the largest number and the largest proportion of recorded mutations (on average 19.3 mutations and 29.1%). Noteworthy, in the *Tpo1*-like case, again genetic background proved to play a role. A general trend that Scarlett mutants carried more mutations than Brenda's was not obvious for *Tpo1*-like as demonstrated by 9.3 mutations in Brenda and 7.8 in Scarlett as well as respective proportions 18.5% and 18.8%.

Mutants harboured from 12 to 29 mutations, which corresponded to 11% - 27%. Nevertheless, no clear correlations among mutation abundance and morphological changes were found. A largest number i.e., 25 and more mutations, and a similarly high percentage (above 23%) were typical of all Scarlett mutants in addition to 070B (Fig. 2). This agreed well with numerous morphological changes in mutants with the exception of 070B differing from Brenda only in quantitative traits. Intermediate position was occupied by a *brachytic* type mutant, 054B with 20 mutations (18%). This may indicate that most changes in leaf, spike, grain and root morphology accompanying by the *brachytic* mutation are partly due to pleiotropic effects. The lowest number (12 - 16) and proportion (11% - 15%) of mutations were discovered in a group of four Brenda mutants, 011B, 033B, 051B and 052B. This was surprising because all of them expressed large degenerative traits including colour spots on leaves, light green leaves, aberrant leaf and root morphology and had been expected to carry many mutations.

**Table 2.** Activity of *BARE-1* and *Tpo1*-like transposons based on the gain and loss of SSAP insertion sites.

| Mutant line | Total | | | *BARE-1* | | | *Tpo1*-like | | |
|---|---|---|---|---|---|---|---|---|---|
| | Insertion N$^{o}$ | New Insertions | Deletions | Insertion N$^{o}$ | New Insertions | Deletions | Insertion N$^{o}$ | New Insertions | Deletions |
| Brenda | 84 | | | 46 | | | 38 | | |
| 011B | 87 | 9 | 6 | 49 | 3 | 0 | 38 | 6 | 6 |
| 033B | 80 | 4 | 8 | 44 | 2 | 4 | 36 | 2 | 4 |
| 051B | 92 | 12 | 4 | 54 | 9 | 1 | 38 | 3 | 3 |
| 052B | 90 | 11 | 5 | 52 | 7 | 1 | 38 | 4 | 4 |
| 054B | 93 | 12 | 8 | 50 | 7 | 3 | 43 | 5 | 5 |
| 070B | 95 | 19 | 8 | 53 | 10 | 3 | 42 | 9 | 5 |
| Mean$_{\text{Brenda}}$ | 89.5 | 11.2 | 6.5a | **50.3** [B] | 6.3a | **2.0**a [A] | **39.2** [A] | 4.8 | 4.5 [B] |
| ±SD | +2.9 | ±2.0 | ±1.1 | ±1.8 | ±1.3 | ±0.7 | ±1.8 | ±1.3 | ±0.7 |
| Scarlett | 83 | | | 45 | | | 36 | | |
| 016S | 94 | 20 | 9 | 57 | 18 | 6 | 37 | 2 | 3 |
| 026S | 91 | 17 | 9 | 55 | 14 | 4 | 36 | 3 | 5 |
| 029S | 91 | 17 | 11 | 58 | 15 | 4 | 33 | 2 | 7 |
| 038S | 74 | 8 | 17 | 41 | 6 | 10 | 33 | 2 | 7 |
| Mean$_{\text{Scarlett}}$ | 87.5 | 15.5 | 11.5b | 52.8 [B] | **13.3**b [B] | 6.0b | 34.8 [A] | 2.3 [A] | 5.5 |
| ±SD | +3.5 | ±2.5 | ±1.4 | ±2.2 | ±1.6 | ±0.9 | ±2.2 | ±1.6 | ±0.9 |
| Mean$_{\text{All}}$ | 88.7 | 12.9 | 8.5 | **51.3** [B] | **9.1** [B] | 3.6 | **37.4** [A] | 3.8 [A] | 4.9 |
| ±SD | ±6.7 | ±5.2 | ±3.6 | ±5.5 | ±5.2 | ±2.9 | ±3.3 | ±2.3 | ±1.5 |

Different letters mean significant differences between means of Brenda and Scarlett mutants at P = 0.05. Different capitals in brackets mean significant differences between means of *BARE-1* and *Tpo1*-like at P=0.05, Mean$_{\text{All}}$ – an average of all ten mutants.

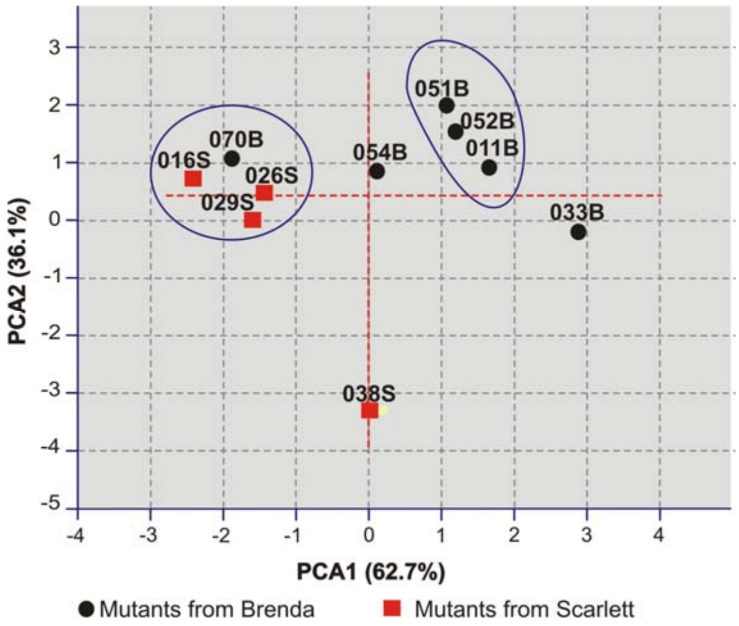

**Fig. 2.** Relationships of ten barley mutants revealed by the PCA analysis of SSAP data derived from *BARE-1* and *Tpo1*-like transposons.

### 3.3 Assessment of *BARE-1* and *Tpo1*-like activity after mutagenic treatment

Visualizing the gain and the loss of fragments, SSAP fingerprints allowed to detect reorganisation in transposon fraction in response to mutagenic treatment. Almost identical number of insertion sites (84 and 83) in cultivars did not evidence any unusual transposon activities. Interestingly, a number of SSAP fragments increased in mutants (on average 89) indicating that new insertion sites appeared (Table 2). Likely, these new insertion sites were indicative of transposition. Comparable results were obtained for Brenda and Scarlett mutants with respect to insertion sites and new insertions. Inspecting new insertions, in particular *BARE-1* seemed to rise more rapidly than *Tpo1*-like (9.1 and 3.8 new insertions, respectively). In Scarlett mutants, *BARE-1* proliferated even five-fold faster than *Tpo1*-like. Among mutants, numerous transposition events were detected in three mutants, 016S, 026S and 029S (14-18 new insertions).

Further rearrangements in transposon sequences were indicated by SSAP fragment losses. On average, 8.5 deletions were observed but they were more frequent in Scarlett mutants with the maximum in 038S (17 deletions). Despite *BARE-1* is a retrotransposon while *Tpo1*-like is a DNA transposon, their deletions were equally frequent (3.6 and 4.9). Relative ratios of new insertions and deletions inform about transposon contribution to an increase of genome sizes. An excess of *BARE-1* new insertions over deletions (250% more) suggested *BARE-1* specific genome rearrangements associated with mutagenic treatment. Recombinational loss is a major mechanism protecting genome from a retrotransposon burst. The more insertions in relation to the loss the more active are transposons. Thus, *BARE-1* was highly active in eight mutants with the highest activity in 051B and 052B as demonstrated by seven to nine-fold more insertions. At this point, 033B and 038S mutants with inverted relationships (twice more *BARE-1* deletions than insertions) were unusual. Their distinctiveness was also clear in the PCA plot (Fig. 2). Presumably, recombination processes acted against a transposon burst in these mutants. Expectedly, a balance between new insertions and deletions was generally noted for the DNA transposon, *Tpo1*-like (3.8 insertions vs. 4.9 deletions). Exceptions involved three Scarlett mutants with fewer insertions then deletions.

### 3.4 SSAP diversity of induced barley mutants

Estimates of Nei’s genetic similarities between mutants and their parent cultivars were relatively low, well within the range of values normally associated with species at the early stages of divergence (Table 3). As expected, given the mutation numbers, Brenda mutants with the average Nei’s index, 0.898 were more similar to their parent cultivar than Scarlett ones with the value of, 0.839. These estimates classified barley mutants as semispecies (Brenda’s) or even subspecies (Scarlett’s). Such an observation demonstrated the role of transposons activation through mutagenic treatment in population divergence.

**Table 3.** Nei’s genetic similarities and distances between barley mutants and their parent cultivar assessed by *BARE-1* and *Tpo1*-like transposons.

| | Brenda | | | | | | Scarlett | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 011B | 033B | 051B | 052B | 054B | 070B | 016S | 026S | 029S | 038S |
| Similarity | 0.912 | 0.923 | 0.909 | 0.908 | 0.884 | 0.849 | 0.836 | 0.851 | 0.828 | 0.841 |
| Distance | 0.092 | 0.080 | 0.095 | 0.097 | 0.123 | 0.164 | 0.179 | 0.161 | 0.188 | 0.173 |
| Common similarity values (Polok, 2007) | | | | | | | | | | |
| Populations | >0.925 | | | | | | | | | |
| Semispecies | >0.873 | | | | | | | | | |
| Subspecies | >0.759 | | | | | | | | | |

## 4 DISCUSSION

It has been long suggested that mutagenic treatment can cause large diversity in plant genomes. Difficulties in mutant distinguishing on molecular levels and a huge number of mutant-derived cultivars differing only in a trait of interest, all these have seemed to confirm that mutants are alike near isogenic lines. This view has been dominant for years despite many obvious contrasting arguments including high variation of quantitative characters. The situation has only started to change recently when high-throughput molecular technologies targeted at different genomic elements became available. Mutation densities from one per 140 kb in *Arabidopsis* to one per 400 kb in maize discovered by TILLING (Targeting Induced Local Lesions In Genomes) are only some of early examples questioning the near isogenic status of induced mutants. In the barley case, these values correspond to approximately 197 mutated genes per diploid genome (Till *et al*., 2004; Talame *et al.,* 2008).

The overall picture from the present analyses is that mutagenic treatment induces huge genome changes likely driven by activation of transposons. Revealed SSAP fingerprints are characterized by more than a hundred of insertional loci of which about 40% are polymorphic among mutants and their parent cultivar. Mutants had up to 27% mutated loci what is much higher than it has been thought before. Given these numbers in gene equivalents, they are equal to nearly 10000 mutated genes assuming 53 453 as the estimated gene number in barley (Sreenivasulu *et al*., 2008). This implies 50-fold more mutated genes in comparison with TILLING data (Talame *et al*., 2008) but it is consistent with the transcriptional changes in 11000 genes of irradiated rice mutants (Batista *et al*., 2008). Although demonstrative, this comparison may be somehow biased because the TILLING strategy is aimed at rapid identification of allelic series with point mutations at coding gene segments up to 1 kb – 1.5 kb (Till *et al*., 2004). Conversely, SSAP profiles inform about the sites in whole genomes, in which transposons are inserted. It should be pointed however, that transposon insertions are coupled with point mutations at target sites.

New insertion sites in barley mutants suggest that *BARE-1* and *Tpo1*-like transposons have played a role in inducing some morphological changes. Presumably, an insertion itself or resultant point mutations underlie behind at least some of morphological aberrations in barley mutants, for instance colour spots on leaves (033B, 038S). This hypothesis has strong roots in transposon insertions responsible for different expression of *a1* alleles encoding purple anthocyanin pigments in maize (Pooma *et al*., 2002). In rice, the 607 bp transposable element that inserted into the gene encoding Mg-protoporphyrin IX methyltransferase, generated an 8 bp target site duplication and finally an albino mutation (Fujino *et al*., 2005). Similar explanation may be valid for a *tigrina* mutant (029S) derived from Scarlett.

A potential difficulty arising from the "transposon-origin of morphological mutants" is the lack of clear connections between insertional polymorphism and morphological phenotypes. From one side, all Scarlett mutants characterized by large morphological changes carry highest numbers of mutations but this is not true for Brenda mutants. Surprisingly, the highest proportion of mutated loci was a feature of the 070B mutant, which otherwise do not differ in major traits from Brenda. In this case, because differences are rather quantitative than qualitative, transposon insertions could take place in genes responsible for quantitative traits. This pattern explains well background mutations earlier postulated on the basis of significant differences in yield parameters in induced mutants as well as heterosis effects in crosses involving mutants originated from the same parent cultivar. Among postulated reasons dominant mutations increasing values of yield parameters and new interactions among non-allelic genes have been assumed (Polok *et al*., 1997; Polok, 1997). Hypothesis of transposon origin of mutations in genes controlling quantitative characters complements results from other plants. Eight from ten yield enhancing QTLs (Quantitative Trait Loci) from rice contain transposons from the CACTA superfamily and all classes of retrotransposons (Reddy *et al.,* 2006). An intriguing association between QTLs and *Tpo1* insertions as well as Ty1-*copia* like elements is observed on the *Lolium perenne* x *L. multiflorum* genetic map (Polok, 2007). Although a transposon role in shaping plant architecture needs further experimentations, the present studies demonstrate that new techniques may provide new insights into old phenomena.

Particularly intriguing is the presence of active transposons in barley mutants as deduced from high frequency of mutations (new insertions and deletions). Transpositional activity of mobile elements has persisted at a high level for million years in multiple cereal species (Leigh *et al.,* 2003). This implies that transposons have a potential to move under certain conditions. For instance, drought stress in Evolution Canyon favours an increase in the *BARE-1* copy number and a simultaneous decrease in the relative lost in *Hordeum spontaneum* (Kalendar *et al*., 2000). Similarly, retrotransposons are activated in wheat under *Fusarium* stress (Ansari *et al*., 2007). Then, why transposons could not be mobilised in response to mutagenic treatments. Postulated mechanisms may resemble these observed in yeasts, in which chemical mutagens activate a Ty1 retrotransposon in two ways, by direct DNA damaging effects and increased levels of reactive oxygen species (ROS). The last seems to play the major role in Ty1 mobilisation (Stoycheva, 2009). The activation of transposons from the CACTA superfamily in rice mutants (Batista *et al.,* 2008) suggests that DNA transposons may also be activated by mutagenic treatments. However, this process may depend on background genotypes as deduced from different level of insertional mutations in barley cultivars. It can not be excluded that variation in sensitivity of different genotypes to mutagenic treatment is connected with different activities of transposons. However, this hypothesis needs more cultivars and mutants to study.

The transposons, *BARE-1* and *Tpo1*-like are distinct in their pattern of mobility after mutagenic treatment. This results from the transposon nature itself as well as from differences in their activity. Certainly, more insertion sites followed by more new insertions and mutated loci in a case of *BARE-1* are related to extremely high abundance of this retrotransposon in barley genome (Leigh *et al*., 2003). In contrast, DNA transposons, to which the *Tpo1*-like element belongs, are several times less frequent in plant genomes. A fact that there is only 27% less insertion sites than for *BARE-1*, the *Tpo1*-like transposon owes the membership to the CACTA superfamily, relatively abundant in cereals. An excess of new insertion sites over deletions in a case of *BARE-1* is attributed to the “copy and past” mechanism of retrotransposon movement while deletions can be explained by recombinational loss. By contrary, the DNA, *Tpo1*-like elements transpose through the “cut and past” mechanism, which is reflected by a balance among new insertions and deletions. Notwithstanding differences between *BARE-1* and *Tpo1*-like elements, they seem to be equally active in response to mutagenic treatments as similar frequency of mutations suggests.

And last but not least, the present data demonstrate that mutant diversity is comparable with differences at the early stages of speciation. When applying commonly used similarity index, Brenda mutants could be classified as semispecies while Scarlett ones even as subspecies. This is not to say that we should treat them as new species. Induced mutants still belong to a species from which they are derived. This is rather a suggestion that induced mutants can provide a good model to study evolution, speciation and role of transposons in these processes. Frequency of spontaneous mutations in natural populations is believed to be higher at earlier stages of evolution. Similarly, many transposons, mobile in the past are stable at present. There are many uncertainties how the biological diversity has been created. Obviously, natural environment now and in the past has been a stressor to which plants would have had to adapt. This depends on genome adaptive abilities which are demonstrated by different responses of cultivars to chemical mutagens. Similarities of processes in the present and Evolution Canyon studies (Kalendar *et al*., 2000) rely on activation of transposons as adaptation to stress. This way mutagenic treatment can provide not only better crops but also mimic selective forces operating on a plant level.

## 5 CONCLUSIONS

1. High proportions of mutations in induced barley mutants deny their near-isogenic stage and confirm the view that a lot of mutations are induced during mutagenic treatment. Some of them can result from direct damages of DNA, however, the majority can be attributed to transposons’ activation in response to stress.

2. Transposons can be distinct in their pattern of activity, but the majority of differences mirror differences between retrotransposons and DNA transposons.

3. The activity of transposons after mutagenic treatment can lead to substantial diversity of mutants, comparable to differences between species at early stages of divergence. This result confirms the role of transposons in speciation.

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**Agris category code**: F61, Q04

# Selenium uptake and distribution in chicory (C*ichorium intybus* L.) grown in an aeroponic system

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## ABSTRACT

Cultivated plants generally are a poor source of dietary selenium (< 0.1 mg/kg wet weight). In this work the influence of sodium selenate on selenium distribution in the chicory (*Cichorium intybus* L.) cultivars 'Anivip' and 'Monivip' was studied in an aeroponic system in a greenhouse. The plants roots were moistened every fifteen minutes with a Resh nutrient solution with added selenium (10 mg $L^{-1}$) in the form of sodium selenate. The content of total selenium in chicory roots and leaves was studied after different periods of time and different temperatures of exposure to the selenium enriched nutrient solution. In two separate experiments chicory was treated with selenium enriched nutrient solution for 40 days at 10 ºC in the slow growing phase, and for 5, 7 and 10 days at 20 ºC in the developmental phase at the beginning of formation of the flower stem. The concentration in leaves increased with time during plant growth, and in Se treated groups was 370 and 139 mg $kg^{-1}$ in 'Anivip' and 460 and 205 mg $kg^{-1}$ in 'Monivip' leaves after 40 and 10 days of exposure, respectively. A smaller increase in selenium content was obtained in roots, namely to 73 and 46 mg $kg^{-1}$ in 'Anivip' roots and to 87 and 46 mg $kg^{-1}$ in 'Monivip' roots after 40 and 10 days of exposure, respectively. All results are expressed on a lyophilised matter basis. In long and short term treatment, the selenium content in 'Monivip' cultivar was higher than in 'Anivip'. No visible toxic effects on the chicory plants were observed.

**Key words:** aeroponics, chicory, selenium, distribution, uptake

## IZVLEČEK

### PRIVZEM IN PORAZDELITEV SELENA V RADIČU (*Cichorium intybus* L.), GOJENEM V AEROPONSKEM SISTEMU

Gojene rastline so skromen vir selena (<0,1 mg/kg ). Raziskovali smo vpliv selenata na privzem selena in njegovo porazdelitev v radiču (*Cichorium intybus* L.) kultivarjev 'Anivip' in 'Monivip'. Rastline so bile gojene aeroponično v rastlinjaku. Korenine rastlin smo škropili s Reshevo hranilno raztopino, ki je vsebovala 10 mg Se(VI) $L^{-1}$ v obliki natrijevega selenata, vsakih petnajst minut. Naredili smo dve pilotni študiji. V prvi študiji so bile rastline, ki so ob začetku poskusa imele 6 listov, izpostavljene 40 dni hranilni raztopini, ki je vsebovala Se(VI) pri temperaturi 10 $^{0}$C, v drugi pa 5, 7 in 10 dni pri temperaturi 20 $^{0}$C in v razvojni fazi tik pred cvetenjem. Vsebnost Se je bila večja v listih kot v koreninah in je naraščala s časom izpostavljenosti. Masni delež selena v listih kultivarja 'Anivip' se je povečal od 55 na 139 mg $kg^{-1}$ med petim in desetim dnevom izpostavljenosti, v kultivarju 'Monivip' pa od 78 na 205 mg $kg^{-1}$. Vsebnost Se v koreninah je tudi naraščala, vendar so bile razlike med kultivarjema manjše. Pri 40 dnevni izpostavljenosti je bil masni delež Se 370 mg $kg^{-1}$ v listih in 73 mg $kg^{-1}$ v koreninah kultivarja 'Anivip' ter 460 mg $kg^{-1}$ v listih in 87 mg $kg^{-1}$ v koreninah kultivarja 'Monivip'. Vsi rezultati so izraženi na liofilizirano snov. Tako pri dolgi kot pri kratki izpostavljenosti rastlin hranilni raztopini s selenom je bila vsebnost Se v kultivarju Monivip večja kot pri kultivarju Anivip. Na rastlinah nismo opazili znakov zastrupitve.

**Ključne besede:** Aeroponsko gojenje, radič, selen, privzem, porazdelitev

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## INTRODUCTION

Selenium is an essential micronutrient for animals, some prokaryotes and some eukaryotes, but its essentiality for the growth of higher plants has not yet been demonstrated (Ellis and Salt, 2003). Selenium intake in humans is low in many European countries such as Spain (Diaz-Alarcon *et al.*, 1996), Turkey (Foster and Sumar, 1997), Poland (Wasowicz *et al.*, 2003), Austria (Pfannhauser *et al.*, 2000), Slovenia (Pokorn *et al.*, 1998) and Croatia (Matek *et al.*, 2000), and in some other parts of the world such as some regions of China (Combs and Combs, 1986). Plants generally contain low amounts of selenium, and besides this, contents vary depending on the plant species and the selenium level in the soil where they are grown.

Several studies have been carried out dealing with supplementing the amount of selenium in various plants. Poggi *et al.* (2000) studied the effect of foliar spraying of 0, 50 and 150 g of Se ha$^{-1}$ in the form of sodium selenate and sodium selenite on potatoes. The selenium content in such potatoes exposed in this way markedly increased. Wheat, alfalfa and sunflower seeds were germinated for 5 and 7 days in solutions containing 0.78 to 800 mg Se L$^{-1}$ in the form of sodium selenate (Lintschinger *et al.*, 2000). The production of sprouts containing a higher amount of selenium represented an additional increase in the generally high nutritional value of such sprouts. Stadlober *et al.* (2001) investigated if the selenium content of different kinds of cereals could be raised after application of fertilisers containing 30 mg Se kg$^{-1}$ soil as selenate. The selenium content in cereals increased from the range 0.006-0.024 mg kg$^{-1}$ in the control group to the range 0.168-0.238 mg Se kg$^{-1}$ in the exposed group. Elles *et al.* (2000) tested if plants could serve as a source of more available selenium than inorganically based mineral supplements. For this purpose Indian mustard was grown under hydroponic conditions and the accumulated selenium possessed better solubility than mineral supplements. Hu *et al.* (2001) determined the effects of selenium treatments on the sensory and chemical quality of green tea. Sodium selenite and organically bound Se solutions were used as fertilizer sprays. The sensory quality e.g. astringent taste, bitterness, sweetness and aroma, and the chemical quality e.g. vitamin C content, total amino acid and polyphenol content, were significantly improved after treatment with selenium. By increasing the amount of selenium in plants a defensive role against external stress factors (UV-B radiation), a growth promoting response manifested as a decrease in lipid peroxidation, an increase in glutathione peroxidase activity and the enhanced growth of senescent ryegrass (Hartikainen *et al.*, 2000) and lettuce (Xue *et al.*, 2001) were confirmed. Seppänen *et al.* (2003) cited positive effects of selenium on photooxidative stress tolerance in selenium enriched potato.

In the literature only few data on selenium uptake in chicory could be found (Germ *et al.*, 2007). Scotti *et al.* (1999) studied the effect of fly ash on the availability of metals like Zn, Cu, Ni and Cd to chicory.

Chicory has abundant growth and is usually cultivated for the production of inulin from leaves or roots. It is a plant whose roots store inulin with a high fructose content (about 94 %). It is also used as salad during the whole year and has many benefits such as low calorific properties and the ability to regulate intestinal flora (Figueira *et al.*, 2004). It is very appropriate for different types of cultivation such as conventional cultivation, hydroponic and aeroponic cultivation; also after harvest plants can be stripped of their green leaves, stored in the cold and finally forced by warmth and humidity in complete darkness to develop chicory heads. Furthermore, it is insensitive to transport. Its consumption is very popular in Slovenia and in northern part of Italy. In Slovenia in the year 2008 an area of 204 ha was devoted to chicory cultivation and was for 16% bigger than in year 2002. (Statistical Yearbook of the Republic of Slovenia, 2010, http://www.stat.si/letopis/2010/16_10/16-12-10.htm)

The purpose of our work was to study the uptake and distribution of selenium in the chicory cultivars 'Anivip' and 'Monivip' after various periods and at various temperatures of treatment with selenium enriched nutrient solution. These are the two most frequently grown cultivars in Slovenia and are resistant to low temperatures, down to –12 ˚C.

## EXPERIMENTAL

### Plant material and growth conditions

We used the aeroponic system for chicory cultivation because of its many advantages. Aeroponics is a form of hydroponic plant cultivation in which the plant roots are suspended in a closed chamber and misted with a complete nutrient solution. Aeroponics requires no solid or aggregate growing medium and allows for easy access to the roots. The chamber and misting system provide complete control of the root zone environment, including temperature, nutrient level, pH, humidity, misting frequency and duration, and oxygen availability. For preparation of 100 L of Resh nutrient solution, containing the macro- and microelements essential for the growth of leafy vegetables, Fe-EDTA (4.17 g), $CuSO_4{\cdot}5H_2O$ (0.0393 g), $MoO_3$ (0.0075 g), $MnSO_4$ (0.203 g), $ZnSO_4{\cdot}7H_2O$ (0.0044 g), $H_3BO_3$ (0.286 g), $Na_2SO_4$ (1.0 g), $MgSO_4{\cdot}7H_2O$ (40.5 g), $KH_2PO_4$ (22.0 g), $K_2SO_4$ (32.8 g), $Ca(NO_3)_2$ (82.1 g), $NH_4NO_3$ (14.2 g) were used (Resh, 1995). $Na_2SeO_4$ (Sigma) (2.3949 g) was dissolved in water in a 100 mL flask and then transferred a 100 L plastic container, as used in the food industry, to reach a selenium concentration of 10 mg selenium per litre of nutrient solution. Chicory (*Cichorium intybus* L.) cv. 'Monivip' and 'Anivip' (National list of varieties, p. 34-35, 2002, Ministry of Agriculture, Forestry and Food, Republic of Slovenia) were used and two experiments were performed under different climatic conditions and in various developmental phases of the plants.

Long term treatment

Seeds were cultivated in peat (Potgrond H, Klasman, Geeste, Germany) and after 60 days 84 plants were transferred to the aeroponic system, where plants were placed in small pots, fixed with peat, but the roots left free in the air. The roots were sprinkling every 15 minutes with nutrient solution. After 90 days, plants were divided into two groups, 42 plants in control and 42 plants in exposed group. For treated group Se was added to the nutrient solution for the next forty days. At the time of Se addition to the nutrient solution, the plants had approximately 6 leaves. The average temperature in the greenhouse during winter was maintained around 10 ˚C, by additional hot air heating when necessary. The temperature of the nutrient solution was maintained at 8 – 10 ºC. Slow growth occurred because of the low outside temperatures and light conditions in the winter period. At the end of experiment, each plant in the control or in the treatment group had approximately 10-12 leaves. 6 plants of each cultivar were taken for analysis. The nutrient solution in the plastic vessel was sampled on the first day and at the end of the trial.

Short term treatment

Plants were grown in the same way as in the long term experiment. Stone wool was used for fixation of plants. At the beginning of the short term treatment, the seedlings had been 180 days in the aeroponic system and 14 plants was used for each type of treatment. They had approximately 16 leaves and were in the development phase at the beginning of formation of the flower stem when selenate was added to the nutrient solution for 5, 7 and 10 days. The average temperature was 20 ºC with periodic increases above 20 ºC. 4 plants of each cultivar were harvested for analysis. The nutrient solution in the plastic vessel was sampled every day during the trial and acidified with conc. HCl to pH 1 and stored at 4 $^0$C until analysis to prevent adsorption on the walls (Gomez Ariza *et al.*, 2000).

### Sample preparation

Plant leaves and roots were thoroughly washed with tap water to remove all superficial dirt as suggested by Markert (1995). Composite samples of 6 (first study) or 4 plants (second study) from both control and treated groups (~ 300 g) were lyophilised (Christ, Alpha 1-4) at –50 ºC and 0.050 mbar pressure and then milled in an agate mill (Fritsch, Pulverisette 7) (speed: 6; time: 6 min). The particle size was less than 0.2 mm.

### Selenium determination

Plants

The digestion procedure is described in detail elsewhere (Smrkolj and Stibilj, 2004).

0.150-0.200 g of sample was weighed in a Teflon tube. After that 0.5 mL conc. $H_2SO_4$ and 1.5 mL $HNO_3$ was added and heated for 60 min at 130 ºC in the screw-capped tubes in an aluminium block. After cooling, 2 mL of 30 % $H_2O_2$ was added and the tube reheated for 10 min at 115 ºC, then 0.1 mL of 40 % HF was added and heated for 10 minutes at 115 ºC, and again after addition of 2 mL $H_2O_2$ the solution was kept at 115 ºC for 15 min. By adding 2.5 mL of conc. HCl reduction of Se (VI) to Se (IV) at 100 ºC followed. The solutions were than diluted, depending on the foreseen selenium concentration in the samples.

Nutrient solution

2 g of nutrient solution was weighed in a Teflon tube and then 2 mL of conc. HCl was added and reduction from Se (VI) to Se (IV) was performed in 6 M HCl media for 10 min at 100 ºC and than diluted to 50 g.

Precipitate occurring in the closed chamber with nutrient solution in the long term experiment

0.8 g of precipitate was weighed in a Teflon tube. After that 1 mL of fuming $HNO_3$ was added and the solution was heated for 30 min at 115 ºC. Then 5 mL of $H_2O_2$ was added and heated for 30 minutes at 115 ºC, and finally 2.5 mL of conc. HCl was added and heated for 10 min at 100 ºC for reduction of Se (VI) to Se (IV). The solution was than diluted to 50 g.

In all cases selenium was detected in the digested solutions by HG-AFS (Smrkolj and Stibilj, 2004).

The accuracy of selenium determination was checked by analysing the standard reference material Trace Elements in Spinach Leaves, NIST 1570a. The mean result for 4 determinations was $113 \pm 4$ ng $g^{-1}$ and the certified value is $117 \pm 9$ ng $g^{-1}$. The repeatability of the determination of selenium was under 10 % and the reproducibility ($RSD_R$), calculated by ANOVA (Farrant, 1997), over a period of 8 months (38 determinations), for the determination of selenium in SRM Trace Elements in Spinach Leaves was 9 %. The detection limit was 0.14 ng $g^{-1}$ solution.

**Determination of macro and micro elements by $k_0$-instrumental neutron activation analysis ($k_0$-INAA)**

For $k_0$-INAA, all irradiations were made in channels of the TRIGA Mark II reactor at the JSI: for short irradiation (ca 1 minute) in the pneumatic tube (PT) at a thermal flux of $3.5 \cdot 10^{12}$ $n \cdot cm^{-2} \cdot s^{-1}$, and for long irradiations in the carousel facility (CF) at a thermal flux of $1.1 \cdot 10^{12}$ $n \cdot cm^{-2} \cdot s^{-1}$ (irradiation time for each sample was 20 h).

Aliquots of about 200 mg of lyophilized samples were pressed into pellet form with a diameter of 10 mm. A sample and standard (Al-Au(0.1%) IRMM-530R disk 6 mm in diameter and 0.2 mm high) were stacked together and fixed in the polyethylene ampoule in sandwich form and irradiated long time in the CF of the TRIGA reactor, while for short irradiation only a sample and standard were irradiated together in the PT. Determination of Al, Cl, Mg, Mn and V were made in the PT channel after short irradiation, while other elements were obtained after long irradiation in the CF of the TRIGA reactor. For peak area evaluation, the HyperLab program was used. For elemental concentrations and effective solid angle calculations a software package KAYZERO/SOLCOI® was applied. More details about the method used are given elsewhere (Jaćimović *et al.*, 2002).

## RESULTS AND DISCUSSION

**Long term treatment**

The results of the study showed that the selenium content in roots and leaves greatly increased after 40 days treatment with selenium enriched solution (Table 1). In leaves of the cultivar 'Anivip' the selenium content increased from 0.057 in the control group to 370 mg $kg^{-1}$ in the Se exposed group, and in the cultivar 'Monivip' from 0.047 in the control group to 460 mg $kg^{-1}$ in the Se exposed group. The selenium enrichment rate was lower in roots than in leaves; in the cultivar 'Anivip' the Se content increased from 0.022 to 73 mg $kg^{-1}$ and in 'Monivip' from 0.069 to 87 mg $kg^{-1}$. The cultivar 'Monivip' had a higher ability to take up selenium than 'Anivip'. The majority of the selenium was transported from roots to leaves. And in both cultivars a similar ratio (5-fold) between the Se content in leaves and roots was found.

Table 1. Selenium content in chicory after 40 days treatment

| **Group** | **Se content in chicory after 40 days treatment (mg $kg^{-1}$ lyophilised sample)[a]** | | | |
|---|---|---|---|---|
| | **'Anivip'** | | **'Monivip'** | |
| | **Roots** | **Leaves** | **Roots** | **Leaves** |
| Control | 0.088 ± 0.036 | 0.285 ± 0.010 | 0.276 ± 0.076 | 0.235 ± 0.025 |
| Treated | 73 ± 15 | 370 ± 90 | 87 ± 3 | 460 ± 70 |

[a] – average ± SD; The analysis of each sample was performed by fivefold determinations

The growth of treated plants was not diminished in comparison with control plants, and no reduction in dry matter content was found. The average content of moisture in leaves was 80 % and in roots 75 %, irrespective of the group. From the Table 2 it is shown that the contents of essential elements Fe, K, Mn and Zn decreased and Na content increased in exposed chicory leaves, regardless of the cultivars. Fe is required in the formation of chlorophyll and acts as an activator of many biochemical processes (found in ferrodoxin and enzymes such as peroxidise, oxidation-rection processes). Excess Zn, Mn, Cu or Mo encourages Fe deficiency (Marchner, 2002). In our case, the Zn, Mn and Fe contents decreased, only Mo content slightly increased in Se exposed chicory leaves. The literature dealing the effect on distribution of essential metals according to Se accumulation in plants is scarce. Vogrinčič *et al.* (2010) found minor concentration changes of Cd, Mo, Fe, Mn, S in leaves, Cu in husks, Mo and Mn in inflorescences between buckwheat foliarly sprayed with 10 mg Se(VI) $L^{-1}$ and control group. Addition of Se(VI) did not have any effect on concentration of Zn. Cuderman (2010) reported that the Fe content was approximately 30 times lower in Se enriched vegetables (chicory, dandelion, rocket, wild rocket), twice foliary sprayed with Se(VI) solution (10+50) mg $L^{-1}$ in comparison to control group. She found linear correlation between Zn and Se as well as between Se and Mo was obtained for all samples investigated. The Zn content was two or three times higher compared to non exposed leafy vegetables. No interdependence was observed by comparing control and Se enriched values obtained for Mn and Cd. However, Pedrero *et al.* (2006) reported that radish exposed to 1 mg $L^{-1}$ Se(VI) or Se(IV) showed a slight decrease in the translocation of the Mn, Mo and Cu when compared to control plants. Depending on the element, the decrease was more or less noticeable, but a tendency to reduce the translocation was observed when Se was supplied as Se(IV). No correlation was observed for Cu and Zn. It was demonstrated that the chemical form of Se can influence the uptake and translocation of essential metals in radish plants, which could be the cause of the growth reduction of those plants grown in selenite media, in which the translocation were slightly poor (Pedrero *et al.*, 2006).

**Table 2**: The element composition in non exposed and 40 days aeroponiccaly exposed chicory to nutrient solution with 10 mg Se/L, obtained by $k_0$-INAA
Results are expresed in µg/g of lyophilised sample.

| Element | Leaves | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Non exposed | | | | Exposed | | | |
| | ANIVIP | | MONIVIP | | ANIVIP | | MONIVIP | |
| | Content | Unc. | Content | Unc. | Content | Unc. | Content | Unc. |
| Al | 58.4 | 3.9 | 44.0 | 2.6 | 38.5 | 2.5 | 44.4 | 2.5 |
| Ba | 4.35 | 0.58 | 2.77 | 0.17 | 1.68 | 0.42 | 1.16 | 0.35 |
| Br | 12.0 | 0.4 | 10.9 | 0.4 | 10.6 | 0.4 | 8.74 | 0.31 |
| Ca | 9329 | 329 | 6942 | 246 | 6540 | 308 | 8495 | 308 |
| Cl | 10187 | 399 | 7763 | 330 | 8752 | 317 | 5995 | 214 |
| Cr | 0.10 | 0.02 | 0.13 | 0.02 | < 0.16 | | 0.13 | 0.04 |
| Fe | 68.9 | 3.4 | 66.8 | 3.4 | 29.3 | 1.2 | 29.8 | 1.6 |
| K | 55596 | 1962 | 52626 | 1858 | 46282 | 1655 | 35446 | 1282 |
| Mg | 2826 | 117 | 2764 | 114 | 2764 | 126 | 3611 | 152 |
| Mn | 56.0 | 2.0 | 39.8 | 1.4 | 17.2 | 0.6 | 13.9 | 0.5 |
| Mo | 0.62 | 0.05 | 0.79 | 0.05 | < 1.2 | | 2.42 | 0.27 |
| Na | 644 | 23 | 527 | 19 | 732 | 26 | 1321 | 48 |
| Sr | 49.1 | 2.4 | 37.9 | 2.1 | 43.0 | 1.7 | 57.3 | 2.5 |
| V | < 0.5 | | < 0.4 | | < 0.4 | | < 0.3 | |
| Zn | 111 | 4 | 137 | 5 | 51.7 | 1.8 | 44.1 | 1.6 |

**Short term treatment**
To estimate the selenium uptake in 180 day old chicory plants, fixed in pots with stone wool under advantageous climatic conditions (higher temperature (20 ºC) and more light), short term treatment was applied.

The selenium content in leaves increased with time during plant growth; the ratio between selenium contents in the groups treated for 5 and 10 days was the same for both cultivars (2.5 and 2.6) in the flowering period (Table 3).

In roots the selenium content increased with time during plant growth from 27 to 46 mg $kg^{-1}$ for 'Anivip' and from 33 to 46 mg $kg^{-1}$ for 'Monivip', all on a lyophilised basis (Table 3). Similar ratios between Se content in roots of plants treated for 5 and 10 days (1.7 for 'Anivip' and 1.4 for 'Monivip') were obtained. Sample preparation of roots was very difficult, since they were hard to clean due to their branched shape, so the representativeness of the sample was very hard to assure. This could be a possible reason for the lower selenium content found after 7 days of exposure than after 5 days for the cultivar 'Monivip'. In both treatments, selenium uptake was greater by the 'Monivip' than by the 'Anivip' cultivar. No difference in dry matter content in leaves and in roots was observed. Despite the high selenium concentrations, none of the organoleptic toxic effects described in the literature such as a smell of garlic, chlorosis, withering and drying of leaves, bleaching or yellowing of younger leaves, pinkish spots on roots or premature death of the plant (Kabata Pendias, 2001; Terry *et al.*, 2000) were found in chicory plants, either in the short or in the long treatment group. In a very short time (5 days) a rather high Se accumulation was obtained and after 10 days the concentration approximately doubled in comparison to 5 days exposure. In 10 days approximately half of the concentration found after 40 days was accumulated. However, in the comparing the accumulation of Se in chicory in the both experiments, it should be considered that in the 40 day treatment the Se concentration in nutrient solution decreased due to Se containing precipitate.

Table 3. Selenium content in chicory leaves and roots after 5, 7 and 10 days of treatment with selenium enriched solution

| **Time of treatment with Se enriched solution (days)** | **Se content in chicory (mg kg$^{-1}$ lyophilised sample)$^a$** | | | |
|---|---|---|---|---|
| | **‘Anivip’** | | **‘Monivip’** | |
| | **Roots** | **Leaves** | **Roots** | **Leaves** |
| 5 | 27 ± 1 | 55 ± 1 | 33 ± 2 | 78 ± 1 |
| 7 | 32 ± 2<br>(1.2) | 123 ± 3<br>(2.2) | 23 ± 1<br>(0.7) | 130 ± 8<br>(1.7) |
| 10 | 46 ± 1<br>(1.7) | 139 ± 16<br>(2.5) | 46 ± 2<br>(1.4) | 205 ± 19<br>(2.6) |

$^a$ – average ± SD (ratio = treated 7 or 10 days : treated 5 days ); The analysis of each sample was performed by fivefold determinations

Literature data on Se accumulation in vegetables are scarce, so for comparison of our results the studies on cultivation of vegetables in soil (Wrobel *et al.*, 2004; Hartikainen *et al.*, 1997) and in hydroponic system (Ximenez-Embun *et al.*, 2004) with addition of various concentrations of selenium in the form of sodium selenate were used. The Se contents in leaves of hydroponically cultivated sunflower and lupine in selenate nutrient solution containing 5 mg Se L$^{-1}$ were in the same range ( inside 100-800 µg g$^{-1}$) as Se contents in chicory leaves after 10 days of selenium treatment in aeroponic system. (Table 4).

**Table 4**. Comparison of results of this work with literature data for the leaves of exposed plants

| **Plant leaves** | **Type of cultivation** | **Se addition** | **Se content (µg g$^{-1}$)** | **Ref.** |
|---|---|---|---|---|
| Onion<br>(*Allium cepa)* | in soil | $Na_2SeO_4$ (5 mg L$^{-1}$)<br>8 days | 601 ± 7 | Wrobel *et al.*, 2004 |
| Ryegrass<br>(*Lolium multiflorum*) | in soil | $Na_2SeO_4$ (33 µg kg$^{-1}$)<br>21 days | 5.05 | Hartikainen *et al.*, 1997 |
| Lettruce<br>(*Lactuca sativa*) | | $Na_2SeO_4$ (33 µg kg$^{-1}$)<br>46 days | 4.90 | |
| Lupine<br>(*Lupinus albus*) | hydroponically | $Na_2SeO_4$ (5 mg L$^{-1}$)<br>14 days | 609 ± 12 | Ximenez-Embun *et al.*, 2004 |
| Sunflower<br>(*Helianthus annus*) | | | 724 ± 187 | |
| Chicory cv. ‘Anivip’<br>(*Cichorium intybus*) | aeroponically | $Na_2SeO_4$ (10 mg L$^{-1}$)<br>10 days | 139 ± 16 | This study |
| Chicory cv. ‘Monivip’<br>(*Cichorium intybus*) | | | 205 ± 19 | |

Furthermore, we also studied the Se speciation in leaves of chicory ('Anivip') (Mazej *et al.*, 2006). 64 % of Se was accumulated in the water soluble form, irrespective of the time of exposure to Se enriched solution. Besides inorganic Se (63-73 %), in the extracts after enzyme hydrolysis SeMet (4.2-8.4 %) and some unidentified peaks were observed. The compound SeMeSeCys was obtained (to 0.7 %) only in leaves of chicory that was 40 days exposed to Se enriched nutrient solution.

### Losses of Se during the experiments

Although it was not the purpose of the experiments to perform a quantitative or mass balance study of selenium, we monitored the concentration of selenium in the nutrient solution during the short and long term treatments. In the latter, the actual concentration of Se in the nutrient solution fell considerably due to absorption on the contact surfaces of the chamber holding the nutrient solution and formation of a precipitate from an initial 10 to 4.52 mg $L^{-1}$. We found that this precipitate contained around 20 % of Se in a form of $Se^0$ or in strong organic complex, soluble only in fumic nitric acid. These forms of Se could be a result of reaction with the humic substances present e.g. humic acids, fulvic acids and humins, that are known to be redox reactive and capable of reducing elements (Chen *et al.*, 2003). Also the organic acids as root exudates are capable of reducing low levels of selenite or selenate in the immediate vicinity of roots (Shanker *et al.*, 1996).

In the short term experiment, where the plants were fixed in pots only in stone wool, the Resh nutrient solution was sampled every day. The mean value of selenium content over the 10 days was found to be 7 ± 1 mg $L^{-1}$, pH=7.8 and no precipitate was found. The decrease in selenium concentration in the nutrient solution was due to adsorption on plastic tubes and on the walls of reservoir at pH 7-8. This was confirmed by a laboratory experiment, in which we prepared a 10 mg $SeL^{-1}$ solution in a polyethylene reservoir and found 6.8 ± 0.2 mg $L^{-1}$ after 10 days.

To conclude, chicory has the capability to take up a great amount of Se(VI) independently of the cultivation temperature and development phase. To cultivate selenium enriched chicory as a potential beneficial supplement aeroponically, more experiments are needed to define the appropriate duration of cultivation and the concentration of selenium in the nutrient solution.

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**Agris category code**: C30

# Topics related to social sciences by authors from Slovenia in agriculture-and-life-sciences database CAB Abstracts

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## ABSTRACT

Documents related to social sciences are processed/indexed by several field-specific (e.g. Sociological Abstracts) and general (Web of Science, Scopus) bibliographic databases. These topics are scattered also among other specialized databases and information systems in agriculture, biomedicine, and other life-sciences, for example Agris, Agricola, FSTA, Medline (PubMed), etc. Agriculture can also involve social issues such as rural sociology, public services, settlements, demography, tourism (agritourism). Relevant documents may thus elude attention of researchers which seek information in a limited number of databases. We used CAB Abstracts (CABI/CAB International), the leading global database for agriculture, environment, veterinary sciences, applied economics, food science and nutrition. In a bibliometric/scientometric analysis, we used the classification based on subject categories CABICODES (CC), which enable identification of general research subject areas, in order to assess publishing patterns by researchers from Slovenia in 1991-2010. We assessed growth, and identified database records which had been classified with the social-sciences-related CC. We assessed co-classification or co-occurrence of these categories with other general subjects, such as Economics, Plant Science and Protection, Food Science and Produce, Animal Science, Forestry and Wood, Pathogen, Pest, Parasite and Weed Management, Soil Science, Human Health and Nutrition, Education, Extension, Information, Training, Natural Resources. 336 records were published by Slovenian authors, and classified with social sciences CC in CAB Abstracts. In total, 1313 different CC had been assigned to the records. The social-sciences-related research co-occurs with the following subject matter, in a decreasing order: economics, forestry/wood science, natural resources (e.g. water, meteorology, pollution), human biology/health/nutrition, food, plant, animal science/protection.

**Keywords:** agriculture, sociology, social sciences, bibliographic databases, information systems, subject headings, category codes, categories, indexing, co-occurrence, co-classification, CAB Abstracts, bibliometrics, scientometrics

## IZVLEČEK

### DRUŽBOSLOVNA TEMATIKA V PRISPEVKIH SLOVENSKIH AVTORJEV PO PODATKIH BIOTEHNIŠKE/KMETIJSKE ZBIRKE CAB ABSTRACTS

Dokumente v povezavi z družboslovjem oz. sociologijo obdelujejo in indeksirajo različne specializirane (npr. Sociological Abstracts) in splošne (Web of Science, Scopus) bibliografske podatkovne zbirke (baze podatkov). Ta tematika je razkropljena tudi med drugimi specializiranimi zbirkami in informacijskimi sistemi v kmetijstvu (biotehniki), biomedicini in drugih disciplinah, npr. Agris, Agricola, FSTA, Medline (PubMed). Kmetijstvo se navezuje na ruralno sociologijo (sociologijo podeželja), javne storitve, demografijo, naselja, turizem (kmečki turizem) ipd. Raziskovalci, ki uporabljajo samo določene zbirke, lahko spregledajo številne relevantne dokumente. V bibliometrični/scientometrični analizi smo uporabili zbirko CAB Abstracts (CABI/CAB International), ki je vodilna zbirka za kmetijstvo, okolje, veterino, uporabno ekonomiko, živilstvo in prehrano, in sicer glede na klasifikacijo, temelječo na predmetnih kategorijah CABICODES (CC), ki omogočajo identifikacijo splošnih raziskovalnih področij in na tem temelju ocenili objave avtorjev iz Slovenije v 1991-2010. Ocenili smo letno rast objav in identificirali vse zapise v zbirki, ki so bili klasificirani s kategorijami iz sekcije družboslovje (social sciences). Ovrednotili smo soklasifikacijo-sopojavnost teh kategorij z drugimi splošnimi kategorijami, kot so ekonomika, rastlinska

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pridelava in varstvo rastlin, živilstvo, znanost o živalih in varstvo živali, gozdarstvo in lesarstvo, bolezni, škodljivci in pleveli, pedologija, zdravje, biologija in prehrana ljudi, izobraževanje, svetovanje, informacije, naravni viri ipd. Slovenski avtorji so v tem obdobju objavili 336 dokumentov, ki so bili klasificirani z družboslovnimi kategorijami zbirke CAB. Ti dokumenti so bili skupno opremljeni s 1313 različnimi kategorijami. Družboslovna tematika se najbolj prepleta z ekonomiko, sledijo gozdarstvo/lesarstvo, naravni viri (npr. voda, meteorologija, onesnaževanje), zdravje, biologija in prehrana ljudi, živilstvo, rastlinska proizvodnja in varstvo ter živalska proizvodnja.

**Ključne besede:** kmetijstvo, biotehnika, sociologija, družboslovje, bibliografske podatkovne zbirke, informacijski sistemi, predmetne oznake, kategorije, indeksiranje, sopojavnost, soklasifikacija, CAB Abstracts, bibliometrija, scientometrija

## 1 INTRODUCTION AND BACKGROUND

In the age of an increasing interdisciplinary research it is getting progressively more difficult to identify relevant documents, on account of the scatter of information among many different resources. It is possible to employ general, global information systems, such as Web of Science (Thomson Reuters, formerly ISI) or SciVerse Scopus (Elsevier). These systems and respective databases place strong importance on tracking citations. But in order to carry out comprehensive searches, end-users need to consult also discipline-specific databases. These databases excel in complex classification of subject matter. Such additional analysis and organization of knowledge is performed by information professionals which take into account characteristics of each particular information system. Interdisciplinary topics are scattered across many different databases, served by particular systems of subject headings (descriptors, preferred terms, identifiers, category codes, classifications etc.). This is the case with many life-sciences or social-sciences sub-disciplines. For example, the topics of agricultural economics or rural sociology can be scattered among social-sciences or agriculture-related information systems.

Social sciences are organized in several discipline-specific databases, for example Sociological Abstracts. But some particular social-sciences-related research can also be strongly represented in agricultural databases, such as Agricola, Agris, CAB Abstracts or FSTA (Food Science and Technology Abstracts). Each of these systems is organized in a specific way. In our bibliometric (scientometric) analysis we wish to identify, on the example of authors from Slovenia, patterns of documents/publications which have been classified as pertaining to social sciences in an agricultural database. To this end, we will focus on the leading international database CAB Abstracts (CABI/CAB International) which contains several thousand agriculture-and-life-sciences-related documents by Slovenian authors. This database contains millions of records which are relevant not only to agriculture but also other topics in life sciences, such as environment, human development and health. We wish to assess the context of documents classified as social sciences also in relation to other research topics, such as animal, plant, forestry/wood and food sciences. At the same time we will also assess characteristics of the CABI classification system (CABICODES). This research thus also seeks to offer some methods for better search techniques.

CABI subject categories (CABICODES, henceforward CC) were examined, in an early study, in order to identify literature on agricultural economics and rural sociology (Stanton, 1991). The use of CC in combination with keywords would lead to better searching precision (Greider, 2002; Stephens, 2002). CC, along with some other classifications and vocabularies, were also used in classifying and describing learning resources on organic agriculture and agroecology (Ebner et al., 2009, Kastrantas et al., 2009). Some basic usage of Agris Subject Category Codes and CC was presented on an example of an agricultural journal by Bartol (2009). CAB Abstracts (henceforward CABA), and social-sciences databases, such as Sociological Abstracts (henceforward SocAb) or Social Science Citation Index have frequently been used to assess specific fields related to social sciences and economics, most notably agricultural economics, communications, consumers, public health, rural sociology, tourism, etc. SocAb and CABA were used in a research on the perception of health foods (Paquette, 2005). The scatter of journal literature in the field of agricultural economics and agribusiness was also investigated on the models of CABA, and some other information systems, such as Web of Knowledge, Google Scholar and EconLit (Salisbury and Takewade, 2006). Zumalt (2007) used CABA and Communication Abstracts to tackle agricultural communications and concluded that it is necessary to include also many other information resources to retrieve information in such a widely scattered field. Consumer perception of food labels was also investigated, on the examples of CABA and SocAb, along with several other life sciences and social sciences databases (Grunert and Wills, 2007). Social Science Citation Index and CABA were compared with regard to the concepts in titles and abstracts (Pautaso, 2010). CABA has frequently been used as a good bibliographic source of tourism-related scholarly information. CABA was identified as an important resource for the field of leisure and tourism in an early study that also listed SocAb (Leighfield, 1987). Another study employed CABA and database ABI-Inform to assess information on travel and tourism (Ojala, 1990). CABA was also identified as an important source of information related to sport and tourism by Allen (2005). Leading scholarly tourism journals were assessed through CABA, Scopus and JSTOR in research by Baggio et al. (2008). Tourism research communications through journals, on the example of CABA, SocAb, Social Science Citation Index, and some other databases in the field of social sciences, were assessed by Xiao and Smith (2010). CABA and SocAb were sometimes used to evaluate national production, and were identified as important resources for information on public health-related scientific production in Latin America (Coimbra and Carlos, 1999). CABA and SocAb, and several other databases were also compared with regard to the scientific production by research institutions in the capital cities of the new European Union countries (Bartol and Hocevar, 2005). Coverage of national production in Mexico was also investigated through CAB Abstracts, Social Science Citation Index (SSCI), and some other databases (Bravo-Vinaja and Sanz-Casado, 2008)

## 2 MATERIALS AND METHODS

We employed database CAB Abstracts and its classification scheme CABICODES in order to assess publishing patterns by researchers from Slovenia in the period 1991-2010. We placed emphasis on topics related to social sciences, which we also investigated in relation to other major research topics indexed by CAB Abstracts (henceforward CABA). CABA is compiled by CAB International (CABI), and is the most comprehensive global database involving agriculture (animal and plant sciences, forestry), environment, veterinary sciences, applied economics, food science and nutrition (CAB, 2011). It contains 6.3 million records from 1973 onwards. 300,000 new records are added each year. The database includes documents from international (English-language) as well as non-English-language journals and publications from over 116 countries in 50 languages. English abstracts are included in most records. All records are also indexed and classified with indexing terms and classification codes (CABICODES, henceforward CC or codes). There are more than 90,000 indexing terms (descriptors or preferred terms, keywords) in CABI's controlled indexing vocabulary (CAB Thesaurus). These terms are structured on several hierarchical levels and are used to describe very specific subject concepts. The codes, which are divided into 23 subject areas or sections, however, are used for subject classification of broad or general concepts. Each section includes codes that divide a concept into more specific subjects (Advanced, 2011; Ison, 2011). The utility of CABICODES is very useful in retrieving major subjects or topics which would be difficult to identify with indexing terms (keywords) alone. The codes consist of double letters in combination with three digits, for example BB500 - *History and Biography*, FF061 - *Plant Nutrition*, LL300 - *Animal Behaviour*, etc. In total, there are some 250 codes. The CC categories are based on the broad classification system used in the Agricola database (Subject Category Codes, NAL-National Agricultural Library) and AGRIS database (Agris Subject Categories, FAO of the United Nations), adapted for the subject emphasis of the CAB Abstracts database.

In our study we used these codes to identify different topics classified as pertaining to social sciences in the CABA database, which is principally an agricultural database. In the database we retrieved records co(authored) by researchers from Slovenia, and which had been in the period 1991-2010 classified with any of the codes pertaining to the section UU *(Social Sciences)*. Currently, there exist 17 specific codes in the section UU, such as *Housing and Settlement, Demography, Tourism and Travel* etc. Nine (9) codes, such as *Urban Regeneration, Community Development, Recreation and Sport*, etc. were discontinued in 2000. Most have been substituted with other codes. For example, *Recreation and Sport* (UU620) has been replaced with *Sport and Recreational Activities* (UU625). Our study, however, spanned two decades so we employed all the codes that had been during this period assigned to the documents. Namely, older, discontinued codes must currently still be used to retrieve older documents in back-file. These UU section codes are later assessed in the Figure 2.

We set up an experimental database, based on the downloaded records, in order to carry out further analyses. We identified all codes pertaining to the section UU. We assessed the growth of records classified with any of the codes from the section UU. We identified the other 22 major categories or major sections, such as *Economics...* (EE), *Forestry...* (KK), *Plant Science...* (FF), *Animal Science...* (LL), and assessed co-occurrence or co-classification of the UU codes with any of these 22 major sections.

## 3 RESULTS AND DISCUSSION

In the period 1991-2010, there were 213,060 records in CABA which had been classified with the codes pertaining to the section UU (*Social Sciences*). In the same period there were 9,013 records affiliated with the authors from Slovenia. The section UU was assigned to 336 records which had been co(authored) by researchers from Slovenia. In the initial ten-year period, up to 15 yearly records were classified with the UU codes. In the more recent period, almost twice as many records are classified with these codes. Noticeable increase can be observed after 2002 (Figure 1). Some decrease in 2010 can probably be attributed to a delay in inclusion of records in the database.

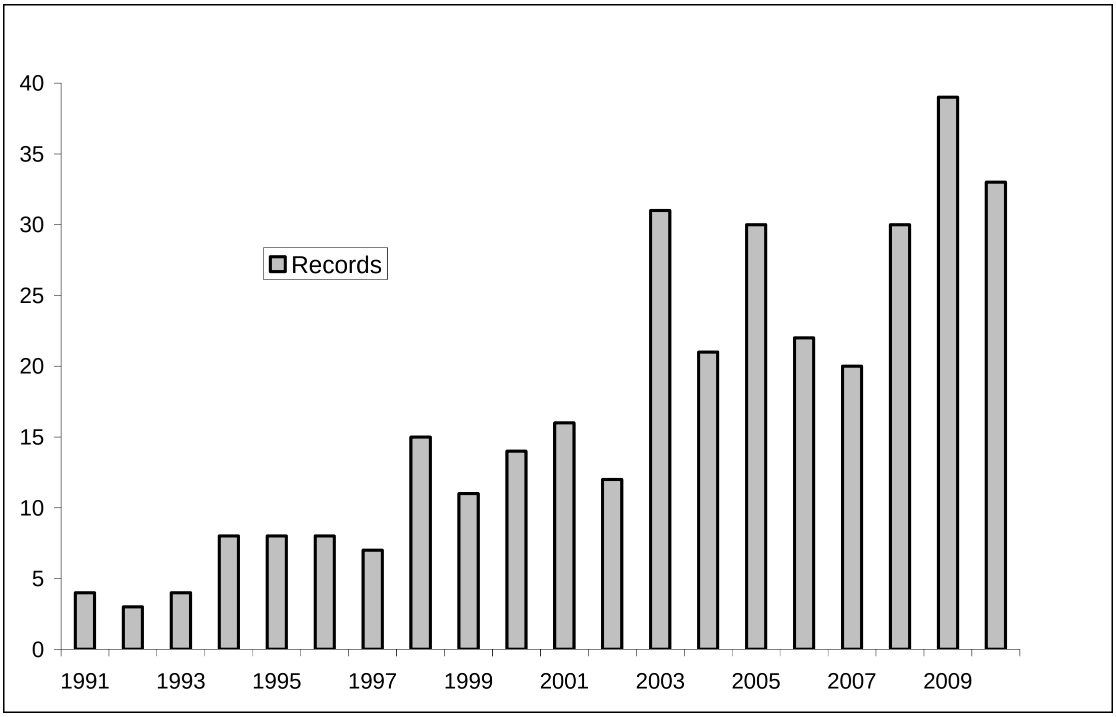

**Figure 1:** Growth of documents published by Slovenian authors in the period 1991-2010, as classified with any social-sciences-related CABICODE (UU)

In CABA several codes are usually assigned to each document. Sometimes, a document is also classified with different codes from the same section so in our case 430 different UU codes were assigned to the 336 records. These UU codes are presented in the Figure 2. As many as 108 documents were classified with the code *Social Psychology and Social Anthropology* [UU485]. This is a rather general concept which can be attributed to many different topics. It is followed by 88 documents classified as *Tourism and Travel* [UU700]. This is a more specific code than UU485. We can also observe that some special emphasis is placed on selected topics or concepts. There are several codes which denote the concepts of leisure, sport and tourism. This subject matter is namely an important subset of the CAB Abstracts database. Two particular codes are dedicated to rural topics, *Rural Development* [UU850] and *Rural Sociology* [UU800]. The two concepts are nevertheless very related but the later concept [UU800] is more general in meaning.

Some codes were discontinued in 2000, as we mentioned before. These codes are in the Figure 2 marked with (D). They must, however, still be used for retrieval of older documents.

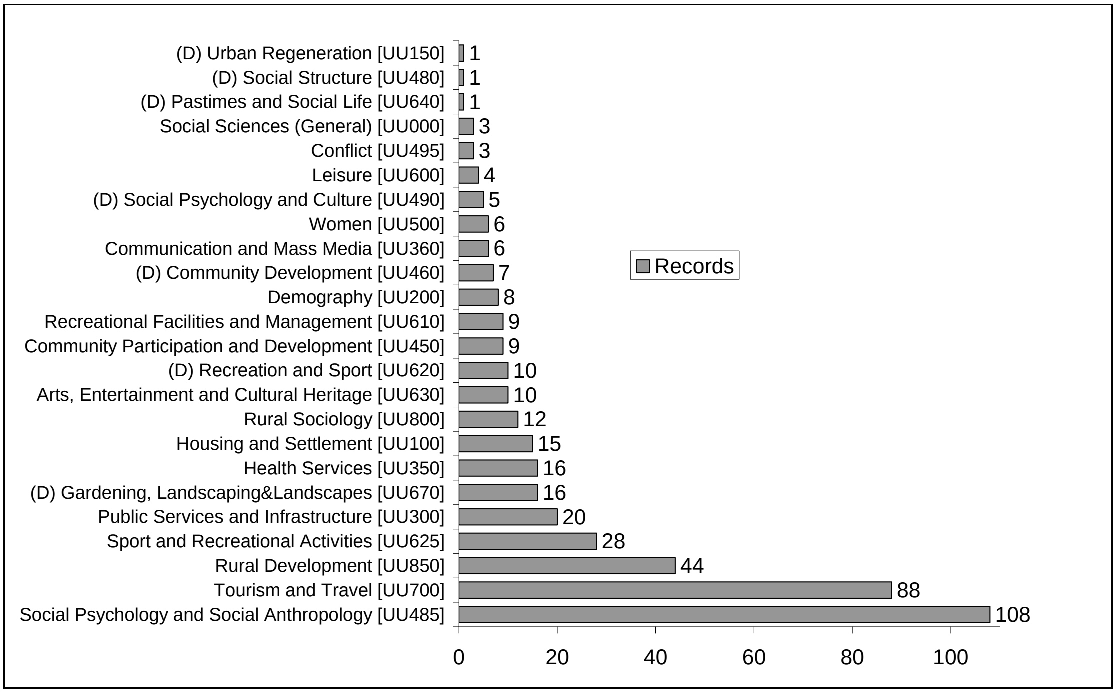

**Figure 2:** Specific social-sciences-related CABICODES, assigned to document published by Slovenian authors in the period 1991-2010

In our further analysis we measured the level of interdisciplinarity by way of co-classification or co-occurrence of *Social Sciences* [UU] codes with the codes from the other 22 sections from the CABICODE system, such as *Economics* [EE], *Forestry and Wood Science* [KK], *Plant Science, Protection* [FF], A*nimal Science, Protection* [LL] etc. (Figure 2, Table 1). Altogether, 1314 different codes were assigned to the 336 records. Thus, on average, every document was classified with four different codes. Some 140 records were classified with only three codes or fewer. However, ten documents were classified with at least 10 codes, and one document with as many as 13 different codes.

It is interesting to examine the document which was classified with 13 different codes. This is a monograph contribution with a rather general title 'Slovenia' and was published in an international book dedicated to Mediterranean forests. The document was classified with the following CABICODES:

*Biological Resources (Plant) [PP720], Social Psychology and Social Anthropology [UU485], Sport and Recreational Activities [UU625], Agencies and Organizations [DD100], Forestry Economics [EE112], Policy and Planning [EE120], Structure, Ownership and Tenure [EE165], International Trade [EE600], Labour and Employment [EE900], Forests and Forest Trees (Biology and Ecology) [KK100], Silviculture and Forest Management [KK110], Non-wood Forest Products [KK540], Land Resources [PP300].*

Such codes can serve as a powerful tool for identification of topics in a particular book, especially in the case of general titles which are not sufficiently indicative of a book content.

We mentioned before that different codes from the same section can be assigned to one document. This is shown in the Table 1 where the second column (Occ.) presents all occurrences of a particular code in the 336 records. The third column (Records) presents the records which were classified with any of the codes in the section. The records are also presented in Figure 2. The difference is the highest in *Economics* [EE] where there are 152 records, classified with 319 different codes. So, on average, two different codes from the EE group were assigned to EE-classified records.

In the fourth column of the Table 1 we present co-classification of all major sections which co-occurr with the UU codes which can in this case serve as a measure of interdisciplinarity. In this model, all major sections from the CABICODE list co-occur with the UU codes, except for the major section *Medical and Veterinary ...*

*Records* [TT] which was discontinued already in 1995. UU-section codes co-occur with the highest rate (0,45 or 45%) with the codes from the section on *Economics* [EE] (Table 1). The value 0,45 indicates that 152 among the 336 total UU-records contain at least one EE code (152/336=0,45). This somehow high value can be explained. Namely, some topics in economics are in fact strongly associated with social sciences. In the section [EE] there exist such subsections as *Aid* [EE125], *Structure, Ownership and Tenure* [EE165], or *Income and Poverty* [EE950]. In such cases it may be difficult to disambiguate 'social' topics from purely 'economical' ones. Therefore, both UU and EE sections are frequently assigned by information professionals to a particular document in order to improve search results (search recall). Such assessment of co-occurrence is probably more relevant in the case of specific agricultural disciplines or technologies, and can imply a certain level of interdisciplinarity. There are 119 *Forestry and Wood Science* [KK] records, or 35 %, among the 336 records. The codes for *Natural Resources* [PP] come about in 24 % of records. But the [PP] is a rather general section which contains quite diverse subsections such as *Energy* [PP100], *Water Resources* [PP200], *Land Resources* [PP300], *Wetlands* [PP320], *Grasslands and Rangelands* [PP350], *Meteorology and Climate* [PP500], *Pollution and Degradation* [PP600] or *Biological Resources* [PP700]. *Food science* is represented in 26 records (8 %), however, some important information on social aspects of human nutrition may also be contained in the section *Human Health, Biology, Nutrition* [VV] which accounts for 10 % of records. *Plant science and protection* [FF] occurs in 9 %, and *Animal science and protection* [LL] in 5 % of cases. Some codes, such as those in the section *Pathogen, Pest, Parasite and Weed Management* [HH] apply to plant-, animal- or human-related topics alike.

Some major sections exhibit a rather low co-occurrence with the UU codes. Sections *Non-food/Non-feed Plant Products* [SS], *Forage and Feed Products (Non-human)* [RR] and *Aquaculture (Animals)* [MM] thus come about in less than 1 % of UU-classified records.

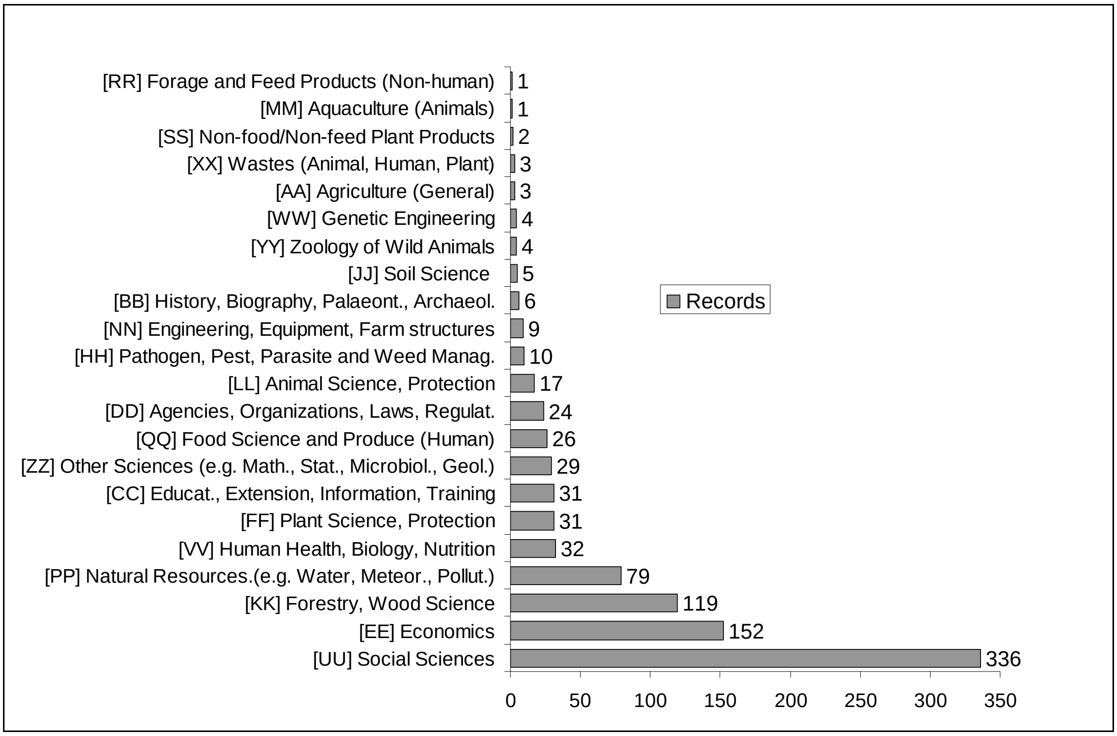

**Figure 3:** General CABICODES, assigned to document published by Slovenian authors in the period 1991-2010, which occur along social-sciences CABICODES.

**Table 1:** General CABICODES, all occurrences of Codes (Occ.), records (documents) classified with the Codes, co-occurrence of other Codes with UU Codes.

| Category (CABICODES) | Occ. | Records | Co-occ. |
|---|---|---|---|
| *[UU] Social Sciences* | *430* | *336* | |
| [EE] Economics | 319 | 152 | 0.45 |
| [KK] Forestry, Wood Science | 145 | 119 | 0.35 |
| [PP] Natural Resources.(e.g. Water, Meteor., Pollut.) | 106 | 79 | 0.24 |
| [VV] Human Health, Biology, Nutrition | 44 | 32 | 0.10 |
| [FF] Plant Science, Protection | 52 | 31 | 0.09 |
| [CC] Educat., Extension, Information, Training | 34 | 31 | 0.09 |
| [ZZ] Other Sciences (e.g. Stat., Microbiol., Geol.) | 33 | 29 | 0.09 |
| [QQ] Food Science and Produce (Human) | 35 | 26 | 0.08 |
| [DD] Agencies, Organizations, Laws, Regulat. | 26 | 24 | 0.07 |
| [LL] Animal Science, Protection | 32 | 17 | 0.05 |
| [HH] Pathogen, Pest, Parasite and Weed Manag. | 14 | 10 | 0.03 |
| [NN] Engineering, Equipment, Farm structures | 10 | 9 | 0.03 |
| [BB] History, Biography, Palaeont., Archaeol. | 6 | 6 | 0.02 |
| [JJ] Soil Science | 7 | 5 | 0.01 |
| [YY] Zoology of Wild Animals | 6 | 4 | 0.01 |
| [WW] Genetic Engineering | 4 | 4 | 0.01 |
| [AA] Agriculture (General) | 3 | 3 | 0.01 |
| [XX] Wastes (Animal, Human, Plant) | 3 | 3 | 0.01 |
| [SS] Non-food/Non-feed Plant Products | 2 | 2 | 0.01 |
| [MM] Aquaculture (Animals) | 1 | 1 | 0.00 |
| [RR] Forage and Feed Products (Non-human) | 1 | 1 | 0.00 |

## 4 CONCLUSIONS

Scientific information is scattered in an increasing number of information resources and databases, also on account of a growth in interdisciplinary research. All databases, however, exhibit some particular characteristics so a certain level of information expertise is required for the discovery of this knowledge. The many different research topics within the scope of social sciences are scattered among some major life-sciences databases. This is for example the case with agricultural economics, communications, consumers, public health, rural sociology, rural tourism etc. These topics are strongly represented in agricultural databases, such as CAB Abstracts, which served as a research model in our study. Because many authors publish social-sciences-related research in agriculture-related publications, such document may escape the attention of end-users which consider social-sciences databases only.

General subject categories may serve as a useful tool for retrieval of major subjects or topics which would be difficult to locate with very specific keywords. Such codes can be, for example, especially practical in the case of books or book-chapters which sometimes provide only very general titles. This was shown on the example of the chapter published in a book dedicated to Mediterranean forests, and which was classified with as many as 13 different categories or codes. Subject categories can thus serve, along with keywords (descriptors) and abstracts, as a valuable tool for knowledge discovery. Our research identified some patterns in interdisciplinary research by scientists from Slovenia. The main topics in the UU category are rather diverse even though they are all classified as social sciences. Our subsequent examination of the records in an experimental database shows that there are in fact several rather consistent research themes, related to economics, health, sport and tourism, and several general agricultural disciplines. If considering the particular broader agricultural disciplines, we found that an important share of social-sciences-related research was related to forestry, food and human nutrition, followed by plant and animal sciences. It also possible to identify the social aspects of some particular fields, such as meteorology or pollution-related issues.

It is important to point out that all codes have the same classification value. Some are rather general, such as *Social Psychology and Social Anthropology* [UU485] and are less indicative of a specific topic in a document, and are thus also less applicable in an end-user searching. Some other codes, however, such as *Tourism and Travel* [UU700], are more specific and can be used with a better search precision. On the other hand, there are several codes, which denote associated concept, such as *Rural Development* [UU850] or *Rural Sociology* [UU800]. User wishing to identify relevant documents should thus combine several topically similar codes with a Boolean OR in order to improve search results. Users wishing to find documents preceding the year 2000 must pay special attention to the fact that some new codes were introduced in that year. Most new codes replace previous similar concepts but older codes should still be used for the retrieval of older information.

We need to mention that information professionals and specialists play a major role in assigning classification codes and descriptors to the documents in databases. Each database or information system possesses a particular subject system which is database-specific and standardized on some set principles. Subjective influence of information professionals, which assign subject headings, can nevertheless not be excluded. But the issues of classification subjectivity are a research field in its own right and could not be tackled in this analysis which was based on state-of-the-art classification system by one information service (CABI). We have thus employed this system according to the set of fields and headings which are constant in this database, thus facilitating a possible further research and comparison of our results by other authors. At the end we wish to conclude that the end-users should pay more attention to the selection of information resources, especially in the case of interdisciplinary research which is scattered among many information systems. Users need to get acquainted with characteristics and classification principles in databases if they wish to use information systems to the full capacity.

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**Agrovoc descriptors:** women, role of women, social consciousness, social structure, social values, social groups, value systems, decision making, management, human behaviour, incentives, plant production, potatoes, solanum tuberosum

**Agris category code**: E50, E20

# Rural women's attitudes toward their participation in the decision-making process and production of potato crops in Shoushtar, Iran

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## ABSTRACT

The main purpose of this study was to evaluate rural women's' attitudes to their participation in the decision-making process and production of potato crops in Shoushtar, Iran. It is an applied research for which a descriptive-correlation method is chosen. Using the documentary study methodology and field study, required data have been collected and then described in terms of frequency, percentage and standard deviation. According to the size of population, the number of samples was determined to be 288 people. According to the results, there is a significant correlation between the level of attitude and participation in the decision-making process and production. Also, the rural women's' higher levels of attitude to their participation in decision-making processes regarding crop production and farm work improves their self-confidence, sense of independence and power and, therefore, encourages them to participate in those activities effectively.

**Key words:** participation, rural women, decision-making, attitude, potato

## IZVLEČEK

### DRŽA PODEŽELSKIH ŽENSK GLEDE SODELOVANJA PRI ODLOČANJU IN PRIDELOVANJU KROMPIRJA V SHOUSHTAR-JU, IRAN

Cilj raziskave je ovrednotiti držo podeželskih žensk do njihove udeležbe v procesu odločanja in pridelave krompirja v Shoushtarju v Iranu. Gre za aplikativno raziskavo, pri kateri je uporabljena deskriptivno-korelacijska metoda. Želeni podatki so bili zbrani s pomočjo študija metodološke literature in terenske raziskave, predstavljeni pa so s frekvencami, odstotki in variancami. Glede na velikost populacije je bil izbran vzorec, ki je zajemal 288 ljudi. Rezultati so nakazali močno povezavo med odnosom do dela ter participacijo v procesih odločanja in pridelave. Rezultati so prav tako pokazali, da boljša drža žensk do njihove participacije v procesu odločanja glede pridelovanja in dela na kmetiji izboljša njihovo samozavest, občutek neodvisnosti in moči ter jih tako spodbuja k učinkovitemu sodelovanju v teh dejavnostih.

**Ključne besede:** udeležba, podeželske ženske, odločanje, drža, krompir

## 1 INTRODUCTION

In many societies rural women serve as the main work force involved in agricultural activities. These activities may include farm works, from planting to harvest, animal husbandry and some marketing. Involvement of women in the labor market is believed to be one of the main indicators of a country's development that reflects women's interests in different economic and social activities. The extent of rural women activity is far more influenced by economic, social, cultural and ecological factors than it is in case of men. Rural women, either directly or with indirect engagement in agricultural

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activities, are considered to have significant potential effects on a society.

The concept of participation and cooperation is an old one which has originated from public affairs management and religious beliefs. The concept is mentioned in Islamic teachings as “Shora”. The managers have been required clearly by the Holy Quran to consult with others before making decisions about the issues which need research and investigation.

In many countries, women make up 60-80% of workforces involved in the agriculture sector. Provided that 75-90% of the population are dependent on agriculture in the Third World countries, a great proportion of women are involved in agricultural activities. Women not only prepare all, or most of the family food requirement, but also play a significant role in production of those kinds of commercial products which are exclusively produced by men (Liming, 1991).

As the most critical products in a society, agricultural products play a significant role in economic development and food security. The world population growth and overuse of cultivable lands have led farmers to improve their efficiency and performance instead of expanding the cultivated land area. In this regard, farm management plays an important role: it is aimed at optimizing the use of resources, improving the performance and, as a result, improving the economic efficiency of production. The purpose of farm management is to improve resource-use efficiency by understanding the possibilities and/or limitations of production (Zaree, 2000).

The family is considered to be the main social unit in which one experiences the first stages of socialization and also the first instances of sexual inequalities. The kind of relationship between parents, their share of economic, social, cultural and symbolic resources makes an unequal image of different sexes in one’s mind and gradually this inequality seems natural to a normal person. If the traditional sexual patterns are accepted by the woman, two consequences arrive: first, the woman accepts and affirms her low position and the inequality and second, she as the main person involved in the children’s socialization, passes the belief on, to the next generation. Both the consequences maintain and reproduce the inequality inside the family and then in the society (Garousi, 2005).

Multiple responsibilities of women, as a result of their activities out of the home, have doubled their expected tasks but have not led to an improvement in their rights. In other words, an increase in their participation has reduced their leisure time and well-being (Cowan, 1983). Classic conservative sociologists believe that women should have a defined fixed role in the family and they should not participate in other activities outside of the home. They have a God-given responsibility to take care of their husbands and children (Saroukhani, 1991).

Housewives therefore, always experience a series of economic or social dependencies on men. However, deep economic and social changes occurred during the industrial revolution, leading to definition of new values and norms and destruction of old traditional patterns that used to limit women's activities to the family and inside the homes enabling women to have their opinions about subjects out of their families and homes and, thus, to make their own decisions. Women's employment, therefore, helped them to acquire their own right to participate and to make decisions about their families (Bagheri et al., 2008).

The important roles of women in the processes of production and marketing have always been ignored by the development designers. They are not included in the educational programs regarding new planting strategies, food stuff production, and use of technology in managing the workforce, animal husbandry, small industries, marketing and other services. Official credits are rarely given to women and often the men are those who can apply for cooperatives and benefit the loans (Dulyapach, 1985).

According to the Agricultural Research Institute, men mainly make decisions about farming and women have more important roles in managing the family (Anony, 1991). Researchers have found that in South Asia, women from big landowner families who supervise daily work processes in the farms tend to give information to the farmer women that are beneficial for both sides. Women landowners may serve as good contact farmers. Therefore, participation of rural women in decision- making processes is not an inevitable easy to accept fact in many areas and may conflict benefits of many rural men and women (FAO, 1991).

One may consider three levels of women's participation and decision-making in farming activities:

- Social and national levels in which their role in production of commercial non-commercial products are determined.
- Family level, in which they prepare food, clothes, fuel and care for the elderly.
- Intra-generation level in which they satisfy children’s educational and emotional demands (Suman, 2008). In a research entitled 'Evaluating the economic

participation of rural women in Fars province, Iran', Lahsaii Zadeh et al. (2005) suggested that there is a reverse relationship between the level of women's education and their economic participation. Also, their participation is improved as they become older. Their participation is also reduced when the men's education and income level increase. Using mass media, men's perception of women's economic role and women's self-confidence, can explain 33.8% of changes in the dependent variables.

- In a research entitled 'Factors influencing women's empowerment', Rahnavard and Hosseini (2008) found that the level of women's empowerment exists at a moderate level and the results of factor analysis highlights following factors: 1) management attitude; 2) group structure; 3) inter-group values; and 4) information communication. Furthermore, regression analysis shows that only the first three factors determine 63% of variability in women's empowerment.

The main purpose of this study was to evaluate rural women's' attitudes to their participation in the decision-making process and production of potato crops in Shoushtar township, Iran. The objectives of the study were as follows:

1. Describe socio-personal characteristics of women who participated in potato production,
2. Determine the level of women's attitude to participation in decision-making and production

## 2 MATERIALS AND METHODS

The researchers conducted a study using a survey research design and it was descriptive-correlation according to the methodology. Required data is collected using documentary and field studies and obtained results were described in terms of frequency, percent, mean score and standard deviation. The target population involves all girls and women over 15, living in Shoushtar Township rural areas who participating in potato crop production, from the planting to the harvesting. Shoushtar Township is made up of two regions, six rural districts and 196 villages. The population of women involved in potato production is 1150 people who are considered as target population which 288 samples were selected randomly. The data-collection instrument was a closed-form questionnaire. A panel of experts reviewed the instrument for face, content, and constructs validity. A pilot study was conducted to establish the reliability of the instrument. Reliability coefficient (Cronbach's alpha) for the competencies was 0.81. All members of the population were interviewed by a researcher-designed questionnaire to solicit attitudes toward their participation in the decision-making process and production of potato crops.

Descriptive statistics such as mean score, percent, standard deviation and Spearman rank correlation were used to analyze this survey data.

## 3 RESULTS

The data collected in the study include the subject's age, education, income, job, number of family members, work history, owned land area, level of technical knowledge and level of attitude, but the values regarding level of education, number of family members and level of attitude are mentioned here:

According to the results, 25.3% of women (n=73) were illiterate, 58.7% (n=169) were able to read and write, 8.7% (n=25) have high school diploma and 7.3% (n=21) have higher diploma degrees (table 1).

**Table 1**: Distribution of women participating in potato production according to level of education (f=frequency).

| Level of education | f | percent | cumulative percent |
|---|---|---|---|
| Illiterate | 73 | 25.3 | 25.3 |
| Able to read and write | 169 | 58.7 | 84.0 |
| High school diploma | 25 | 8.7 | 92.7 |
| Higher that diploma | 21 | 7.3 | 100.0 |
| Total | 288 | 100.0 | |

According to the results, the number of family members of the subjects is as follows: 77.1% (n=222) were in 3-6 member families, 20.5% (n=59) in 6-10 member families and 2.4% (n=7) in 10-13 member families. The smallest families have 3 members and the biggest ones have 13 members (table 2).

**Table 2**: Distribution of women participating in potato production according to the number of family members (f=frequency).

| Number of family members | f | Percent | cumulative percent |
|---|---|---|---|
| 3-6 members | 222 | 77.1 | 77.1 |
| 6-10 | 59 | 20.5 | 97.6 |
| 10-13 | 7 | 2.4 | 100.0 |
| Total | 288 | 100.0 | |

Representative: 5 maximum: 13 minimum: 3

Twelve statements were prepared to evaluate the women’s attitude to participation in decision-making and production and they were asked to give their desired answers. Table 3 shows the frequency and percentages of the answers. The answers were given values from 1 to 5 to group the women according to their attitude, and everyone’s results were divided by 12. If the resultant number was 1, then it was mentioned in a very low attitude group, if the number was from 2 to 3, it was mentioned in an average attitude group, if the number was from 3 to 4, it was mentioned in a high attitude group and in a very high attitude group if the resultant number was 4 to 5.

**Table 3**: Distribution of women participating in potato production

| N | Statements | Strongly agree | | Agree | | Undecided | | Disagree | | Strongly disagree | | M | SD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | f | % | f | % | f | % | f | % | f | % | | |
| 1 | Does not improve self-confidence in women | 61 | 21.2 | 41 | 14.2 | 41 | 14.2 | 56 | 19.4 | 7 | 2.4 | 3.60 | 1.09 |
| 2 | Does not help family members to cooperate with each other | 52 | 18.1 | 38 | 13.2 | 38 | 13.2 | 41 | 14.2 | 17 | 5.9 | 3.58 | 1.11 |
| 3 | Encourages women to accept farm management responsibilities | 77 | 26.7 | 83 | 28.8 | 29 | 10.1 | 55 | 19.1 | 13 | 4.5 | 3.64 | 1.19 |
| 4 | Is not a good method to improve performance and the quality of potato | 27 | 9.4 | 56 | 19.4 | 56 | 19.4 | 52 | 18.1 | 28 | 9.7 | 3.24 | 1.14 |
| 5 | Improves women position in the family and society | 21 | 7.3 | 114 | 39.4 | 75 | 26 | 90 | 13.3 | 19 | 6.6 | 2.98 | 1.07 |
| 6 | Helps women get access to the credits and loans | 19 | 6.6 | 56 | 19.4 | 67 | 23.3 | 147 | 51 | 20 | 6.9 | 2.60 | 1.01 |
| 7 | Does not help reduce women’s dependence on men. | 50 | 17.4 | 39 | 13.5 | 39 | 13.5 | 56 | 19.4 | 36 | 12.5 | 3.27 | 1.30 |
| 8 | Helps avoid time and money loss | 13 | 4.5 | 58 | 20.1 | 79 | 27.4 | 111 | 38.5 | 39 | 13.5 | 2.59 | 1.05 |
| 9 | Does not help women’s ideas and role to be noticed | 35 | 12.2 | 31 | 10.8 | 31 | 10.8 | 69 | 24 | 44 | 15.3 | 3.07 | 1.31 |
| 10 | Helps family get more income and benefits | 20 | 6.9 | 35 | 12.2 | 35 | 12.2 | 137 | 47.6 | 40 | 13.9 | 2.57 | 1.15 |
| 11 | Helps reform old traditional thoughts | 7 | 2.4 | 41 | 14.2 | 43 | 14.9 | 118 | 41.0 | 63 | 21.9 | 2.39 | 1.11 |
| 12 | Helps women accept the responsibilities | 39 | 13.5 | 46 | 16 | 29 | 10.1 | 100 | 34.7 | 62 | 21.5 | 2.69 | 1.36 |

(f=frequency).

The results shows that 39.9% (n=115) of people are in an average attitude group and 60.1% (n=173) in a high attitude group (table 4).

According to each variable scale, the correlation coefficient was calculated to determine the correlation between the variables and the degree of significance.

**Table 4**: Distribution of women participating in potato crop production according to level of attitudes (f=frequency).

| Level of attitude | f | Percent | Cumulative percent |
|---|---|---|---|
| Very low attitude | 0 | 0 | 0 |
| Low attitude | 0 | 0 | 0 |
| Average attitude | 115 | 39.9 | 39.9 |
| High attitude | 173 | 60.1 | 100 |
| Very high attitude | 0 | 0 | 0 |
| Total | 288 | 100.0 | |

**Table 5**: Results of correlation analysis

| First variable | | Second variable | | $r_s$ | p |
|---|---|---|---|---|---|
| Variable | Scale | variable | scale | | |
| Education | ordinal | Participation[1] | ordinal | 0.385** | 0.01 |
| Number of family members | interval | Participation | ordinal | -0.104 | 0.39 |
| Attitude toward production activities | ordinal | Participation | ordinal | 0.450** | 0.01 |

[1] attitude towards women's participation in decision-making

**$p \leq 0.05$

There was a significant relationship between the rural women's education level and their degree of participation in decision-making and in potato production. According to the Spearman rank correlation value of 0.385 for the two variables and 0.01 level of significance, there should be a significant correlation between them, therefore the supposed assumption is verified.

There is also a significant relation between the number of family members and the women's participation in decision-making processes and producing of potato crops. According to the Spearman rank correlation value of -0.104, between the two variables there was no significant correlation between the variables (p=0.39), therefore, the supposed assumption is rejected.

According to the results, there was a significant relation between women's levels of attitudes toward production activities and participation. According to the Spearman rank correlation ($r_s$=0.450) between the two variables there was a significant relationship, therefore the supposed assumption is verified.

## 4 DISCUSSION AND CONCLUSIONS

It was found that there was a significant correlation between the rural women's level of attitude toward production activities and their participation in potato crop production. The results obtained by other researchers like Lahasai Zadeh et al (2005), Rahnavard and Hosseini (2007) and Maghsoudi et al. (2005) also support these findings. Therefore, attitude-derived faculties like the senses of power, dignity and independence are improved in the women and these bring them a more strong self-confidence and also encourage them to participate in different economic activities. Jarollahi (1992) in a research called "woman's employment in Iran" states that social, economical and cultural changes of Iranian society has changed women's social conditions and consequently their decision making power throughout history so that when society put importance on women's social condition and status, she was able to initiate and by decreasing its importance, decision making power has decreased, too. Romito and Volpato (2005), Also, state that lack of consideration to their needs, sexual orientation in designating responsibilities as limited access to power resources, options, designation, promotions and influence on top organization hierarchy, would lead to their deprivation of conditions and job's options and decrease in motivation.

Rural women either directly or in terms of help to Agriculture part are considered significant potentials. But their roles have been affected more by different social, economical, cultural and ecological conditions and factors. So, attitudes indexes such as power, status and independence would be reinforced in women in order to participate at different affaires especially at productive activities by more self-confidence.

According to the present study results, educational and cultural programs are suggested to improve rural men's perception of women's capabilities and their significant roles. Also, it is suggested to prepare required facilities to balance different economic, social and cultural roles of rural men and women. It is also recommended to consider suitable positions for rural women's participation while developing and designing agricultural programs. Building women groups and help-committees inside rural societies are assumed to be basic and practical solutions in this regard.

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**Agrovoc descriptors:** tractors, vehicles, equipment, farm equipment, cultivation equipment, forestry equipment, occupational hazards, health hazards, mortality, death, accident prevention, working conditions, safety, legislation

**Agris category code**: T10, D50, A01

# The comparison of number of deaths in accidents with the agriculture and forestry tractors among European countries

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## ABSTRACT

Agricultural and forestry tractors are very often involved in accidents with overturning because of their construction with a high centre of gravity. Such accidents are caused by various factors such as drivers, vehicles, driving conditions, or landscape/terrain. The data related to the number of deaths in accidents with agriculture and forestry tractors was collected from several European countries. The landscapes (or terrain) of the countries for tractor traffic was assessed by the coefficient of the topography and the economic development level of the countries was evaluated in terms of the GDP. The correlation analysis among the factors showed us that neither the relief of the country landscape nor the economic development level of the countries have a significant influence on the number of deaths in such accidents. The essential influence is in the legislation for the tractors and drivers. The comparison of three countries (Austria, Slovenia, and Serbia) showed the implementation of legislation in this area and time are needed for reducing the number of deaths by half.

**Key words:** agriculture tractors, forestry tractors, accidents, deaths in traffic accidents, influence of factors

## IZVLEČEK

### PRIMERJAVA ŠTEVILA MRTVIH V NESREČAH S KMETIJSKIMI IN GOZDARSKIMI TRAKTORJI PO EVROPSKIH DRŽAVAH

Kmetijski in gozdarski traktorji so zelo pogosto vpleteni v nesreče s prevračanjem, saj imajo zaradi njihove konstrukcije zelo visoko težišče. Take nesreče se dogajajo zaradi različnih vplivov kot so vozniki, vozila, vozne razmere ali površina/teren. Zbrani so bili podatki o številu mrtvih v nesrečah s kmetijskimi in gozdarskimi traktorji za več evropskih držav. Oblika površine držav, po katerih poteka vožnja traktorja, je bila ocenjena s koeficientom topografije, stopnja gospodarskega razvoja držav pa je bila ocenjena z BDP. Korelacijska analiza med faktorji je pokazala, da niti oblika površine države niti stopnja gospodarskega razvoja držav nimata močnejšega vpliva na število mrtvih v tovrstnih nesrečah. Bistveni vpliv imajo predpisi za traktorje in voznike. Primerjava treh držav (Avstrije, Slovenije in Srbije) je pokazala, da sta potrebna implementacija predpisov na tem področju in čas, da se število mrtvih prepolovi.

**Ključne besede:** kmetijski traktorji, gozdarski traktorji, nesreče, mrtvi v prometnih nesrečah, vpliv faktorjev

## 1 INTRODUCTION

Agricultural and forestry tractors, so-called off-highway vehicles, have significantly impacted the safety and health on road and in working places as their numbers increase in rural areas. Because of their working nature and construction with a higher centre of gravity, tractors are often classified as a high-risk vehicle. The major cause of accidents related to agricultural and forestry tractors is overturning. Researchers have collected the number of deaths in accidents with agriculture and forestry tractors with the help of international authorities and organisations in other European countries since the year 1990.

The number of motor vehicles has been growing continuously due to industrialisation in the Republic of Slovenia just like in other developed countries. The traffic is increasingly dense, and our surroundings are polluted by toxic gases and noise. Consequently, many

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traffic accidents occur as well. Although the causes of accidents vary, in Slovenia the majority of them are due to unsuitable speed (19% of accidents) and the use of alcohol (15% of accidents) (Hribernik, 1995). In many other states in Europe the situation is very similar. It is impossible to prohibit selling alcohol and avoid speeding. Therefore, we must make changes in other fields. In addition to building better roads, we can also improve the safety of motor vehicles in terms of other strategies. For these reasons, experts all around the world carry out a large number of investigations, perform tests on vehicles and their parts, and then develop better technology and legislation to make improvements on motor vehicles. Besides the legislation, there are also the rules that regulate the implementation of equipment and specify vehicles' safety features in use.

In the European Union there are a lot of regulations and rules regulating the conditions and technical prescriptions for the producers of motor vehicles and their trailers and all types and variants of those vehicles have to be type-approved before selling in the market (The Acquis..., 2006). However, these regulations and rules are valid not only for road motor vehicles and their trailers, but also for agricultural and forestry tractors. These vehicles can be operated on roads, on fields, and in forests. Therefore, accidents with tractors happen very often for a variety of reasons.

Tractors are nowadays present on almost every farm in Slovenia and in the world. According to the statistical data (FAO, 2004), there are 116 tractors per 100 farms in Slovenia while there are different rates of tractor population in other developed states such as Portugal – 51, Italy – 59, Austria – 132, France – 158, and Great Britain – 205. Furthermore, there are 0.25 tractors / ha in Slovenia, which is ahead of some EU Member States such as France with 0.08 tractor/ha, and Austria with 0.10/ha).

A lot of drivers or their attendees are killed every year in tractor accidents in the agricultural and forestry industries. There were 821 fatalities related to tractor accidents in the Republic of Slovenia from 1981 to 2006 (Jerončič, 2008). Fortunately, the number of victims has been declining yearly. As the tractors are used on public roads, macadamised roads, and fields, these accidents could happen anywhere. Particularly, because a tractor is a slow vehicle, it is often in danger when it is on congested public roads. Even worse is that the tractors run mainly on rural roads where the driving conditions are rough and unpredictable.

We divide the accidents related to agriculture and forestry tractors into traffic accidents and working accidents. A traffic accident occurs when a tractor is on public roads, while a working accident occurs at the workplace on a farm, field, meadow, or forest etc (Hribernik, 1995).

## 2 THEORETICAL CONSIDERATIONS

There are many reasons that cause agricultural and forestry tractors accidents. We will divide them into those related to:

(1) the driver (inexperience, alcohol, incorrect reaction of the driver …),
(2) the tractor (technical conditions of the vehicle, maintenance …),
(3) the driving conditions (slippery driving surface, bad weather conditions …).

The most frequent cause of tractor accidents in agricultural and forestry industries is unprofessional operation, such as driving a tractor with inappropriate technique, speeding or overturning on a slope or incompetent maintenance and repairs, replacement of pneumatic tyres, or fuel filling.

Accidents resulting from overturning of tractors represent about 40% of all tractor related accidents in Slovenia (Hribernik, 1995). A tractor may be unstable on an uneven surface because the tractor has a relatively high centre of gravity, a short wheelbase, and a narrow track width. The stability of the tractor (static and dynamic) changes due to several reasons such as the slope and characteristic of the driving terrain, inappropriate speed in certain conditions, skidding of the driving wheels, the amount of the force on pulling rod, dramatic changes of movement of the tractor like standstill – driving, driving – inappropriate stopping.

Although tractor manufacturers build safer tractors, they cannot, presently, build a tractor that can "recognize" a dangerous situation. Implementation of ROPS, safety arcs, cabins, or safety belts reduces the number of victims, but the development of new tractors also continues to challenge safety structure capabilities. Overturning could still happen because of fast driving, sharp turning, sudden obstacles such as rocks and holes on the road, driving over canals, pushing by towing trailer, and inappropriate use of front loading devices (Ayers, 1992; Hoppe, 2005; Hunter, 1991).

In order to evaluate the influence of terrain on a tractor accident, the authors have developed a coefficient of the topography based on topography or on the mountain area out of the country area (Jerončič, 2008). The topography is defined:

$$\text{Coefficient of the topography} = \begin{cases} 0; \text{when the terrain} \\ \text{ is all of flat} \\ 1: \text{when the areas} \\ \text{ are all in the mountain} \end{cases}$$

The diagrams below demonstrates the definition of the coefficient of the topography. For instance, if the coefficient of the topography is 0.4, this means that the approx. 40% of the country area is less than 200 meters high, and approx. 60% of the country area is over 200 meters high (above the sea level). The topography is included as a variable in this study. The average altitude of the Netherlands is very low, a few meters above sea level. In such case the coefficient of the topography is close to 0 (Fig. 1). The average altitude of Poland is medium, there are flat and low places and there are also mountains. In this case the coefficient of the topography depends of the percentage of the mountain area versus the flat and low areas (Fig. 2). The average altitude of the Switzerland is very high. There are a lot of mountains, therefore the coefficient of the topography is close to 1 (Fig. 3).

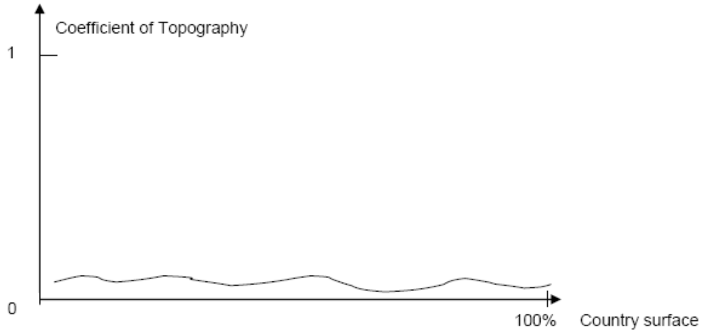

**Figure 1:** Example for a very flat country (i.e. the Netherlands)

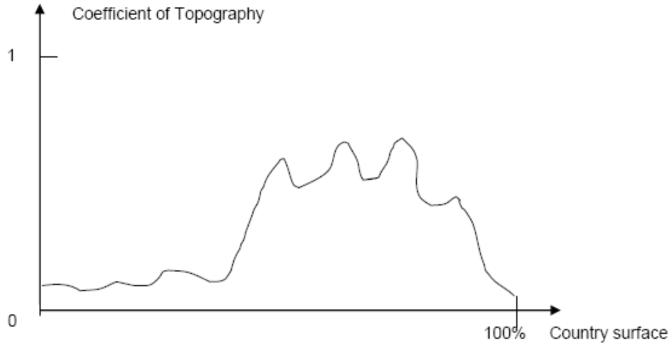

**Figure 2:** Example for a country with flat places and some mountains (i.e. Poland)

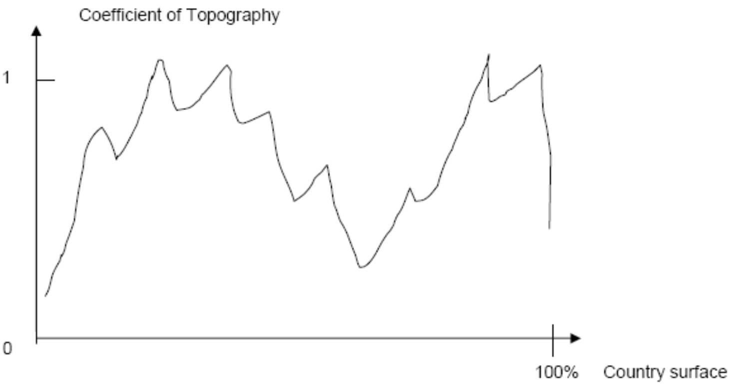

**Figure 3:** Example for a country with a lot of mountains (i.e. Switzerland)

## 3 MATERIALS AND METHODS

Most of the data about the number of deaths in accidents related to agricultural and forestry tractors was gathered with the inquiry, the others was acquired from the Web-based EU data base CARE (Jerončič, 2008). Questionnaires were sent to the authorities and/or statistical institutions in other EU Member States.

As mentioned before we have to estimate the topography of countries that the data of deaths in accidents related to agriculture and forestry tractors was gathered. We are able to estimate the coefficient of the topography. This may be a subjective estimation or presumption, but we took it into account as a real estimation of the form of the landscape. For this reason we took into account that the confidence of this data is only 90%. The coefficient has values between 0 and 1. For a country with a lot of mountains it is closer to 1, and for a very flat country it is closer to 0. Values were estimated and defined based on the maps of the countries (Table 2) (Jerončič, 2008). From the value of the GDP, we could make inferences about the economic development level of the country where technical characteristics of the tractors and qualification of the drivers are considered. The coefficient of the development of the country was calculated, relative to the biggest value for Luxemburg from selected countries (Table 3)..

**Table 1:** Deaths in traffic accidents with agricultural and forestry tractors

| | 1990 | 1991 | 1992 | 1993 | 1994 | 1995 | 1996 | 1997 | 1998 | 1999 |
|---|---|---|---|---|---|---|---|---|---|---|
| **SLOVENIA** | 18 | 8 | 11 | 12 | 13 | 8 | 6 | 9 | 7 | 6 |
| **FINLAND** | - | 2 | 7 | 3 | 1 | 5 | 5 | 4 | 1 | 3 |
| **AUSTRIA** | - | 24 | 13 | 18 | 20 | 20 | 15 | 7 | 13 | 12 |
| **NETHERLANDS** | - | 7 | 1 | 5 | 3 | 1 | 2 | 2 | 2 | 4 |
| **SWEDEN** | - | 5 | 4 | 2 | 1 | 5 | 0 | 3 | 3 | 6 |
| **GERMANY** | - | - | 115 | 112 | 94 | 107 | 134 | 112 | 101 | 106 |
| **LUXEMBURG** | - | - | - | - | - | - | - | 1 | 1 | 0 |
| **GREAT BRITAIN** | - | - | - | - | - | - | - | - | 55 | 44 |
| **PORTUGAL** | - | - | - | - | - | - | 34 | 36 | 40 | 35 |
| **ESTONIA** | 32 | 23 | 12 | 14 | 17 | 9 | 4 | 4 | 9 | 1 |
| **LATVIA** | - | - | - | - | - | 14 | 7 | 12 | 15 | 10 |
| **POLAND** | 175 | 123 | 115 | 111 | 93 | 98 | 68 | 84 | 82 | 72 |
| **SWITZERLAND** | - | - | - | - | - | - | 27 | 26 | 26 | 24 |
| **SERBIA** | 107 | 94 | 80 | 69 | 74 | 66 | 72 | 106 | 81 | 55 |
| **BELGIUM** | 0 | 4 | 2 | 1 | 2 | 4 | 3 | 2 | 3 | 4 |
| **DANMARK** | - | 3 | 3 | 3 | 4 | 2 | 0 | 0 | 3 | 2 |
| **FRANCE** | - | 43 | 37 | 28 | 32 | 32 | 36 | 35 | 22 | 20 |
| **GREECE** | - | 114 | 93 | 79 | 99 | 81 | 44 | 80 | 61 | 71 |
| **ITALY** | - | 51 | 40 | 42 | 47 | 30 | 38 | 31 | 23 | 38 |
| **SPAIN** | - | 43 | 40 | 23 | 26 | 30 | 36 | 33 | 30 | 32 |
| **CROATIA** | - | - | - | - | - | - | 20 | 14 | 8 | 3 |
| **MACEDONIA** | - | - | - | 15 | 11 | 13 | 5 | 14 | 4 | 15 |

| | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 |
|---|---|---|---|---|---|---|---|---|
| **SLOVENIA** | 5 | 10 | 4 | 4 | 9 | 4 | 2 | - |
| **FINLAND** | 4 | 2 | 2 | 3 | 1 | 0 | 2 | 0 |
| **AUSTRIA** | 19 | 15 | 12 | 10 | 7 | 8 | 8 | 9 |
| **NETHERLANDS** | 3 | 1 | 2 | 5 | 5 | 5 | 1 | 2 |
| **SWEDEN** | 4 | 0 | 3 | 2 | 5 | 1 | 3 | 1 |
| **GERMANY** | 95 | 96 | 104 | 110 | 87 | - | - | - |
| **LUXEMBURG** | 2 | 0 | 0 | 0 | 0 | 0 | - | - |
| **GREAT BRITAIN** | 53 | 4 | 38 | 51 | 7 | 0 | - | - |
| **PORTUGAL** | 42 | 38 | 38 | 27 | 35 | 33 | 41 | 32 |
| **ESTONIA** | 2 | 7 | 4 | 2 | - | 2 | 0 | 0 |
| **LATVIA** | 7 | 10 | 9 | 11 | 10 | - | - | - |
| **POLAND** | 72 | 62 | 47 | 61 | 57 | 67 | - | - |
| **SWITZERLAND** | 28 | 23 | 20 | 25 | 18 | 13 | - | - |
| **SERBIA** | 58 | 77 | 74 | 65 | 71 | 58 | 54 | - |
| **BELGIUM** | 5 | 0 | 1 | 2 | 3 | 1 | 2 | - |
| **DANMARK** | 3 | 1 | 0 | 0 | 0 | 3 | 2 | 1 |
| **FRANCE** | 26 | 12 | 20 | 25 | 13 | 12 | 12 | 11 |
| **GREECE** | 54 | 72 | 43 | 46 | 37 | 26 | 29 | - |
| **ITALY** | 28 | 24 | 23 | 24 | 23 | 23 | - | - |
| **SPAIN** | 35 | 28 | 16 | 23 | 40 | 26 | 32 | - |
| **CROATIA** | 10 | 13 | 14 | 8 | 10 | 13 | - | - |
| **MACEDONIA** | 7 | 7 | 10 | 2 | - | - | - | - |

Note: "-" in the table indicates no data available

**Table 2:** The coefficient of the topography (Jerončič, 2008)

| Country | Coefficient of the topography – estimation (*) | Country | Coefficient of the topography – estimation (*) |
|---|---|---|---|
| Netherlands | 0.05 | Greece | 0.5 |
| Estonia | 0.05 | Germany | 0.6 |
| Denmark | 0.05 | Portugal | 0.6 |
| Latvia | 0.1 | France | 0.6 |
| Great Britain | 0.2 | Spain | 0.6 |
| Belgium | 0.2 | Croatia | 0.6 |
| Finland | 0.3 | Slovenia | 0.7 |
| Sweden | 0.4 | Italy | 0.7 |
| Luxemburg | 0.4 | Macedonia | 0.8 |
| Poland | 0.4 | Austria | 0.8 |
| Serbia | 0.4 | Switzerland | 0.9 |

(*) the confidence of the data is 90%

**Table 3:** The GDP of the countries (Tosi et al., 2002)

| Country | The development of the country | | Country | The development of the country | |
|---|---|---|---|---|---|
| | GDP in USD | Coefficient | | GDP in USD | Coefficient |
| Serbia | 900 | 0.039 | Italy | 17180 | 0.753 |
| Croatia | 2640 | 0.116 | Austria | 17500 | 0.767 |
| Latvia | 4480 | 0.196 | Netherlands | 17940 | 0.786 |
| Poland | 4920 | 0.216 | Great Britain | 17980 | 0.788 |
| Estonia | 6460 | 0.283 | Belgium | 18040 | 0.79 |
| Greece | 8870 | 0.389 | Sweden | 18580 | 0.814 |
| Slovenia | 9352 | 0.41 | France | 18670 | 0.818 |
| Portugal | 10190 | 0.446 | Denmark | 19860 | 0.87 |
| Spain | 13120 | 0.575 | Switzerland | 22080 | 0.967 |
| Finland | 16140 | 0.707 | Luxemburg | 22830 | 1 |
| Germany | 16580 | 0.726 | | | |

## 4 RESULTS AND ANALYSIS

As already indicated, the data has been collected in several European countries. Ideally, a more reliable analysis could be performed if we could have information for every accident case and for the essential parameters such as type of tractor, age of tractor, condition of the driver, education of driver etc. As this study is hard to obtain and trace all of the accident information, the analysis was performed on the basis of certain assumptions.

Direct comparison based on statistics about accidents or deaths and injuries is not feasible. We had to take into consideration major factors that influence data comparison. Firstly, the number of tractors in use in the countries was considered, and therefore it is obvious that more tractors in operation consequently result in more accidents. Furthermore, it is also important that the data is based on the number of tractors per area. The data indicates that the density of tractors in an area results in a higher potential rate of accidents and deaths. For example, in Germany there are almost 1.2 million agricultural and forestry tractors in use and the number of deaths in accidents is every year more than 100 (Schauer, 2004; Schelo, 2005). On the other hand, in the Netherlands there are only about 150.000 agricultural and forestry tractors in use and there are only 5 deaths per year in accidents with those vehicles (Jerončič, 2008). The comparison of the economic development level of the countries in terms of GDP can induce several other factors such as technical development of the vehicle fleet, the education of the drivers, the purpose for which the tractors are used, driving culture, and consciousness of the drivers.

Moreover, it is very important that the analysis is based on the number of accidents and deaths pertaining to a landscape in the country. It is clearly shown that flat countries such as The Netherlands and Denmark have a much lower number of tractor overturns than the countries with more mountain (Switzerland, Austria, etc…). Although the number of tractors in use varied with years, the number in a reference year was used for analysis because the change of number of tractors in percentage within a few years was so small that it does not mean a substantial calculating mistake.

### 4.1 INFLUENCE OF FACTORS

We could analyse the relationships from calculations for the purpose of correlations. If the value of the correlation is 1, then it indicates a full linear mathematical connection among variables. If the value of the correlation is 0, then it means that there is not a linear connection among variables.

Since we have the most complete mortality data for 2000, we can accurately calculate the correlation for this year. For the calculations of the correlation the software MATLAB has been used. The correlation for the number of deaths per area is at the coefficient of the topography 0.2735 and at the coefficient of GDP 0.1637. Furthermore, the decision was made to calculate also the correlation for a period of years. If we account the data for a period of 5 years (1996 – 2000), the correlation is at a coefficient of the topography 0.2654. It is unnecessary to calculate the correlation at the coefficient of GDP because the value of GDP changes within only a few years and this causes an additional impact on the calculation. They probably do not change much on a relative scale.

In the next step, calculations of the correlation at the coefficient of the topography were done. The correlation for the year 2000 between the number of deaths and number of tractors is for the coefficient of the topography 0.1762 and at the coefficient of GDP -0.0608. Also here we calculated the correlation for a longer period than one year. If we consider the data for a period of 5 years (1996 – 2000), the correlation will be for the coefficient of the topography 0.0924.

Based on these results, we can conclude that the relief of the surface and the value of GDP have almost no influence on the number of deaths with agricultural and forestry tractors. The significant influence on the number of deaths related to agricultural and forestry tractors are the tractor characteristics and the driver. Indirectly, this indicates the important influence of legislation on traffic safety and the reduction of the number of deaths.

According to these facts, we can now compare the number of deaths in different countries without multiplication of data with the coefficients of the relief and the GDP.

### 4.2 COMPARING OF COUNTRIES

Comparing Austria, Serbia and Slovenia, we can find considerable geographic similarities. In all countries, there is a rugged landscape and an extensive alpine area where the driving conditions for tractors are always very problematic. However, the situation regarding the legislation in these countries is very different. Namely, with legislation the safety elements to be fitted in the tractor are prescribed and therefore tractors are safer for drivers and other participants in road traffic. Austria had started to regulate agricultural and forestry tractors long ago. On the other hand, Serbia is geographically different because there are plains in Vojvodina in the North of Serbia and a very rugged surface in the central and southern regions. Serbia has had a substantial delay for legislation and does not follow the development of legislation. We can compare this with all three types of reference data and divide the number of deaths in accidents with the agricultural and forestry tractors by the number of tractors, by the size of the area that is cultivated, and by the number of residents.

There is a difficulty in defining the correct number of agriculture and forestry tractors in Slovenia. According to the data from the Ministry of the Interior on 31.08.2005, there are 87,617 registered agricultural and forestry tractors in Slovenia (Jerončič, 2008); however, the experts who work in the agriculture field estimate there are actually 130,000 tractors in use (Hribernik, 1995). Therefore, we must compare the countries, take into account both data, and the area between these values.

## 5 DISCUSSION

All three diagrams *(Fig. 6, 7 and 8)* indicate that Austria has a lower death rate in accidents with agricultural and forestry tractors than Serbia does . If we look at the number of deaths divided by the number of tractors we could see Slovenia has an average from 1.82 to 2.69 times (depending on the number of the tractors being calculated) more deaths than Austria. The death rate in Serbia is 4.90 times more than in Austria. If the number of deaths is divided by the area, we could see Slovenia has an average 2.28 times more deaths than Austria, and Serbia is 3.39 times more than Austria. The death rate per citizen in Slovenia is an average 2.27 times more than in Austria, and Serbia is 4.91 times more than in Austria.

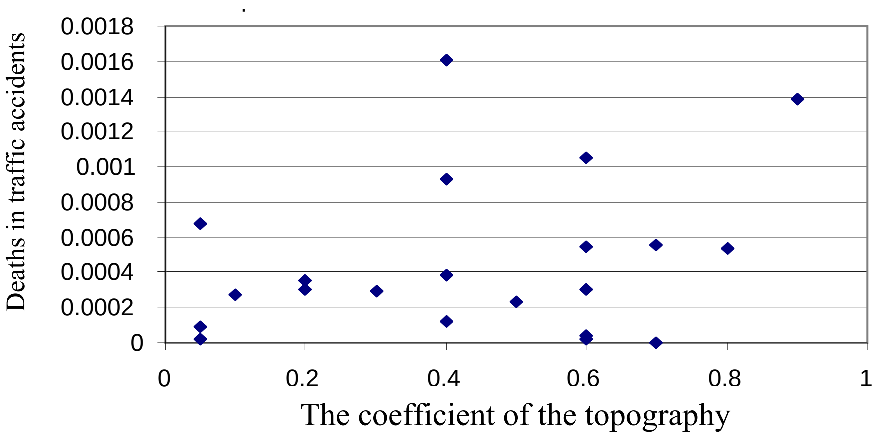

**Figure 4:** The graphical review of the regression between the data (topography)

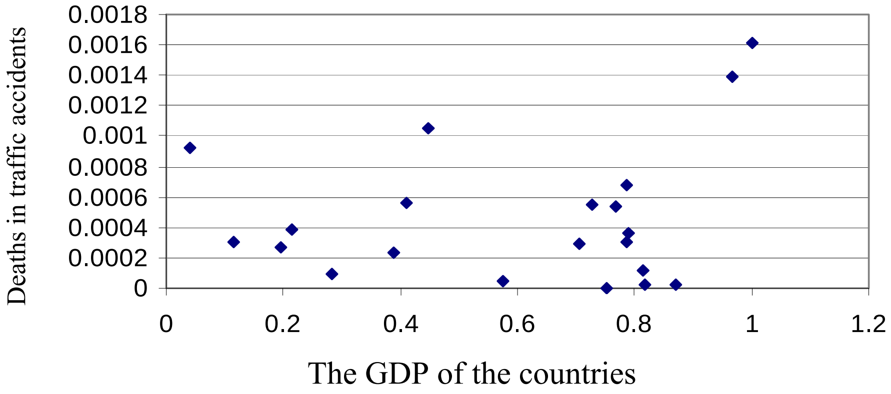

**Figure 5:** The graphical review of the regression between the data (GDP)

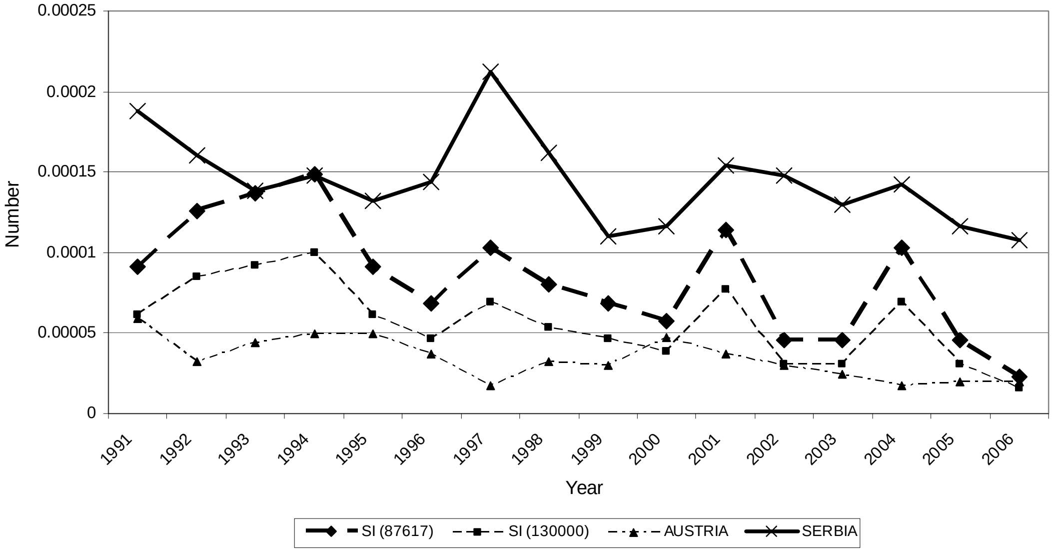

**Figure 6:** The comparison of reference data (deaths dividing by the number of tractors) between Slovenia, Austria and Serbia

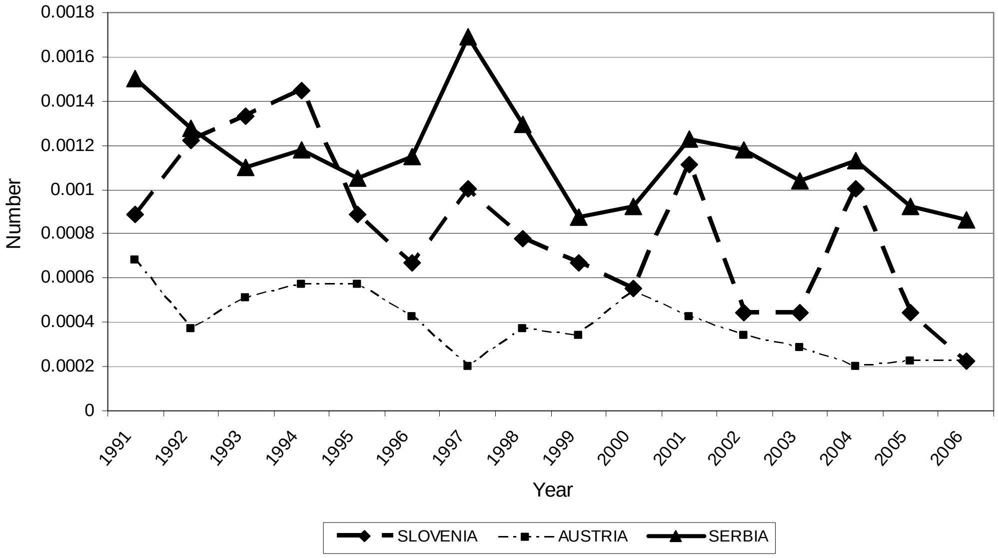

**Figure 7:** The comparison of reference data (deaths dividing by the area) between Slovenia, Austria and Serbia

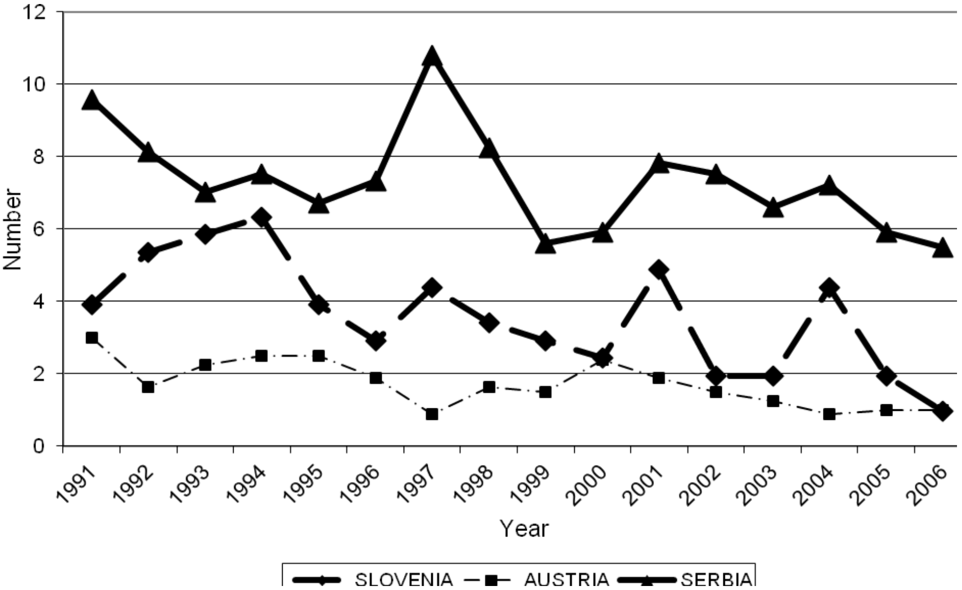

**Figure 8:** The comparison of reference data (deaths dividing by the number of residents) between Slovenia, Austria and Serbia

Although Slovenia and Austria have very similar landscape and therefore also very similar coefficient of the topography, it was indicated that this does not have a significant influence on the number of deaths in accidents with agricultural and forestry tractors. In Slovenia, the number of deaths in accidents with those vehicles is much higher than in Austria. But if we compare the regulation for agricultural and forestry tractors we could find out the following interesting result. Austria started to regulate motor vehicles with the prescriptions in terms of the first act on motor vehicles about 1923 (Wurst, 2004). This was almost 60 years earlier than Slovenia did. This reflects the fact that despite very similar geographic landscape the number of deaths with agriculture and forestry tractors in Austria is significantly less than in Slovenia.

Serbia started to regulate motor vehicles with the prescriptions at the same time as the whole of Yugoslavia and Slovenia. However, Slovenia took a big step in the years after 1993 when the legal system started systematically upgrading and implementing the international UN/ECE regulations into the national legislation. Serbia has remained on the same level as before. Furthermore, in their market as the major tractor manufacturer, IMT, does not follow the development of the technique, and therefore their tractors do not comply with the approved valid prescriptions.

## 6 CONCLUSIONS

This research has conducted a comprehensive analysis of the impact of various factors on the death rate related to tractors in agricultural and forestry industry and demonstrates quite a surprising finding. Neither the relief of the country or the landscape where the agricultural and forestry tractors are used, nor the economic development level of the country have a significant influence on the number of deaths in accidents related to the agricultural and forestry tractors. The most significant influence on the number of deaths is the tractor and the driver. Indirectly, this indicates that the influence of the legislation on motor vehicles is when some countries start to implement conformity assessment of tractors before they offer products to the market and the yearly technical inspections of tractors in use. In the EU Member States each type of tractor with all of its variants has to be type-approved before being offered for sale. That means that every type of tractor undergoes several tests and checks and then the EU Whole vehicle type approval document is issued. This area has gradually been regulated, therefore improving the safety of traffic, and reducing the number of deaths related to tractors.

Furthermore, the implementation of the prescriptions possibly and gradually halves the number of deaths if we compare Austria and Slovenia. With the same approach, it is possible to predict what the future outcome should be in Serbia with the full implementation of all of the relevant prescriptions to agricultural and forestry tractors. After approximately 15 years the number of deaths in accidents related to agricultural and forestry tractors will be reduced by 50%.

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# Evaluation of cacao-pudding as a probiotic food carrier and sensory acceptability properties

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## ABSTRACT

A number of health benefits have been claimed for probiotic bacteria such as *Lactobacillus acidophilus*, *Bifidobacterium* spp. and *Lactobacillus casei*. These benefits include anti-mutagenic effects, anti-carcinogenic properties, improvement in lactose metabolism, reduction in serum cholesterol and immune system stimulation. Because of the potential health benefits, these microorganisms are increasingly being incorporated into dairy foods. Several studies in recent years have shown the benefits deriving from the ingestion of probiotics and a large number of products containing lactobacilli and bifidobacteria formulated. The purpose of this study was to develop a pudding with cacao to which probiotic microorganisms were added and investigate the viability of probiotic microorganisms during the shelf-life along 25 days at 4 °C. Organoleptic properties of the puddings were also evaluated during the storage. *Bifidobacterium animalis* ssp. *lactis* LAFTI B94, *Lactobacillus acidophilus* LAFTI L10 and *Lactobacillus casei* LAFTI L26 cultures were activated and incorporated into the product. Pudding with cacao was shown to be a good vehicle for the delivery of *Bifidobacterium animalis* ssp. *lactis*, *Lactobacillus acidophilus* during 15 and 25 days respectively and these microorganisms did not interfere in the sensorial preferences of the product except *Lactobacillus casei.* The pH values and organoleptic scores of the pudding samples, except *Lactobacillus casei* LAFTI L26 containing ones, did not change for 20 days during the storage period, statistically (P<0.01). The pudding containing *Lactobacillus casei* LAFTI L26 was taken the lowest sensorial scores. The all samples were lost their organoleptic properties at the 25 days of storage period.

**Key words:** Probiotic cacao-pudding, *Lactobacillus acidophilus, Bifidobacterium animalis* ssp. *lactis* , *Lactobacillus casei.*

## IZVLEČEK

### SPREJEMLJIVOST KAKAVOVEGA PUDINGA KOT OSNOVE ZA PROBIOTIČNE PREHRANSKE IZDELKE

Probiotične bakterije kot so *Lactobacillus acidophilus*, *Bifidobacterium* spp. in *Lactobacillus casei* so pomembne za pripravo živil, primernih za prehrano za varovanje zdravja. Varujejo lahko pred mutagenimi in rakotvornimi učinki, izboljšajo presnovo laktoze, prispevajo k zniževanju holesterola in stimulirajo imunski sistem. Zaradi potencialnega pomena za zdravje te mikroorganizme čedalje bolj uporabljajo pri pripravi mlečnih izdelkov. Več raziskav je v zadnjih letih pokazalo prehransko prednost uživanja probiotikov; razvili so vrsto izdelkov, zasnovanih na uporabi laktobacilov in bifidobakterij. Namen te raziskave je bil uvesti kakavov puding z dodatkom probiotskih mikroorganizmov in ugotoviti viabilnost probiotskih mikroorganizmov tekom roka trajanja 25 dni pri 4 °C. V času skladiščenja so bile preverjene tudi organoleptične lastnosti pudingov. Pri pripravi izdelkov smo uporabili kulture *Bifidobacterium animalis* ssp. *lactis* LAFTI B94, *Lactobacillus acidophilus* LAFTI L10 in *Lactobacillus casei* LAFTI L26. Kakavov puding se je izkazal kot primerna osnova za živila z *Bifidobacterium animalis* ssp. *lactis*, *Lactobacillus acidophilus* tekom 15 in 25 dni in razen mikroorganizma *Lactobacillus casei* niso neugodno vplivali na senzorične lastnosti izdelkov. Vrednosti pH in organoleptične lastnosti vzorcev pudingov se, razen enega z *Lactobacillus casei* LAFTI L26, se tekom 20 dnevnega skladiščenja niso statistično značilno spreminjale (P<0,01). Puding z *Lactobacillus casei* LAFTI L26 je imel najnižjo senzorično vrednost. Vsi vzorci so po preteku 25 dni skladiščenja izgubili značilne senzorične lastnosti.

**Ključne besede:** Probiotični kakavov puding, *Lactobacillus acidophilus, Bifidobacterium animalis* ssp. *lactis*, *Lactobacillus casei.*

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## 1 INTRODUCTION

The definition of probiotics has been started during the past decade. In general, it means that a food product which contains live organisms and very beneficial for the consumer's health (Rössle et al., 2010; Lamsal and Faubion, 2009; Jay et al., 2005). The improvement of nutritional value of foods, inhibition of enteric pathogens, and alleviation of diarrhea/constipation, hypocholesterolaemic action, anticancer activity and stimulation of immune systems are beneficial effects of lactic acid bacteria which are determined mainly (Ibrahim et al., 2010; Adams and Moss, 2008; Viena et al., 2008). The most commonly used bacteria in probiotic additives are *Lactobacillus* and *Bifidobacterium*. In *Lactobacillus*, probiotic properties are present only in a restricted number of species (Elahi et al., 2008). Probiotic bacteria like bifidobacteria and lactobacilli are natural inhabitants of the human gut. They affect human health by improving the gut microbiota balance beneficially and the defenses against pathogens. The health benefits derived by the consumption of foods containing *Lactobacillus acidophilus*, *Bifidobacterium* and *Lactobacillus casei* are well documented (Shah, 2007).

One of the well-researched probiotic bacteria is *Lactobacillus acidophilus* and it is able to inhibit enteropathogens, such as *Salmonella, Listeria* and *Campylobacter*. The other most known probiotic is *Bifidobacterium lactis* Bb-12. This bacterium was originally isolated from the feces of a healthy adult and has been marketed for more than 15 years in a wide range of fermented dairy and non-dairy products. *Bf. lactis* Bb-12 has been reported to reduce the incidence of rotavirus diarrhea antibiotic-associated diarrhea and travelers' diarrhea (Espinoza and Navarro, 2010; Kun et al., 2008; Ouwehand et al., 2004). *Lactobacillus casei* has good viability in the matrices and it has probiotic features, e.g. survival in the human gastrointestinal tract and an antimicrobial effect against pathogens (Shah, 2007).

Because of the potential health benefits these microorganisms are increasingly being incorporated into dairy foods. Functional foods are defined as 'foods that contain some health-promoting component(s) beyond traditional nutrients'. Foods can be modified to become functional is by addition of probiotics (Shah, 2007). These products should have acceptable taste and suitable for the production of commercial products, too (Kearney et al., 2008). In 2001, a joint committee Food and Agriculture Organization of the United Nations/ World Health Organization (FAO/WHO) redefined probiotics as "live microorganisms in adequate amounts and confer health benefits on the host. Probiotic microorganism viability and efficacy in food products are also important during the storage. In general, the food industry has applied the recommended level of $10^6$ cfu/g at the time of consumption for *Lactobacillus acidophilus*, bifidobacteria and other probiotic bacteria (Aragon-Alegro, 2007; Ross et al., 2005; Helland et al. 2004).

The dairy industry has found probiotic cultures to be a tool for the development of new functional products. Yoghurts and fermented milks are the main vehicles for probiotic cultures. In some studies have shown that some commercial dairy products such as yoghurts, do not contain adequate populations of viable probiotic bacteria during their shelf-life (Heenan et al., 2004). But nowadays, some new products such as milk-based desserts, powdered milk for newborn infants, ice-creams, butter, mayonnaise, various types of cheese, products in the form of capsules and fermented foods of vegetable origin are being found place in the international markets. There is an increased variety of products available in the market and consumers are getting more used to the probiotic concept (Ranadheera et al., 2010; Ozcan et al., 2010; Cruz et al., 2009).

The purpose of this study was to develop a pudding with cacao to which probiotic microorganisms were added and investigate the viability of probiotic microorganisms during the shelf-life along 25 days at 4 °C. Organoleptic properties of the puddings were also evaluated during the storage. *Bifidobacterium animalis* ssp. *lactis* LAFTI B94, *Lactobacillus acidophilus* LAFTI L10 and *Lactobacillus casei* LAFTI L26 cultures were activated and incorporated into the product.

## 2 MATERIALS AND METHODS

### 2.1 Probiotic Cultures, Media and Growth Conditions

Probiotic cultures of *Lb. acidophilus* LAFTIs L10 and *Bf. animalis ssp. lactis* LAFTI B94 and *Lb. casei* LAFTI L26 were obtained from DSM Food Specialties, Istanbul, Turkey. Ten grams from the each culture was weighed and shaked into 20 mL sterilized water for obtaining a homogenized culture and then immediately added and mixed for 1 kg of of cacao-pudding, separately. The cacao-puddings contained about $10^8$ – $10^9$ cfu probiotic microorganism/g.

MRS (deMann, Rogosa and Sharpe) D-sorbitol (10 g/100 mL) media (Tharmaraj and Shah, 2003) was used for the selective enumeration of *Lb. acidophilus* at 37 °C for 72 h. For selective enumeration of *Bf. animalis ssp. lactis*, RCA (reinforced clostridial agar) with 0.03 g/100mL aniline blue and dicloxacillin (2 mg/mL, Sigma) were used. Plates were incubated under the anaerobic conditions at 37 °C for 48 h (Kailasapathy et al., 2008). MRS-Vancomycine agar was used for enumeration of *Lb. casei*, for preparing MRS-Vancomycine agar, 2 ml of 0.05 g vancomycine (Sigma)/100 ml solution was added to 1 L of MRS broth to obtain 1 mg/L final concentration. Agar powder was added to broth and the media were autoclaved. Inoculated plates in duplicates were incubated under anaerobic conditions at 43 °C for 72 h. The average count of the duplicate plates was used for statistical analysis.

### 2.2 Production of Cacao-Pudding

Full-fat (4.5 % fat) standardized and pasteurized milk, cacao powder, sucrose, corn starch and wheat-flour were used for preparing of cacao-pudding. Four pilot-scale cacao-pudding trials denoted; (C) control, (PA) probiotic cacao-pudding with *Lb. acidophilus* culture, (PB) probiotic cacao-pudding with *Bf. lactis* culture and (PC) cacao-pudding with *Lb. casei* culture, were produced in triplicate. The ingredients and quantities employed for the trials of puddings are seen in Table 1.

**Table 1.** Ingredients and quantities (%) employed for the production the cacao- pudding trials.

| **Ingredients** | **Trials** | | | |
|---|---|---|---|---|
| | **C** | **PA** | **PB** | **PC** |
| **Full fat milk (4.5 % fat)** | 75.10% | 75.09% | 75.09% | 75.09% |
| **Sucrose** | 15.03 % | 15.03 % | 15.03 % | 15.03 % |
| **Cacao powder** | 2.36 % | 2.36 % | 2.36 % | 2.36 % |
| **Flour** | 4.51 % | 4.51 % | 4.51 % | 4.51 % |
| **Corn starch** | 3.00 % | 3.00 % | 3.00 % | 3.00 % |
| ***Lb. acidophilus* LAFTI L10 DSL** | - | 0.01% | - | - |
| ***Bf. lactis* LAFTI B94 DSL** | - | - | 0.01 % | - |
| ***Lb. casei* LAFTI L26 DSL** | - | - | - | 0.01 % |
| **TOTAL** | 100 | 100 | 100 | 100 |

Each batch of cacao-pudding was produced in amounts to obtain 4 kg of the final product. For this purpose, after weighing all ingredients individually, they were all mixed together, and heated to 80–85 °C in a stainless-steel boiler about 20 min, after it was cooled to 40 °C in a water bath being continuously stirred. As soon as the mixture reached the desired temperature, cacao-pudding was separated as 4 batches. One batch was remained as control group without any probiotic culture. The probiotic cultures were added into other three batches and stirred homogenously with a sterile mixer. After the stirring of cacao-puddings, they were packaged in individual plastic cups, each one containing approximately 150 g of cacao- pudding, cooled and then stored at 4±1 °C for up to 25 days.

### 2.3 Microbiological analysis

Viabilities of *Lb. acidophilus*, *Bf. lactis* and *Lb. casei* were monitored during the storage period (1., 3., 5., 10., 15., 20., 25. days) for cacao-puddings PA, PB and PC. Populations of the contaminants yeasts and moulds, coliforms were also monitored for all trials of cacao-puddings studied (C, PA, PB and PC). At each sampling day, portions of 25 g were collected aseptically and blended with 225 mL of 0.1 % peptone water in sterilized blender and submitted to serial dilutions with the same diluents. For the enumeration of probiotic bacteria media and conditions which were stated at section 2.1 were applied. Potato Dextrose Agar (PDA) was used for the enumeration of yeast and moulds in samples at 25 °C for 3-5 days (Ozer et al., 2002). Total coliform counts were determined on Violet Red Bile Agar (VRBA, pH 7.4) incubated at 37 °C for 24 h under anaerobic conditions according to Martinez-Villaluenga et al. (2008). All data belonging to counts were calculated as logarithms (log cfu/g) prior to the statistical analyses.

### 2.4 Physico-chemical analysis

The pH value was recorded using a pH meter (Hanna HI221 Microprocessor, Hanna Instruments Inc., Woonsocket, Rhode-Island), at each sampling day. The moisture content was determined according to AOAC (1995) procedure.

### 2.5 Sensory Analysis

The sensory characteristics were carried out according to Metin (2006) on each day of sampling. A panel composed of 10 experienced members from our university was used to evaluate the puddings for external appearance (color), flavor, taste and texture with a point scale from 0 to 5 (0 spoiled sample and unfit for human consumption; 5, very good). PA, PB and PC samples were compared with control group puddings. The results were analyzed statistically as described in the next section.

### 2.6 Statistical Analysis

SPSS 15.0 software for windows (SPSS Inc., Chicago, Illinois, USA) was used for the statistical analyses. One-way analysis of variance (ANOVA) test was done for determining mean differences. The level of significance between means was determined by the Tukey HSD test (Ozdamar, 2004).

## 3 RESULTS AND DISCUSSION

### 3.1 Physico-chemical analysis results

Average moisture of puddings was varied between 38.9 ±0.2 – 39.2±0.3 % during the storage period.

The pH values of puddings were given in Figure 1. There were no significant differences among the PA, PB and C trial groups during the storage ($p > 0.05$). But, pH value of PC group was different significantly ($p < 0.01$) after the 15th days, because of the high growth and acid production ability of *Lb. casei* than the other bacteria throughout the storage period. The optimum pH for growth of *Bifidobacterium* is 6–7 and they could not growth at pH 4.0–5.0 or below. In similarly, survival of *Lb. acidophilus* is affected by the low pH of the environment, too. But, we suppose that the milk and other protein rich ingredients such as wheat flour increase the buffering capacity of the puddings and all the bacteria's viability didn't affect from pH (Ranadheera et al., 2010; Helland et al., 2004) in the study.

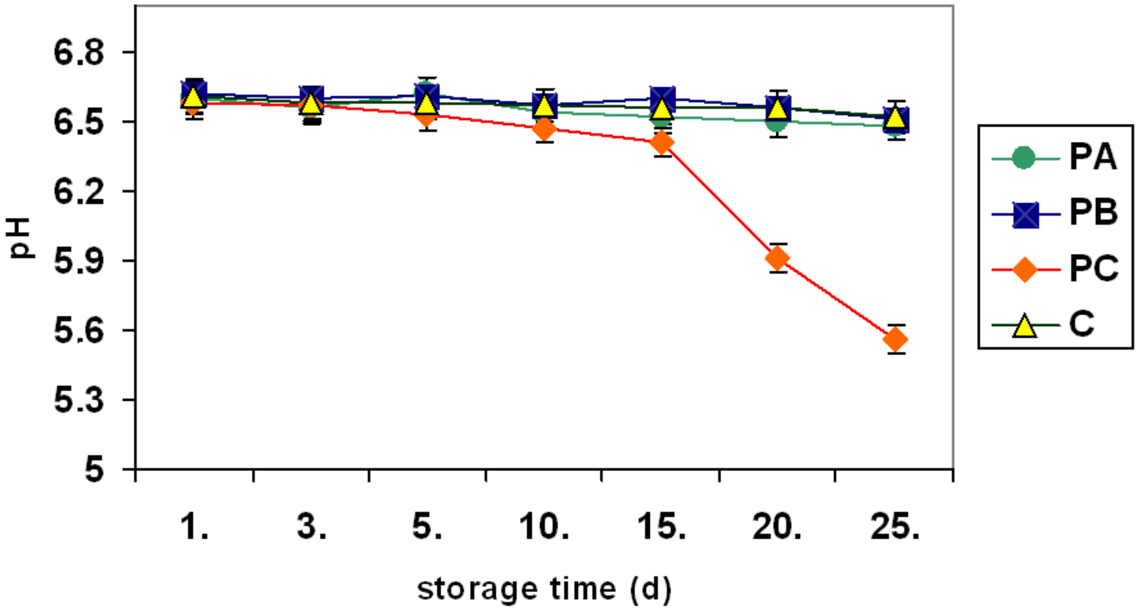

**Figure 1.** The pH values of cacao-puddings during the storage at 4°C. Data represent mean values of triplicate measurements, and error bars are indicated.
PA; Cacao-puddings are incorporated with *Lb. acidophilus*
PB; Cacao-puddings are incorporated with *Bf. lactis*
PC; Cacao-puddings are incorporated with *Lb. casei*

### 3.2 Microbial analysis results

The population of the contaminants total coliforms, yeast and moulds during storage of the different trials of cacao-puddings are shown in Table 2. Total coliforms were not detected in all puddings. Yeast and mould counts were at a level of < 1-3.9 log cfu $g^{-1}$ during the storage.

**Table 2.** Population of total coliforms and yeast-mould counts during storage of the trials of cacao puddings studied.

| **Trials** | **Days** | **Total Coliforms** | **Yeast and Moulds** [1] |
|---|---|---|---|
| C | 1. | ND | ND |
|  | 3. | ND | ND |
|  | 5. | ND | <1-3.2 |
|  | 10. | ND | <1-3.0 |
|  | 15. | ND | <1-3.5 |
|  | 20. | ND | <1-3.5 |
|  | 25. | ND | <1-3.6 |
| PA | 1. | ND | ND |
|  | 3. | ND | ND |
|  | 5. | ND | ND |
|  | 10. | ND | ND |
|  | 15. | ND | <1-3.6 |
|  | 20. | ND | <1-3.7 |
|  | 25. | ND | <1-3.8 |
| PB | 1. | ND | ND |
|  | 3. | ND | ND |
|  | 5. | ND | ND |
|  | 10. | ND | ND |
|  | 15. | ND | <1-3.3 |
|  | 20. | ND | <1-3.1 |
|  | 25. | ND | <1-3.9 |
| PC | 1. | ND | ND |
|  | 3. | ND | ND |
|  | 5. | ND | <1-3.4 |
|  | 10. | ND | <1-3.1 |
|  | 15. | ND | <1-3.3 |
|  | 20. | ND | <1-3.5 |
|  | 25. | ND | <1-3.8 |

ND; Not detected

[1] ; Minimum - maximum counts obtained for all samples analyzed.

The viability of *Lb. acidophilus*, *Bf. lactis* and *Lb. casei* during storage of different trials of cacao-puddings studied is shown in Fig.2.

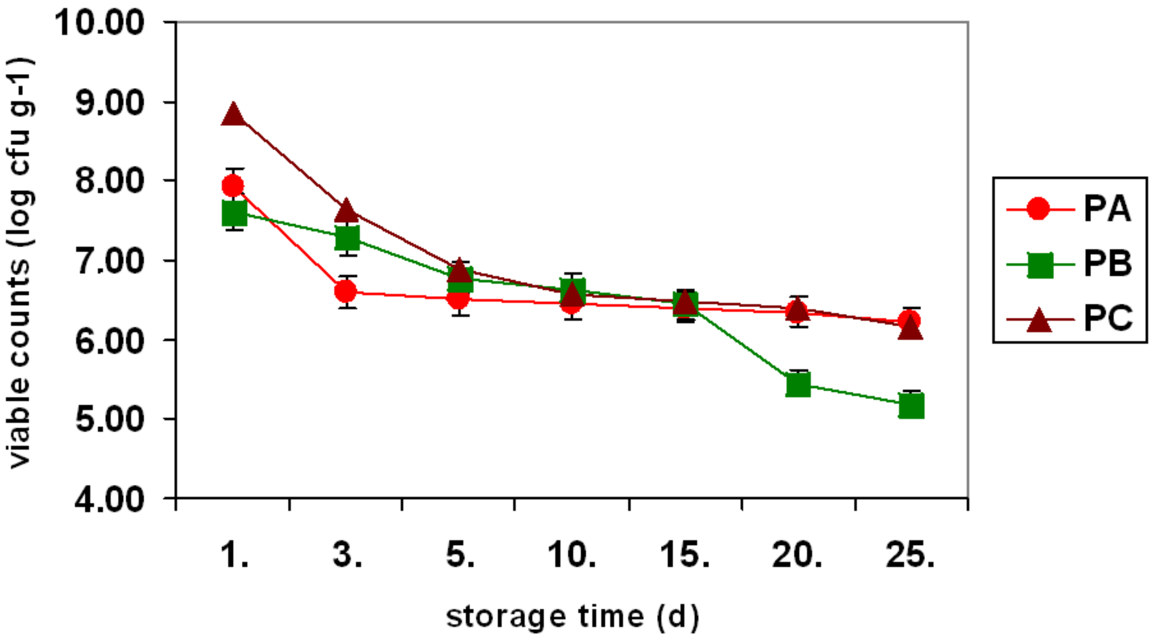

**Figure 2**. Survival of probiotic microorganisms in cacao-puddings stored at 4°C for 25 days. Data represent mean values of triplicate measurements, and error bars are indicated.
PA; Cacao-puddings are incorporated with *Lb. acidophilus*
PB; Cacao-puddings are incorporated with *Bf. lactis*
PC; Cacao-puddings are incorporated with *Lb. casei*

Initial counts of all probiotic bacteria were about 8-9 log cfu mL$^{-1}$ before incorporated into the puddings in our research. *Lb. acidophilus* maintained nearly constant populations after the 3 $^{rd}$ day during the whole refrigerated storage in cacao-puddings and above the 6 log cfu g$^{-1}$. These results indicate good and the highest viability of *Lb. acidophilus* in cacao-puddings. According to Lamsal and Faubion (2009) recommended level of probiotic microorganisms in food should be available in level of $10^6$ cfu g$^{-1}$ at the time of consumption, to provide beneficial effects for consumers. The viability of *Bf. lactis* were decreased from 7.61 to 5.19 log cfu g$^{-1}$ at the end of the storage period. The puddings with *Bf. lactis* didn't maintained probiotic property after the 15th days. There were no significant differences ($p>0.05$) for populations of *Bf. lactis* between the days 5 and 15 in the puddings. *Lb. casei* showed the good viability during the storage period in the pudding trials, too. This samples' population were very close to the counts of *Lb. acidophilus* after the 10 days.

Acid pH tolerance in probiotic bacteria is strain dependent and *Bifidobacteria* strains are more sensitive than *Lactobacillus* strain (Cruz et al., 2009). The viability of probiotics in a food matrix depends, among many factors, on the strain selected interactions between the microbial species present, production of hydrogen peroxide due to the metabolism of bacteria and acidity of the product (Cruz et al., 2009). It was reported that the most important factor affecting loss of cell viability is decreasing pH during storage (Kailasapathy et al., 2008; Shah, 2000). Probiotic bacteria populations are also influenced from the environmental temperatures. In addition to this, agitation caused from mechanical stress might result incorporation of air in a smaller population of viable cells. Most of the *Lactobacillus* and *Bifidobacterium* spp. are gut derived microorganisms. They are microaerophilic and anaerobic, respectively (Cruz et al., 2009). *B. animalis ssp. lactis* is more anaerobic than *Lb. acidophilus* and *Lb. casei*. The decrease observed in *Bifidobacteria* counts depended on incorporation of air in cacao-puddings.

In addition, lactobacilli and bifidobacteria need some complex requirements (carbonhydrates, amino acids, peptides, fatty esters and salts, etc.) to survive and that vary widely from species to species (Lamsal and Faubion, 2009).

Survival of probiotic bacteria added into dairy products were found in the researches Heenan et al. (2004) determined the suitability of non-fermented frozen vegetarian dessert as a food carrier for probiotic cultures. Their research demonstrated that this product may be used as a vehicle for probiotic bacteria especially for *Bifidobacteria* and *Lb. acidophilus*. Possemiers et al. (2010) showed that chocolate was a good carrier for oral delivery of probiotic mixture of *L. helveticus* and *B. longum* in their study. In Helland et al. (2004) study *Lb. acidophilus* La5 and 1748, *Bf. animalis* Bb12 and *Lb. rhamnosus* showed good growth and survival in milk-based puddings at 4-6 ° C for 21 days. In similarly, Ozcan et al. (2010) study showed that high levels of viable *Lb. acidophilus* LA-5 and *B. bifidum* BB-12 in rice pudding is a good source for probiotic bacteria deliver with high sensory quality.

Ranadheera et al. (2010) stated that *Lb. paracasei subsp. paracasei* LBC 82 viability in Minas fresh cheeses increased from 6.61 up to 8.22 at 5 °C during 21 days storage and the same strain of bacteria in chocolate mousse increased slightly from 7.36 up to 7.66 log cfu $g^{-1}$ at the same conditions demonstrating influences of different types of food product on probiotic growth and viability.

### 3.3 Sensory Analysis Results

As seen from sensorial scores in Table 3, the best pudding samples were the puddings with *Bifidobacteria*. The puddings containing *Lb. acidophilus* were scored with slightly lower points than bifidobacteria containing ones. The sensorial scores of control group were higher than the puddings with *Lb. casei* and lower than the puddings with *Lb. acidophilus*.

Generally, the significant changes were observed after 20 days of storage in the all pudding samples (P<0.01). The watery texture (syneresis) was designated by the panelists in the puddings with *Lb. casei* after 15 days of storage. Probably, high proteolytic activity of *Lb. casei* can be related with the texture defects in dairy products. Therefore, the lowest points were obtained from these samples with *Lb. casei*. In terms of flavor and taste, the sensorial scores of the samples with *Bifidobacteria* were even higher than the control samples.

Similarly, Akin et al. (2007) determined high sensorial scores for probiotic ice-cream trials produced with *Lb. acidophilus* and *Bf. lactis*. Cruz et al. (2009) reported that *Lb. acidophilus* showed good sensory properties in artisan strawberry ice-cream, too. But, also they stated that sensory property of the probiotic products respect to its acidity, pH at level 5.6 lead to better flavor and taste.

On the other hand, no significant differences between probiotic chocolate mousse and sweet whey cheese which were produced by addition of *Lb. paracasei* on sensory properties were determined (Aragon-Alegro et al., 2007; Madureira et al., 2008). Contrary, Majchrzak et al. (2010) reported some sensory differences between probiotic and conventional yoghurts. Cardarelli et al. (2008) emphasized that the chocolate mousse dessert is a suitable food for the delivery of bacterial probiotic strains with excellent viability and sensorial attributes. They also stated that, addition of *Lb. paracasei* did not make any effect on taste and aroma at 7 days of storage in chocolate mousses.

**Table 3.** Sensory characteristics of the cacao-pudding trials.

| DAYS | Control | | | | PB | | | | PA | | | | PC | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C$^{1}$ | F$^{1}$ | TA$^{1}$ | TE$^{1}$ | C | F | TA | TE | C | F | TA | TE | C | F | TA | TE |
| 1 | 5.0±0.2$^{a}$ | 4.9±0.3$^{a}$ | 4.5±0.2$^{a}$ | 4.5±0.5$^{a}$ | 5.0±0.6$^{a}$ | 4.8±0.2$^{a}$ | 4.8±0.4$^{a}$ | 4.7±0.4$^{a}$ | 4.9±0.6$^{a}$ | 4.6±0.2$^{a}$ | 4.6±0.5$^{a}$ | 4.5±0.6$^{a}$ | 4.7±0.3$^{a}$ | 4.4±0.3$^{a}$ | 4.4±0.5$^{a}$ | 4.2±0.1$^{a}$ |
| 3 | 4.9±0.3$^{a}$ | 4.9±0.2$^{a}$ | 4.5±0.6$^{a}$ | 4.3±0.2$^{b}$ | 5.0±0.4$^{a}$ | 4.8±0.5$^{a}$ | 4.8±0.2$^{a}$ | 4.7±0.3$^{a}$ | 4.9±0.3$^{a}$ | 4.5±0.5$^{a}$ | 4.4±0.3$^{b}$ | 4.4±0.2$^{a}$ | 4.7±0.4$^{a}$ | 4.3±0.3$^{a}$ | 4.3±0.2$^{a}$ | 4.2±0.3$^{a}$ |
| 5 | 4.9±0.4$^{a}$ | 4.5±0.5$^{b}$ | 4.4$^{a}$±0.3 | 4.3±0.2$^{b}$ | 4.9±0.3$^{a}$ | 4.8±0.3$^{a}$ | 4.6±0.6$^{b}$ | 4.7±0.6$^{a}$ | 4.6±0.4$^{b}$ | 4.3±0.4$^{b}$ | 4.4±0.6$^{b}$ | 4.4±0.2$^{a}$ | 4.6±0.6$^{ab}$ | 4.2±0.6$^{a}$ | 4.3±0.4$^{a}$ | 4.1±0.4$^{ba}$ |
| 10 | 4.9±0.6$^{a}$ | 4.4±0.6$^{b}$ | 4.3±0.2$^{ba}$ | 4.2±0.3$^{b}$ | 4.8±0.4$^{b}$ | 4.6±0.6$^{b}$ | 4.4±0.2$^{c}$ | 4.5±0.5$^{b}$ | 4.6±0.3$^{b}$ | 4.3±0.5$^{b}$ | 4.3±0.4$^{b}$ | 4.2±0.6$^{b}$ | 4.5±0.2$^{b}$ | 4.2±0.2$^{a}$ | 4.1±0.3$^{b}$ | 4.0±0.2$^{c}$ |
| 15 | 4.7±0.2$^{b}$ | 4.3±0.2$^{b}$ | 4.1±0.5$^{c}$ | 4.0±0.5$^{c}$ | 4.8±0.3$^{b}$ | 4.3±0.2$^{c}$ | 4.4±0.5$^{c}$ | 4.5±0.2$^{b}$ | 4.3±0.5$^{c}$ | 4.3±0.2$^{b}$ | 4.1±0.3$^{c}$ | 4.1±0.2$^{b}$ | 4.2±0.3$^{c}$ | 4.0±0.5$^{b}$ | 4.0±0.2$^{b}$ | 4.0±0.5$^{c}$ |
| 20 | 4.5±0.4$^{c}$ | 4.0±0.4$^{c}$ | 4.0±0.6$^{c}$ | 4.0±0.4$^{c}$ | 4.8±0.5$^{b}$ | 4.1±0.4$^{d}$ | 4.2±0.2$^{d}$ | 4.0±0.4$^{c}$ | 4.3±0.1$^{c}$ | 4.1±0.5$^{c}$ | 4.1±0.4$^{c}$ | 4.0±0.1$^{b}$ | 4.0±0.5$^{d}$ | 4.0±0.2$^{b}$ | 3.7±0.5$^{c}$ | 3.3±0.3$^{d}$ |
| 25 | 4.4±0.5$^{c}$ | 3.4±0.4$^{d}$ | 3.1±0.2$^{d}$ | 3.1±0.4$^{d}$ | 4.6±0.4$^{c}$ | 3.8±0.5$^{e}$ | 3.5±0.3$^{e}$ | 3.3±0.5$^{d}$ | 4.2±0.2$^{c}$ | 3.5±0.4$^{d}$ | 3.3±0.6$^{d}$ | 3.2±0.5$^{c}$ | 3.0±0.5$^{e}$ | 3.1±0.4$^{c}$ | 2.9±0.2$^{d}$ | 2.7±0.4$^{e}$ |

PB; Cacao-puddings are incorporated with *Bf. lactis*
PA; Cacao-puddings are incorporated with *Lb. acidophilus*
PC; Cacao-puddings are incorporated with *Lb. casei*

Values (± standard deviation) in a column with same superscripts are not significantly different ($P<0.01$).
*n: 5
[1] C: Color, F: Flavor, TA: Taste, TE: Texture
Scoring scale: very good=5, good=4, fair=3, poor=2, very poor=1, unfit for human consumption=0.

## 4 CONCLUSIONS

Cacao-pudding is an advantageously food can be consumed by all age groups and especially by children. In the present study cacao-pudding was shown to be a good vehicle for the incorporation of *Lb. acidophilus* during 25 days storage. The same organoleptical properties in cacao-puddings with *Bf. lactis* was only preserved for 15 days. This problem may be removed by increasing of initial counts of *Bf. lactis* into the puddings or addition of some prebiotics supplements. Although, *Lb. casei* showed good viability in the puddings, they were taken low sensorial scores during the storage. Further researches are needed to develop probiotic-cacao pudding by addition some prebiotics to improve viability of *Bf. lactis*.

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# Contrastive responses of spring and winter wheat cultivars to chilling and acclimation treatments

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## ABSTRACT

Photosynthesis and antioxidant defense system were investigated under chilling stress without (Ch, 25-4 ºC) and with acclimation (AcCh, 14-4 ºC) in winter (Sabalan) and spring (Zagros) wheat (*Triticum aestivum* L.) cultivars. Maximum quantum efficiency of photosystem II and $CO_2$ assimilation rate decreased in AcCh ‘Zagros’ but not in ‘Sabalan’, and in contrast, an increase in non-photochemical quenching was observed in ‘Sabalan’ but not in ‘Zagros’. Reduction of leaf starch content was observed in both cultivars while total soluble carbohydrates increased only in ‘Sabalan’ under both Ch and AcCh treatments. Activity of superoxide dismutase was significantly higher in Ch plants and activity of ascorbate peroxidase and catalase was slightly higher in Ch and AcCh plants of both cultivars compared with control. Activity of peroxidase increased in Ch and AcCh plants of ‘Zagros’ while phenylalanine ammoialyase (PAL) activity increased in AcCh ‘Sabalan’. Increase in the leaf content of $H_2O_2$ and malondialdehyde (MDA) was more prominent in ‘Zagros’ than in ‘Sabalan’. According to our results, chilling tolerance in winter cultivar was associated with greater thermal dissipation, higher soluble carbohydrates content, greater PAL activity and lower $H_2O_2$ and MDA content. Furthermore, acclimated plants were not more protected against chilling injury compared with non-acclimated ones.

**Key words:** Antioxidant enzymes, leaf photochemistry, gas exchange, phenylalanine ammoialyase

## IZVLEČEK

### RAZLIČEN ODZIV KULTIVARJEV JARE IN OZIMNE PŠENICE NA MRAZ IN AKLIMATIZACIJO

Fotosinteza in antioksidantna obramba sta bili raziskovani v razmerah hladnega stresa brez aklimatizacije (Ch, 25-4 ºC) in z aklimatizacijo (AcCh, 14-4 ºC) pri ozimni pšenici *Triticum aestivum* L. (cv. Sabalan) in jari pšenici (cv. Zagros). Maksimalna učinkovitost fotosistema II in asimilacije $CO_2$ sta se zmanjšali pri AcCh za ‘Zagros’, toda ne pri kultivarju ‘Sabalan’. Nasprotno od tega se je povečalo nefotokemično gašenje pri kultivarju ‘Sabalan’, a ne pri ‘Zagros’. Pri obeh kultivarjih je bilo ugotovljeno manj škroba v listih, medtem ko se je vsebnost celokupnih topnih ogljikovodikov povečala pri ‘Sabalan’ pri obeh tretiranjih, Ch in AcCh. Aktivnost superoksidne dismutase je bila značilno večja pri rastlinah Ch, aktivnost askorbatne peroksidaze in katalaze je bila pri obeh kultivarjih malo višja pri razmerah Ch in AcCh v primerjavi s kontrolo. Aktivnost peroksidaze se je povečala pri rastlinah Ch in AcCh pri kultivarju ‘Zagros’ medtem ko se je aktivnost fenilalanin ammoialiase (PAL) povečala pri AcCh cv. ‘Sabalan’. Povečanje koncentracije $H_2O_2$ in malondialdehida (MDA) je bilo bolj izrazito pri kultivarju ‘Zagros’ v primerjavi s kultivarjem ‘Sabalan’. Glede na naše rezultate je toleranca na mraz pri ozimnem kultivarju povečana z večjim termalnim trošenjem in večjo vsebnostjo večjih topnih ogljikovodikov. Odpornost na mraz je pri ozimnih kultivarjih povezana z večjo aktivnostjo PAL ter nižjo vsebnostjo $H_2O_2$ in MDA. Nadalje, aklimatizirane rastline niso bile nič bolje zaščitene pred poškodbami zaradi mraza v primerjavi z neaklimatiziranimi.

**Ključne besede:** Antioksidantni encimi, fotokemija listov, izmenjava plinov, fenilalanin amoialiaza

## 1 INTRODUCTION

Low temperature is one of the most important stress factors limiting the growth and productivity of cereals (Janda *et al.*, 2003). Photosynthesis is highly sensitive to cold stress, which is the main reason for the reduction

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or cessation of growth and productivity of plants under low temperature (Liang *et al.,* 2007). Reduction of photosynthetic capacity in plants exposed to low temperatures is related to photoinhibition as well as oxidative damage (Tambussi *et al.,* 2004). Low temperatures decrease quantum efficiency of PS II, the activities of PS I, the ATP synthase and the stromal enzymes of the carbon reduction cycle (Allen and Ort 2001). Non-radiative energy dissipation that involve the xanthophyll cycle and indicated by non-photochemical quenching (qN) of fluorescence, represents an important mechanism for protecting the photosynthetic apparatus against potential damage induced by excess excitation energy (Kim *et al.,* 2005).

Chilling temperatures increase the level of reactive oxygen species (ROS) mainly because of chilling-induced photoinhibition. Reduction in the rate of $CO_2$-fixation due to low temperature stress leads to an inadequate supply of natural electron acceptor, NADP, resulting in an over-reduction of the reaction centers. Molecular oxygen may then act as an electron acceptor in place of $NADP^+$, producing superoxide radical ($O_2^{\bullet -}$) (Allen and Ort 2001). Antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) can eliminate toxic oxygen by-products.

Increase in the activity of phenylalanine ammonia-lyase (PAL) is one of the main lines of cell acclimation against cold stress in plants (Leyva *et al.,* 1995, Rivero *et al.,* 2001). Following increased PAL activity, phenylpropanoid derivatives accumulate in chilling stressed plants and are thought to protect plants against cold injury (Rivero *et al.,* 2001, Solecka and Kacperska 2003). Activity of polyphenol oxidase (PPO), catalysing the oxidation and hydroxylation of phenolics, increases in response to different types of stress which is related to the appearance of injuries caused in plants by thermal stress (Dixon and Paiva 1995).

Exposure of plants to a non-injurious low temperature induces a degree of chilling tolerance, allowing them to survive subsequent exposure of plants to more severe low temperatures (Anderson *et al.,* 1995). This acclimation phenomenon involves distinct changes in protein and metabolite synthesis. It was reported that transcript and protein levels of various isozymes of POD were up-regulated in acclimated plants (Anderson *et al.,* 1995).

Short-term, low-temperature stress results in an inhibition of sucrose biosynthesis which leads to a restriction in photophosphorylation. Cold acclimation of cereals results in an increase in photosynthetic capacity following increases in the activities of Rubisco and stromal and cytosolic fructose-1,6-bisphosphatase (Hurry *et al.,* 1995). Winter wheat cultivars can be distinguished from spring cultivars by their ability to adjust their photosynthetic capacity upon cold acclimation which is associated with an increased resistance to photoinhibition (Savitch *et al.,* 1997).

In this work, we examined the effect of chilling stress (4 °C) without and with acclimation (14 °C) treatment on photochemical properties, gas exchange and the photosynthetic end products, sucrose and starch as well as antioxidant defense capacity in the winter wheat cultivar, 'Sabalan', and the spring wheat cultivar, 'Zagros'. We hypothesized that tested cultivars respond differently to chilling stress and cold acclimation regarding photosynthetic characteristics and antioxidative capacity.

## 2 MATERIALS AND METHODS

### *Plant materials and treatments*

Seeds of two wheat (*Ttriticun aestivum* L.) cultivars were surface sterilized and germinated on moistened filter paper in dark. Five-day-old seedlings were transferred to Hoagland nutrient solution (Johnson *et al.,* 1957) and pre-cultured for 5 days. Thereafter, 10-day-old plants with uniform size were selected and subjected to different temperature treatments. Control (Cont) plants were remained at 25 °C and grown for two weeks. The second group (Ch) was grown at 25 °C for one week then exposed to a chilling temperature of 4 °C for subsequent one week. The third group (AcCh) was exposed to an acclimation treatment of 14 °C for one week, followed by a one-week chilling treatment of 4 °C. Defined temperatures refer to that during the light period, night temperatures were 2-3 °C lower. Plants were grown in a germinator at about 200 µmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density, 18/6 h light/dark photoperiod and relative humidity of 60/70%.

### **Determination of chlorophyll fluorescence and gas exchange parameters**

Chlorophyll (Chl) fluorescence parameters were recorded using a portable fluorometer (OSF1, ADC Bioscientific Ltd., UK) on the third youngest, fully expanded and attached leaf. Dark-adapted leaves were used for determination of initial ($F_0$), maximum ($F_m$), variable ($F_v$=$F_m$-$F_0$) fluorescence as well as maximum quantum yield of photosystem II (PS II) ($F_v/F_m$). Light adapted leaves were used for measurement of steady-state ($F_s$) and maximum ($F'_m$) fluorescence. Calculations were made for photochemical quenching (*qP*), non- photochemical quenching (*qN*) and effective quantum yield of PS II ($\Phi_{PSII}$) according to Maxwell and Johnson (2000). Net assimilation rate (*A*), transpiration rate (*E*) and stomatal conductance ($g_s$) were measured in parallel with Chl fluorescence measurements in the same leaf with a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) between 10:00 A.M. and 13:00 P.M under photosynthetic photon flux density of 200 µmol $m^{-2}s^{-1}$ at the leaf surface.

*Determination of carbohydrates*

For determination of carbohydrates, leaves were homogenized in 100 mM phosphate buffer (pH 7.5) at 4ºC and supernatant was used for determination of total soluble sugars whereas the pellets were kept for starch analysis according to the method described in Magné *et al.* (2006).

*Assay of enzymes activity*

Determination of the activity of antioxidant enzymes and concentration of related metabolites were undertaken according to optimized protocols described elsewhere (Hajiboland and Hasani, 2007). Total SOD activity was determined using monoformazan formation test. One unit of SOD was defined as the amount of enzyme required to induce a 50% inhibition of nitro blue tetrazolium (Merck) reduction as measured at 560 nm, compared with control samples without enzyme aliquot. The activity of APX was assayed by recording the decrease in absorbance of ascorbic acid at 290 nm and was defined as the enzyme protein required for oxidation of ascorbic acid $min^{-1}$ at 25˚C. Activity of CAT was assayed by monitoring the decrease in absorbance of $H_2O_2$ at 240 nm $min^{-1}$. Peroxidase activity was assayed using the guaiacol test. The increase in absorbance at 470 nm was recorded at 25 °C over a period of 5 min and the activity was calculated as enzyme protein required for the formation of tetraguaiacol $min^{-1}$. Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobarbituric acid (Sigma) at 532 nm. The concentration of $H_2O_2$ was determined using potassium titanium-oxalate (Sigma) at 508 nm. Soluble proteins were determined using a commercial Bradford reagent (Sigma) and bovine serum albumin (Merck) as standard (Hajiboland and Hasani 2007).

Activity of PAL was determined according to the method of Dickerson (*et al.,* 1984) by measuring the absorbance of *trans*-cinnamic acid at 290 nm after a period of 30 min at 30 ºC. For determination of PPO activity, the increase in the absorbance at 370 nm, based on the disappearance of caffeic acid was followed for 5 min at 30 °C (Ruiz *et al.,* 1999).

Experiments were under taken in complete randomized block design with 4 replications. Statistical analyses were carried out using sigma stat (3.02) with Tukey test (P<0.05).

## 3 RESULTS AND DISCUSSION

Dry matter production of both cultivars was not influenced significantly by temperature treatments likely because of relatively short term exposure of plants to chilling stress. Chilling and acclimation treatments did not affect maximum quantum yield of PS II ($F_v/F_m$) in 'Sabalan', while caused a significant reduction of $F_v/F_m$ in 'Zagros' (Table 1). It is well documented that photosynthetic apparatus is sensitive to several environmental stresses and PS II appears to be preferentially affected by chilling stress (Zhang *et al.,* 2010). Reduction of $F_v/F_m$ in 'Zagros' indicated either damage to PS II or reversible and photoprotective photoinhibition via down-regulation of PS II (Rosenqvist and van Kooten 2003). Although exposure to either low-temperature stress or cold acclimation did not affect *qP* and $\Phi_{PSII}$ in both cultivars, it caused significant increase of *qN* in 'Sabalan' but not 'Zagros'. Because reduction of $F_v/F_m$ in 'Zagros' was not associated with a significant rise of *qN*, it could be suggested that, depressed $F_v/F_m$ was mainly due to photodamage, rather than to a reversible photoinhibition. In contrast to 'Zagros', in 'Sabalan' an ability for effective thermal dissipation reflected in significant rise of *qN*, was likely one of the reasons for unaffected $F_v/F_m$ under low-temperature treatments. Thermal dissipation plays an important role in preventing over-reduction of PS II electron acceptors (Müller *et al.*, 2001).

Net $CO_2$ assimilation rate (*A*) was not significantly influenced by low temperatures in 'Sabalan'. In 'Zagros' in contrast, *A* was diminished that was surprisingly accompanied by significant increase in stomatal conductance ($g_s$) (Table 1). Gas exchange measurements were replicated during following day in this experiment, but the same results were obtained. Nevertheless, regarding $g_s$ values, reduction of photosynthetic capacity in chilled 'Zagros' could be ascribed only to the non-stomatal limitation of photosynthesis including reduction of $F_v/F_m$ following damage to photosynthetic apparatus (Allen and Ort, 2001) and impairment in utilization of electrons and absorbed light energy for $CO_2$ fixation (Liang *et al.*, 2007) that in turn accentuates photogeneration of ROS and photooxidative damage to PS II.

**Table 1.** Dry weight (DW, mg plant$^{-1}$) of shoot and root, chlorophyll fluorescence parameters including $F_v/F_m$ (maximum quantum yield of PS II), *qP* (photochemical quenching), *qN* (non-photochemical quenching) and $\Phi_{PSII}$ (effective quantum yield of PS II) and gas exchange parameters including net photosynthetic rate (*A*, µmol m$^{-2}$ s$^{-1}$), transpiration rate (*E*, mmol m$^{-2}$ s$^{-1}$) and stomatal conductance to water vapor ($g_s$, mol m$^{-2}$ s$^{-1}$) in the leaves of two cultivars of wheat (*Triticum aestivum* L. cvs. Sabalan and Zagros) under three temperature treatments including control (25ºC, Cont), chilling (4ºC, Ch) and acclimation treatment followed by chilling stress (14º/4ºC, AcCh). Data of each row within each cultivar followed by the same letter are not significantly different (P<0.05).

| Temperature Treatments | | | | | | Parameters |
|---|---|---|---|---|---|---|
| **Zagros** | | | **Sabalan** | | | |
| AcCh | Ch | Cont | AcCh | Ch | Cont | |
| 67±9$^{a}$ | 72±7$^{a}$ | 73±7$^{a}$ | 75±5$^{a}$ | 81±7$^{a}$ | 76±5$^{a}$ | Shoot DW |
| 0.80±0.01$^{b}$ | 0.82±0.01$^{a}$ | 0.83±0.01$^{a}$ | 0.81±0.02$^{a}$ | 0.81±0.01$^{a}$ | 0.82±0.01$^{a}$ | $F_v/F_m$ |
| 0.90±0.02$^{a}$ | 0.92±0.02$^{a}$ | 0.92±0.01$^{a}$ | 0.94±0.02$^{a}$ | 0.92±0.02$^{a}$ | 0.93±0.01$^{a}$ | *qP* |
| 0.18±0.07$^{a}$ | 0.12±0.09$^{a}$ | 0.08±0.04$^{a}$ | 0.27±0.09$^{a}$ | 0.17±0.09$^{ab}$ | 0.03±0.02$^{b}$ | *qN* |
| 0.71±0.01$^{a}$ | 0.72±0.03$^{a}$ | 0.75±0.04$^{a}$ | 0.73±0.01$^{a}$ | 0.73±0.02$^{a}$ | 0.75±0.01$^{a}$ | $\Phi_{PSII}$ |
| 1.5±0.4$^{b}$ | 2.4±0.7$^{ab}$ | 3.4±1.2$^{a}$ | 1.9±0.4$^{a}$ | 2.3±0.3$^{a}$ | 2.8±1.1$^{a}$ | *A* |
| 0.42±0.09$^{a}$ | 0.56±0.17$^{a}$ | 0.65±0.07$^{a}$ | 0.45±0.20$^{a}$ | 0.33±0.06$^{a}$ | 0.43±0.09$^{a}$ | *E* |
| 1.63±0.31$^{b}$ | 2.37±0.49$^{a}$ | 0.82±0.09$^{c}$ | 0.73±0.21$^{a}$ | 1.09±0.71$^{a}$ | 0.72±0.03$^{a}$ | $g_s$ |

Significant reduction of starch content of leaves (Table 2) indicated strong impairment in the photosynthetic carbon metabolism in both cultivars. Under low-temperature stress conditions, a limited stimulation of the sucrose biosynthetic pathway and a restriction in starch synthesis might lead to reduced rates of $CO_2$ assimilation as a consequence of decreased ATP production (Savitch *et al.,* 1997). Similar extent of reduction in the starch content in winter and spring wheat cultivars indicated that both cultivars experienced a restriction in photosynthetic carbon metabolism. Nevertheless, and in contrast to 'Zagros', total soluble sugars rather increased in chilled 'Sabalan' leaves. It has been assumed that the accumulation of soluble carbohydrates during cold acclimation plays an important role in winter survival and probably results from the differential low-temperature sensitivity of the enzymes of starch and sucrose metabolism (Hurry *et al.,* 1995).

**Table 2.** Content of starch and total soluble sugars (mg g$^{-1}$ FW) in the leaves of two cultivars of wheat (*Triticum aestivum* L. cvs. Sabalan and Zagros) under three temperature treatments including control (25ºC, Cont), chilling (4ºC, Ch) and acclimation treatment followed by chilling stress (14º/4ºC, AcCh). Data of each row within each cultivar followed by the same letter are not significantly different (P<0.05).

| Temperature Treatments | | | | | | Parameters |
|---|---|---|---|---|---|---|
| **Zagros** | | | **Sabalan** | | | |
| AcCh | Ch | Cont | AcCh | Ch | Cont | |
| 327±37$^{b}$ | 375±26$^{ab}$ | 439±40$^{a}$ | 395±39$^{b}$ | 367±33$^{b}$ | 530±28$^{a}$ | Starch |
| 15.2±1.1$^{a}$ | 14.7±1.8$^{a}$ | 13.7±1.7$^{a}$ | 17.3±0.4$^{a}$ | 17.3±0.9$^{a}$ | 12.2±0.4$^{b}$ | Soluble sugars |

Activity of SOD increased significantly in Ch plants, while in the AcCh plants SOD activity did not differ with Cont ones (Figure 1). A slight increase of APX and CAT activity was observed in both cultivars and under chilling temperature with and without acclimation. In contrast, POD activity was increased in 'Zagros' but not in 'Sabalan' by both temperature treatments. Exposure of plants to chilling stress with or without acclimation caused significant accumulation of $H_2O_2$ and MDA not only in 'Zagros' but also in 'Sabalan' (Figure 1). However, increase of $H_2O_2$ and MDA content was only 20% and 29% in 'Sabalan', while the corresponding values for 'Zagros' was 51% and 117% respectively. A rapid, transient increase in the $H_2O_2$ level was detected in wheat plants after cold treatment (Janda *et al.,* 2003). Accumulation of MDA as a common product of lipid peroxidation is a sensitive diagnostic index of oxidative injury caused by chilling stress (Tambussi *et al.,* 2004). These results indicated that higher chilling tolerance in 'Sabalan' in comparison with 'Zagros' is due to less injured membranes. One possible mechanism contributing to lower MDA content in the 'Sabalan' is an efficient thermal dissipation of excess light energy leading to less ROS production and higher efficiency with which scavenging of ROS takes place. Comparison of activity of antioxidant enzymes in two tested cultivars demonstrated, however, that, 'Zagros' was not, at least enzymatically, less capable for scavenging ROS than ‘Sabalan’ and was rather more active because of significant rise of POD activity under low-temperature treatments. Greater accumulation of $H_2O_2$ is likely the result of greater production of $H_2O_2$ that exceeded scavenging capacity of plants. It is also likely that, other reactive oxygen species was not measured in this work such as $O_2^{\bullet -}$, were differentially produced in two cultivars and may be responsible for higher membrane damages in ‘Zagros’.

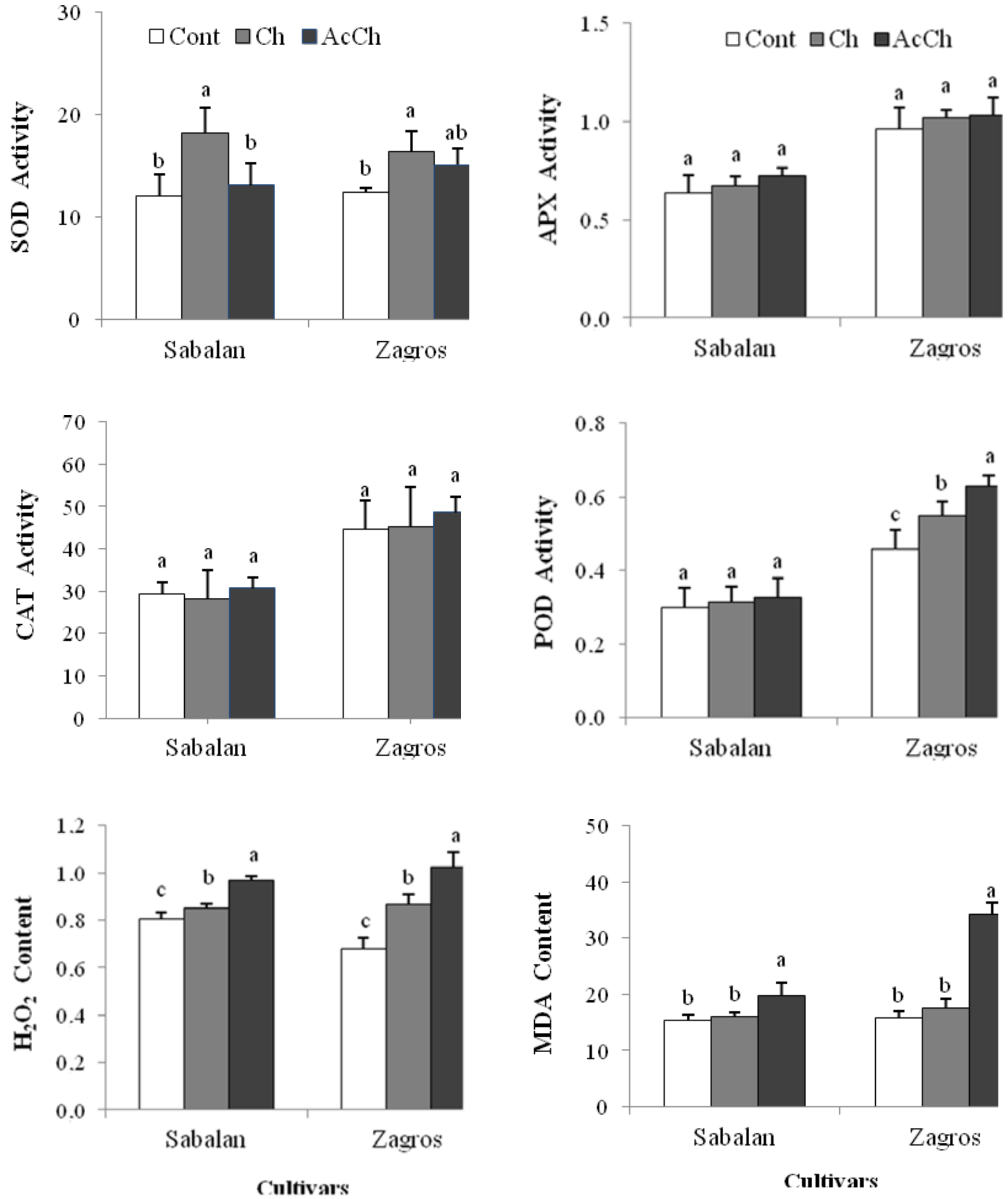

**Figure 1.** Specific activity of superoxide dismutase (SOD, U $mg^{-1}$ Pro. $min^{-1}$), ascorbate peroxidase (APX, µmol $mg^{-1}$ Pro. $min^{-1}$), catalase (CAT, µmol $mg^{-1}$ Pro. $min^{-1}$) and peroxidase (POD, µmol $mg^{-1}$ Pro. $min^{-1}$) and concentration of MDA (nmol $g^{-1}$ FW) and $H_2O_2$ (µmol $g^{-1}$ FW) in the leaves of two cultivars of wheat (*Triticum aestivum* L. cvs. Sabalan and Zagros) under three temperature treatments including control (25ºC, Cont), chilling (4ºC, Ch) and acclimation treatment followed by chilling stress (14º/4ºC, AcCh). Data of each row within each cultivar followed by the same letter are not significantly different ($P<0.05$).

Activity of PAL was influenced by AcCh treatments only in ‘Sabalan’ and PPO activity remained unchanged in both cultivars (Figure 2). In watermelon plants an acclimation against suboptimal temperatures consists of the accumulation of phenolics as a possible form of adapting to this stress (Rivero *et al.*, 2001). Increase in the PAL activity and accumulation of different phenolics are thought to protect plants against various stressors (Solecka and Kacperska 2003). Higher PAL activity that caused likely phenolics accumulation may be one of the reasons for greater tolerance of 'Sabalan' to chilling temperatures.

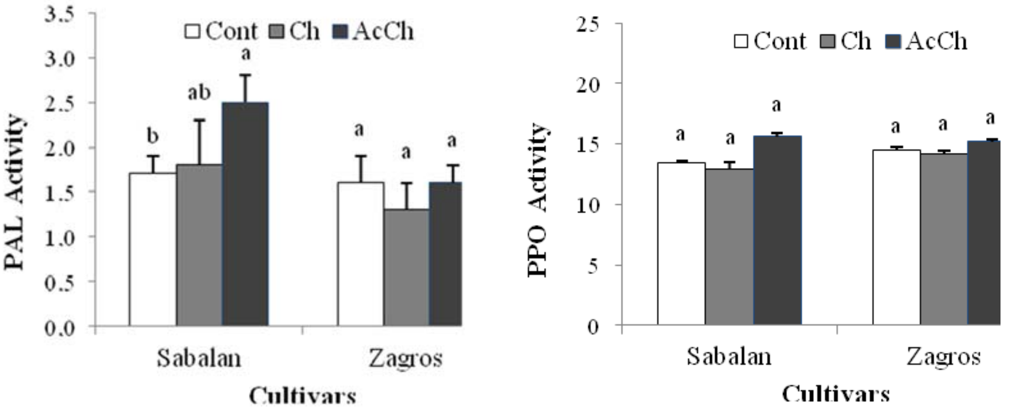

**Figure 2.** Specific activity of phenylalanine ammonia-lyase (PAL, µmol cinnamic acid produced mg$^{-1}$ Pro. min$^{-1}$) and polyphenol oxidase (PPO, µmol caffeic acid oxidized mg$^{-1}$ Pro. min$^{-1}$) in the leaves of two cultivars of wheat (*Triticum aestivum* L. cvs. Sabalan and Zagros) under three temperature treatments including control (25ºC, Cont), chilling (4ºC, Ch) and acclimation treatment followed by chilling stress (14º/4ºC, AcCh). Data of each row within each cultivar followed by the same letter are not significantly different ($P<0.05$).

Unexpectedly, the adverse effects of chilling stress on photochemical events, membrane integrity and $CO_2$ assimilation, as well as its influence on $H_2O_2$ accumulation and activity of APX, CAT and POD in both cultivars were more prominent in AcCh compared with Ch plants. Lower injury of photosynthetic apparatus and membranes in Ch compared with AcCh plants is likely exposure for shorter time (1 week) of former plants to suboptimal temperatures than latter ones (2 weeks). It implicated also that growth at 14º C as acclimation treatment (Anderson *et al.*, 1995) was not effective in protecting plants against chilling (4º C) stress. It was also reported that, not only cold stress but also cold acclimation stimulates feed-back photosynthesis at the level of electron transport (Savitch *et al.*, 1997). However, acclimation treatment would be likely effective if plants were exposed to freezing temperatures.

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**Agris category code**: F30

# Reduction of the lenght of 1 RS.1BL translocation in the bread wheat variety "Yugoslavia"

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## ABSTRACT

This study was conducted to estimate the survival of 1RS.1BL wheat-rye translocations in a bread wheat (*Triticum aestivum* L. em Thell.) variety "Yugoslavia" including "Aurora" as a donor of translocation in its pedigree. The cultivar was cytologically checked by N-banding for the presence of 1RS.1BL translocation. Fluorescence *in situ* hybridization using total genomic biotin labelled rye DNA as a probe was used for identification and localisation of the translocation breakpoints and their size. At metaphase and interphase the introgressed rye segment was observed and photographed. Digital image analysing system with cooled CCD camera was used for overlapping the pictures of the probe signals and counterstained wheat chromosomes and helped to determine the position and extent breakpoint of this translocation. The translocation in this variety is probably restricted to the NOR region only, unlike in other 1RS.BL translocation lines ("Kavkaz") where the translocation carries at least the major part of the 1RS arm.

**Key words:** bread wheat, *Triticum aestivum* L. em Thell., N-banding, fluorescence *in situ* hybridization, 1RS.1BL translocation.

## IZVLEČEK

### ZMANJŠANJE DOLŽINE 1RS.1BL TRANSLOKACIJE PRI KULTIVARJU NAVADNE PŠENICE "YUGOSLAVIA"

Raziskava je skušala ugotoviti preživetje 1RS.1BL translokacije pri kultivarju navadne pšenice "Yugoslavia". Kultivar je imel v rodovniku "Avroro" kot donorja translokacije. Sorta je bila citološko pregledan s pomočjo N-proganja na prisotnost translokacije. Uporabili smo tudi fluorescenčno *in situ* hibridizacijo s celotno genomsko probo rži za natančno opredelitev mesta translokacije in dolžine. V interfazi in metafazi je bil rženi segment na kromosomu opazovan in fotografiran. Slike smo posneli z digitalno CCD kamero in sliki tarče in protibarvanih kromosomov združili z digitalno analizo slike. 1RS.1BL translokacija je bila pri tem kultivarju pšenice omejena na NOR področje kromosoma 1B, z razliko od drugih translokacij („Kavkaz"), kjer je translokacija obsegala vsaj večji del rženega kromosomskega kraka 1RS.

**Ključne beside:** navadna pšenica, *Triticum aestivum* L. em Thell., N-proganje, flurescenčna *in situ* hibridizacija, 1RS.1BL translokacija

## 1 INTRODUCTION

Rye chromatin has been successfully incorporated in many wheat varieties all over the world, especially in eastern Europe and Mexico. Even recent surveys show that sometimes more than 45 % of breeding material may contain those translocations (Zhou et al., 2007) or 55 % of CIMMYT bread wheat germplasms. In Hungary 53 % of wheat cultivars registered during the last twenty years carry the 1RS translocation (Hoffmann, 2008). This translocation has been deemed that it has been incorporated into more than 60 wheat varieties, including the prominent Veery spring wheat lines that occupy more than 50 % of all developing country wheat area, almost 40 million hectares. Their most important phenotypic deviation from common wheat cultivars is the so called wheat-rye desease resistance to races of powdery mildew and rusts (Bartos and Banes, 1971; Zeller, 1972). The desease resistance is linked with decreased breadmaking quality (Zeller et al. 1982), good ecological adaptability and yield performance (Rajaram et al., 1983; Schlegel & Meinel,

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1994; Singh et al., 1990). Indirect sources for 1RS.1BL wheat-rye chromosome translocations in Yugoslav breeding programs were the substitution lines Weique and Neuzhücht, which carried chromatin from Petkus rye. The Soviet cultivars Aurora, Kavkaz and Skorospelka 35 were donors of 1RS.1BL translocations in Yugoslav wheat varieties. During the past 20 years (1967-1986) Yugoslav breeding programs released 148 cultivars of bread wheat (Jošt & Cox, 1990).

Chromosome 1R of rye is a usefull source of genes for desease resistance and enhanced agronomic performance in wheat. The genes encoded on the 1RS chromosome arm of rye carry disease resistance genes (Zeller and Hsam, 1983), including powdery mildew (Pm8) (Zeller, 1973; Zeller and Fuchs, 1983; Heun and Fishbeck, 1987), leaf rust (Lr26), stem rust (Sr31) and tolerance to greenbug (Gb) (Sebesta and Wood, 1978).

Novel tertiary wheat-rye recombinant lines were produced carrying different lengths of of rye chromosome arm 1 RS. The larger root biomass of the 1RS translocation lines contributes to an increased drought resistance, combined with less yield decrease.

The bread wheat variety "Yugoslavia" was introduced by the Institute for Crops, Agricultural Faculty, University in Novi Sad in 1980. The crosses of NS646/Bezostaja1/Aurora are involved in the pedigree of "Yugoslavia". The cultivar has good winter cold resistance, good resistance to leaf rust (*Puccinia graminis*), to stem rust (*Puccinia graminis* tritici) and to powdery mildew (*Erysiphe graminis).* The potential yield of the cultivar was more than 10t/ha. In 1988 on 280.000 ha the winter wheat cultivar "Yugoslavia" was grown (18% of wheat grown areas in Yugoslavia). The cultivar is still nowadays in Slovenia.

Fluorescence *in situ* hybridization (FISH) using total genomic rye DNA, together with an access of unlabelled wheat competitor DNA was used for identification, breakpoint and size of the introgresed rye segment in the bread wheat cultivar "Yugoslavia".

FISH has become an increasingly powerfull diagnostic tool, the plant cell wall can effect the accessibility of the probe to a chromosome preparation and plant cellular debris can cause a relative high background in the commonly used squash preparations. Ambros et al. (1986) have attempted to bypass this problem by the use of protoplast drop technique and modified *in situ* hybridization technique (Bush et. Al., 1994; Lichter & Cremer, 1992). The cultivar "Yugoslavia" was also cytological checked with the differential Giemsa N-banding staining technique (Javornik et al. 1991), for the presence of 1RS.1BL translocations and chromosome identification. B-genome chromosomes of common wheat provided significant N-bands, while the alien 1RS arm none. Our thesis on the basis of N-bands and FISH experiments is that in some Yugoslav varieties, these translocations carry less than the whole chromosome arm.

## 2 MATERIAL AND METHODS

Seeds of bread wheat (*Triticum aestivum* L. em Thell.) cv. "Yugoslavia", were germinated on moist paper at 22°C in the dark. For fluorescence *in situ* hybridization, roots of young seedlings were cut of and stored overnight in ice water, as described in Doležel et al. (1992) & Pan et al. (1993). The material was then fixed in 3:1 (v/v) ethanol:acetic acid and stored at -20ºC untill use. Roots were rinsed in tap water and the root tips (2 mm) cut off for digestion in 250 µl of Pectolyase Y-23 (Kikkoman), Cellulase R 10 (Onozuka) in 75 mM KCl and 7.5 mM EDTA (pH 4.0) at 25°C for 55 minutes after Pan et al. (1993). The lysate of 15 root tips was filtered through a 80 µm mesh net. The protoplasts were resuspended in 75 mM KCL and spun down at 80 G for 5 minutes. The pellet was resuspended four times in fixative and spun down again. The protoplasts were finally resuspended in 120 µl of fixative and dropped on ice-cold, cleaned slides.

### 2. 1 Probe preparation:

Total rye DNA was extracted from first leafs of young plants of cv. "Danko". Competitor wheat DNA was extracted from young leaves of cv. "Chinese Spring". Total genomic competitor DNA from "Chinese Spring" and total genomic rye DNA were enzymaticaly shared using DNAse I, the fragment lengths (100-500 bp) were chequed with electrophoresis on 1% agarose gel (PHW802 Gibco) with ethidium bromide staining. The shared and Sephadex G-50 column purified genomic rye DNA, was labelled by nick translation (nick translation kit, BLR) with Biotin-14-dATP. The rye probe was purified by Sephadex G-50 spin column to remove unincorporated nucleotides and by the ethanol precipitation step.

### 2. 2 *In situ* hybridization and signal detection:

For hybridization 12 ng/µl rye Bio-14 dATP was used together with unlabelled 360ng/µl competitor wheat DNA was dissolved in 50% formamide, 10% dextran sulphate and 2xSSC. Total genomic rye DNA probe and competitor wheat DNA were denatured for 15 minutes at 80°C and cooled on ice.

**Slide preparation:**

Slides were incubated 5 min. in 2XSSC at room temperature (RT), digested with RNAse (1h at 37°C), washed twice for 5 min in 2XSSC at RT, 5 min in 1xPBS at 37°C, digested for

10 min in 0.005% Pepsin according to Wiegant et al. (1991). They were washed twice in 2 x SSC, pH 7.2 for 5 min and then dehydrated in a graded ethanol series (70%, 80%, 90%, 99%), 2 min each. The preparations were denatured in 50% formamide-2xSSC mixture (pH 7.0) at 70°C for 2 minutes. The slides were transferred to ice cold ethanol (70%, 80%, 90%, 99%) 3 minutes each and air dried.

Fifteen µl of DNA probe were loaded per slide, sealed with a 24x36 mm cover slip and hybridized in a moist chamber at 37° C overnight. After hybridization the coverslips were removed in 50% formamide 2xSSC (pH 7.2), at 42°C for 15 minutes in a shaking water bath. The samples were then washed in 50% formamide-2xSSC at 45°C three times for 5 min and 5 min in 2xSSC at 45°C. On the drained slides 180 µl of blocking solution 4xSSC and 3% BSA (fraction V) was added. The slides were covered with a 24x36mm cover slip and incubated for 30 min. at 37°C. The cover slips were taken off, excess fluid drained and 80 µl detection solution was added.

**Signal detection**:
Signal detection of biotinylated genomic rye probe was performed according to Pinkel et al. (1988) with Avidin-Cy3 (1µg/ml). After the final washing in 0.05% Tween-20, 4XSSC, pH 7.2, slides were rinsed twice in phosphate-buffered saline at room temperature and dehydrated in graded ethanol series. Chromosomes were counterstained with 0.5 µ L/ml DAPI and mounted in Vector-Shield antifade solution. The Zeiss filter set 487909 (Avidin Cy-3), the Zeiss filter set 487901 (DAPI) and Zeiss-Axioplan epiflurescence microscope, equipped with cooled CCD camera and digital image analysing system was used for the interpretation of results and generation of counterstained chromosome and probe signals.

### 2. 3 N-banding

For N-banding the root tips were incubated in bromonaftalene for 4h at room temperature, fixed in 3:1 (v/v) ethanol:acetic acid and stored in deep-freeze.

N-banding of mitotic metaphase chromosomes was carried out as described by Gerlach (1977) and improved by the technique of Endo and Gill (1984), which allows the recognition of 16 wheat chromosome pairs out of 21, including the whole B-genome of wheat. Approximately nine root tips from three plants were cytologicaly checked and photographed with Zeiss-photomicroscope at 1000x magnification. Results are based on three spread 1B or 1RS.1BL wheat chromosomes. From the spread chromosomes a typical B-genome karyotype of "Yugoslavia" bread wheat cultivar was prepared, with bands compared to the cultivar "Chinese Spring" (Endo and Gill, 1984). Because of heterochromatin polymorphism among different cultivars, best results were obtained in combining bands with arm ratios. We used the non-occurrence of the telomere band on the short arm of the 1B chromosome as N-band marker for the 1RS.1BL translocation.

## 3 RESULTS AND DISCUSSION

The recognition of normal and translocated common wheat chromosomes by N-banding is based on the distribution of bands and arm ratios. Normal 1B chromosomes posses strong centromeric bands, telomeric bands on the short, and long arms. They have some interstitial bands on the long arm and are submetacentric (Fig. 1.B). Translocated 1RS.1BL chromosomes from the cultivar "Yugoslavia" were identified by similar bands on the 1BL arm and similar arm ratio, but no bands on the telomeric part of the 1RS arm (Figure 1. A).

**Figure 1.** Normal 1B (A) from the cultivar "Dukat" and translocated 1RS.1BL bread wheat chromosome (B) from the cultivar "Yugoslavia", determined by N-banding (magnification ca. 3000x)

Figure 1. Normal bread wheat B-genome karyotype from the cultivar "Dukat" (Figure 1.A) and translocated 1RS chromosome (Figure 1.B) from the cultivar "Yugoslavia", determined by N-banding (magnification ca. 2000x)

Typical B-genome karyotype from the bread wheat cultivar "Dukat" was prepared from B-genome chromosomes spreads (Figure 2) and the chromosomes were measured.

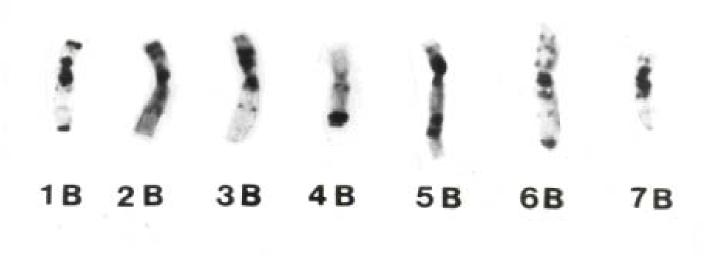

**Figure 2**. Normal haploid bread wheat B-genome karyotype from the cultivar "Dukat", determined by N-banding (magnification ca. 2000x)

The B-genome karyotype is based on measurements of six B-genome chromosome spreads from different cells. Our measurements are based on 4 hours of monobromonaftalene pretreatment.

The translocated 1RS satellite was identified by the unbanded short arm on the 1B chromosome, and characteristic N-bands on the long arm of this wheat chromosome. We observed an extra pericentromeric band on the 1RS arm as evidence for that this translocation carries less than the whole 1RS arm. The translocation was in a homozygous state.

However the exact location of translocation breakpoint is difficult to pinpoint with banding techniques alone (Cai and Liu, 1989). The coupling with the use of fluorescence *in-situ* DNA hybridization using biotin labelled total genomic rye DNA was needed to confirm categorically the translocation breakpoint, localization and size. The reduced length of the 1RS1BL translocation is probably the reason of relative good bread making quality of the cultivar "Yugoslavia"

Genomic *in situ* DNA hybridization:
Probe hybridization sites were detected by Avidin-Cy3 conjugate with red fluorescence under yellow light excitation (Zeiss filter-set 487915) and allowed visualisation of the introgressed rye segment. Chromosomes were counterstained with DAPI (UV excitation with Zeiss filter-set 487901). A Zeiss-Axioplan epifluorescence microscope and digital image analysis system was used for generating signals of probe and counterstained chromosomes. The biotin labelled rye segment (red signal with Avidin Cy-3 conjugate) was restricted to the 1B NOR region only (Figure 3.A) and was in a homozygous state. Figure 3.B shows the typical distribution of rye signals in interphase nuclei of wheat cultivar "Yugoslavia" after the FISH technique.

Figure 3. Hybridization sites of biotin labelled total rye genomic probe in the bread wheat cultivar "Yugoslavia", on metaphase chromosomes (A) and on interphase nucleus (B), after FISH.

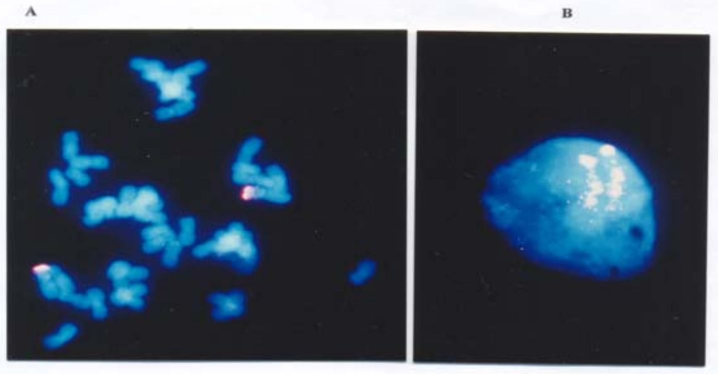

**Figure 3.** Hybridization sites of biotin labelled total rye genomic probe in the bread wheat cultivar "Yugoslavia", on metaphase chromosomes (A) and on interphase nucleus (B) after FISH.

Translocated 1RS arms have probably recombined with 1BS arms of normal wheat during breeding to form short arms composed of centromeric regions of the 1BS near the centromere and satellited 1RS region. Further analyses of storage proteins (HMW and LMW glutenin subunits), using SDS PAGE electrophoresis would be reasonable for this wheat cultivar.

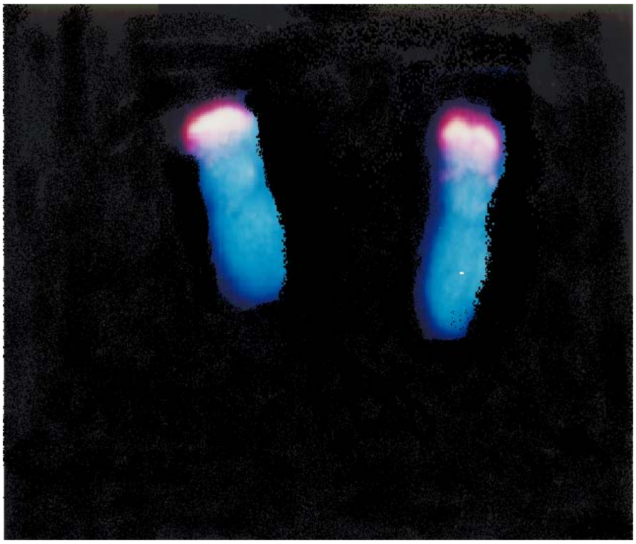

**Figure 4**. The 1B/1R translocations are restricted to the 1B chromosome SAT region. The two translocated chromosomes are from one metaphase cell of the bread wheat cultivar “Yugoslavia”. Red dots represent biotin labelled rye signals (Avidin – Cy3, magnification ca. 3000x).

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**Agrovoc descriptors:** brassica napus, rapeseed, oil crops, crop yield, varieties, genotypes, wild plants, crops, cross pollination, reproduction, fertilization, sexual reproduction, gene transfer, purity, quality

**Agris category code**: F30, F01

# Cultivation, varietal structure and possibilities for cross-pollination of *Brassica napus* L. in Slovenia

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## ABSTRACT

Oilseed rape, *Brassica napus* L., is one of the most important oil plants of the moderate climatic zone and a typical industrial plant. In the past, dynamics of oilseed rape production in Slovenia was reflected in flexible agricultural market situation because of introduction of new crops into rotation and due to financial supports from European Union. In 2010, it was cultivated on 5351ha, accounting for about 1.1% of the total production areas in Slovenia. It is mainly grown in the eastern part of Slovenia (Pomurje, Podravje) and in the Spodnje-Posavska region. From 1984 until 2010, 58 different genotypes of oilseed and fodder rape were grown in Slovenia, of which a total of 28 were registered in the National List of Varieties in each year. Average yield of oilseed rape was ranged between 1.8 and 2.9 t/ha. Under Slovenian fragmented property structure the cross-pollination between *B. napus* and volunteer or feral populations (within and outside the production area) can occur. In addition to that, the presence of some sexually compatible relatives which have a high affinity to cross-pollination with *B. napus* are found (*B. rapa, B. oleracea, B. nigra, Hirchfeldia incana, Raphanus raphanistrum, Sinapis arvensis, Diplotaxis erucoides, D. tenuifolia, D. muralis, S. alba, R. sativus* and *Rapistrum rugosum*). Uncontrolled gene flow between different forms of *B. napus* or sexually compatible wild relatives in the case of coexistence of different production systems has a direct impact on the varietal purity of seeds and on crop quality.

**Key words**: *Brassica napus* L., oilseed rape, field production, varietal structure, wild relatives, volunteers, feral populations, gene flow

## IZVLEČEK

### PRIDELAVA, SORTNA STUKTURA IN OPRAŠEVALNE SPOSOBNOSTI VRSTE *Brassica napus* L. V SLOVENIJI

Oljna ogrščica, *Brassica napus* L., je najpomembnejša oljnica zmernega klimatskega pasu in tipična industrijska rastlina. V Sloveniji se je v preteklosti obseg njene pridelave zelo spreminjal zaradi prilagajanja razmeram na trgu, uvajanja novih poljščin v kolobar in zaradi neposrednih plačil s strani Evropske unije. V letu 2010 se je pridelovala na 5351ha, kar predstavlja okrog 1,1% vseh pridelovalnih površin. Največ se je pridela v vzhodni Sloveniji, in sicer v Pomurski, Podravski in Spodnje-posavski regiji. Od leta 1984 do 2010 se je na območju Slovenije pridelovalo 58 različnih genotipov oljne in krmne ogrščice, od tega je bilo 28 vpisanih v Sortno listo v posameznem letu. Povprečni pridelki oljne ogrščice se gibljejo med 1,8 in 2,9 t/ha. V slovenskih razmerah razdrobljene posestne strukture obstajajo možnost oprašitve posevkov *B. napus* s samosevnimi in podivjanimi populacijami (znotraj in zunaj pridelovalnih površin). Poleg tega se v pri nas pojavljajo nekateri spolno kompatibilni sorodniki, ki imajo visoko sposobnost oprašitve z *B. napus* (*B. rapa, B. oleracea, B. nigra, Hirchfeldia incana, Raphanus raphanistrum, Sinapis arvensis, Diplotaxis erucoides, D. tenuifolia, D. muralis, S. alba, R. sativus and Rapistrum rugosum*). Nenadzorovan prenos genov med različnimi pojavnimi oblikami *B. napus* ali spolno kompatibilnimi sorodniki v primeru soobstoja različnih sistemov pridelave neposredno vpliva na sortno čistost semenskih posevkov in na kvaliteto pridelka.

**Ključne besede**: *Brassica napus* L., oljna ogrščica, obseg pridelave, sortna struktura, divji sorodniki, samosevci, podivjane populacije, prenos genov

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## 1 INTRODUCTION

*Brassica napus* L. is a widely cultivated plant species which belongs to the diverse cruciferous family (*Brassicaceae*). The species is divided into two subspecies groups. The first group includes swedes (*B. napus* ssp. *napobrassica*), the second one includes winter and spring *B. napus* ssp. *napus* forms which are used for oil production or fodder (Snowdon et. al., 2007). Fodder rape (*B. napus* L. ssp. *napus*) is grown as a fresh forage crop for livestock feeding or as an organic soil fertilizer for the incorporation of green biomass into the soil. Oilseed rape (*B. napus* L. ssp. *napus*, *syn. oleifera)* is predominantely used for oil production having high seed oil content. Good quality oilseed rape contains around 40% of oil and the meal contains around 45% of protein, mostly dependent on the environment, fertilization, agrotechnics and genotype (Lääniste et al., 2004). Oilseed rape is today the world's third-leading source of both, vegetable and oil extraction meal (Friedt and Snowdon, 2009).

Oilseed rape is the most important oil plant of temperate climate zone and a typical industrial plant. The oil is also used in more environmentally friendly and cost effective extraction technologies (biofuels, lubricants, surface coatings, polymers, medicinal). Meal proteins are used in bioplastics, adhesives, cosmetics, encapsulation agents, lawn care products and combustion material (IENICA, 2005).

In the Slovenian climatic conditions winter/biennial oilseed rape varieties (f. *biennis*) are grown (vernalization demands) while the production of spring, one year (f. *annua)* varieties is concentrated in warmer areas (Butruille et al., 1999). Winter *B. napus* is cultivated in most of Europe and Asia, whereas in Canada, northern Europe and Australia only spring forms are suitable (Snowdon et al., 2007).

The increased interest in production in recent years is the consequence of the new agricultural policies due to accession of Slovenia to the European Union (EU) in 2004. EU market conditions dictate useful incorporation of oilseed rape in conventional, integrated and organic farming. Multiple use of oilseed rape products throughout the world is possible due to the successful conventional breeding programmes in the past, which could be nowadays completed with the new methods of plant biotechnology (genetic engineering). Christen and Friedt (2007) proposed four major groups of traits in the *B. napus* breeding programs: 1.) Agronomic traits include tolerance to late planting, winter hardiness, plant height and lodging resistance, early maturity, nutrient efficiency and drought tolerance, shattering resistance and herbicide tolerance. 2.) Disease and pest resistance are the following: *Phoma* and *Vericillium,* clubroot and *Cylindorsporium*, *Sclerotinia,* TuYV virus resistance and other insect and pest resistance. 3.) Yield potential traits include: oil content, seed yield components, harvest index, total and marketable seed yield. 4.) In addition to that, seed quality traits are also included: very low erucic acid content (<0.2%), low glucosinolate content (<18mmol/kg seed), reduced lignin content and improved digestibility (monogastric animals) (Christen and Friedt, 2007).

Oilseed rape is used primarily in industry for the biofuel production, varieties with low content of glucosinolates and erucic acid are used for the production of high quality vegetable oil, industrial residues are used for highly enriched protein livestock feed. Usefulness of raw oils is also widespread in lipochemistry (genotypes with high erucic acid) and phytopharmacy (genotypes with high levels of glucosinolates) (Eastham and Sweet, 2002). One of the very important environmental functions of *B. napus* production is in the time of full flowering, allowing pollinators and especially honey bees to forage and consequently boosting the honey production. From the environmental and economic perspective biofuel from oilseed rape is important as a renewable source of energy due to the continuous raise of the fossil fuel price and their unavailability in the future.

*B. napus* is also a very prospective crop in Slovenia. The aim of this paper is to expose the biological characteristics of *B. napus* that are important for the explanation of *B. napus* pollination relations and possibilities in the specific production conditions of Slovenia. The survey of production capacities and varietal structure of *B. napus* on the landscape and regional level are presented. The influence on the gene flow relations inside *Brassicaceae* family and coexistence of different cropping systems in fragmented *B. napus* small field production is discussed. Uncontrolled inter- and intra-specific hybridization of *B. napus* from different habitats in Slovenia may occur.

## 2 *B. NAPUS* PRODUCTION DYNAMICS IN SLOVENIA

In the period from 1984 to 1994 the organised production was led by one of the biggest oil factories in Slovenia that introduced quality oilseed rape varieties to farmers, leading them throughout the entire production process and at the end purchasing their product. Due to the collapse of the entire production system and nationally unregulated market relationships, the oil factory did not renew the contracts with farmers for further oilseed rape production (Kocjan-Ačko, 1999). This was the reason why the production in 1995 decreased for 87 % in comparison with the previous year (Figure 1). The period from 1995 to 2001 was the so called "dead period" because the oilseed rape production in Slovenia was minimal. The Slovenian market still felt the consequences of the end of the organized production lacking the possibility of selling the yield. Oilseed rape in that period disappeared from the Slovenian conventional crop rotation, but it was on the other hand still sown as a fodder crop or as a natural organic fertilizer for introduction of green biomass into the soil (Kocjan-Ačko, 1999).

The adaptation of the oilseed rape production requirements to the EU standards and demands, which started already in 2002, caused an increase in the production (Figure 1). Due to the introduction of direct payment from EU for the production of crops which are not used for food and feed and for the extension of crop rotation systems with new crops, oilseed rape became more and more interesting for farmers. Therefore, the area under oilseed rape was increasing until 2004 when Slovenia joined the EU. Such a dynamics continued and the production increased until 2007 (Figure 1) when it achieved 1.1% of all agricultural areas in Slovenia (SURS, 2011). The motivation of farmers to plant oilseed rape was strongly encouraged by direct payments, obligatory extension of crop rotation and re-establishement of contractual production by industry. In 2008 direct payments from the EU ceased causing certain decrease in the 2008 and 2009 production.

The oilseed rape yield [t/ha] varied between individual years due to different agro-climatic conditions for a specific year and variety planted. The yields in the period 1991 to 2009 ranged between 1.8 t/ha (2003) and 2.9 t/ha (1991) (Statistical Office of the Republic of Slovenia - SURS, 2011).

The total yield of oilseed rape for the entire Slovenia varied between individual years, dependent on yield and extends of production areas. The total yield ranged between 121 t (1997) and 14740 t (2007) (SURS, 2011).

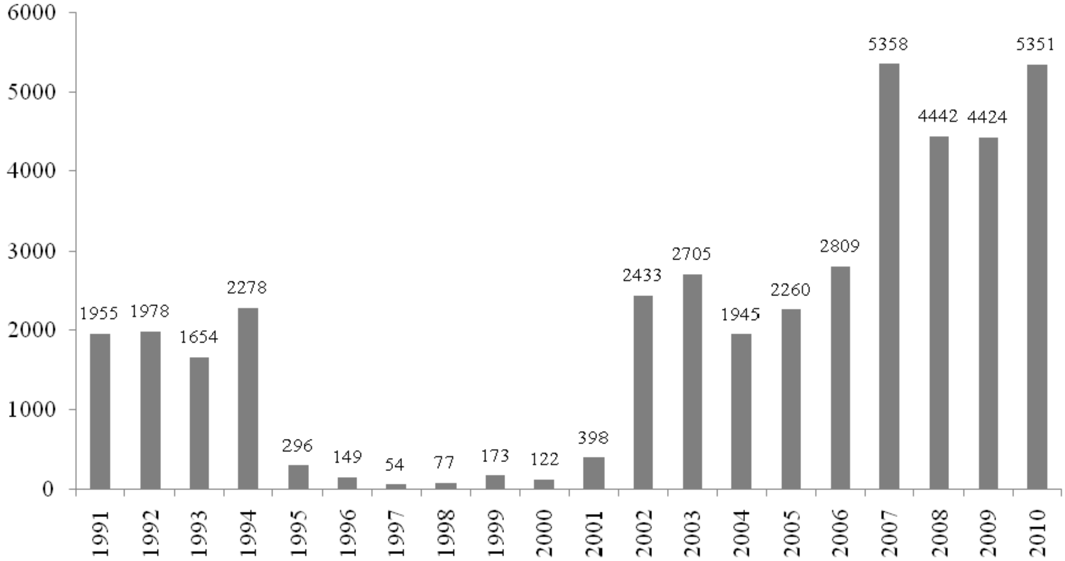

**Figure 1**: Oilseed rape production [ha] in Slovenia from 1991 to 2010 (SURS, 2011)
**Slika 1**: Pridelava oljne ogrščice [ha] v Sloveniji od 1991 do 2010 (SURS, 2011)

### Oilseed rape production on the regional level

Oilseed rape production is concentrated to the east cohesive region of Slovenia. The data about its production from 2007 to 2009 are presented in Table 1 (SURS, 2011).

**Table 1**: Oilseed rape production [ha] for cohesive regions of Slovenia (data for 2010 are not yet available) (SURS, 2011)

**Preglednica 1**: Pridelava oljne ogrščice [ha] po kohezijskih regijah v Sloveniji (podatki za 2010 še niso na voljo) (SURS, 2011)

| | **2007** | | **2008** | | **2009** | |
|---|---|---|---|---|---|---|
| | East Slovenia | West Slovenia | East Slovenia | West Slovenia | East Slovenia | West Slovenia |
| **Oilseed rape production [ha]** | 5235 | 123 | 4281 | 161 | 4293 | 131 |

Slovenian agricultural production areas are represented by 12 statistical regions. The oilseed rape production on the regional level is presented in Table 2. The data are available only from 2007 to 2009. Difference in the total oilseed rape production from SURS in 2009 (4424 ha) and summarized data from GERK (Land Parcel Information System - LPIS) evidence (2503.69) data between areas is derived from different sources and methods of raw data collection (difference between orto-photo pictures and the actual situation on particular field; *B. napus* sown as a main crop in the cropping system, where *B. napus* as a fodder rape is not included; all fields (farms) are not contained in the GERK evidence).

**Table 2**: Oilseed rape production in twelve statistical regions in Slovenia in 2009 (source: GERK evidence by Ministry of Agriculture, Forestry and Food)

**Preglednica 2**: Pridelava oljne ogrščice v dvanajstih statističnih regijah v Sloveniji v 2009 (podatki izvirajo iz GERK evidence na Ministrstvu za kmetijstvo, gozdarstvo in prehrano)

| Statistical regions | Oilseed rape production area | % of regional oilseed rape | Average size of fields with oilseed rape [ha] |
|---|---|---|---|
| **01 Pomurska** | 1205.18 | 48.14 | 0.90 |
| **02 Podravska** | 910.06 | 36.35 | 2.72 |
| **03 Koroška** | 2.6 | 0.10 | 0.43 |
| **04 Savinjska** | 68.27 | 2.73 | 1.90 |
| **05 Zasavska** | 0 | 0 | 0 |
| **06 Spodnje-posavska** | 200.59 | 8.01 | 0.98 |
| **07 Jugovzhodna Slovenia** | 20.22 | 0.81 | 2.53 |
| **08 Osrednjeslovenska** | 56.76 | 2.27 | 2.27 |
| **09 Gorenjska** | 37.01 | 1.48 | 3.36 |
| **10 Notranjsko-kraška** | 0.28 | 0.01 | 0.28 |
| **11 Goriška** | 0.56 | 0.02 | 0.28 |
| **12 Obalno-kraška** | 0 | 0 | 0 |
| **00 Non-defining areas** | 2.16 | 0.09 | 0.43 |
| **Total** | 2503.69 | - | - |
| **Average** | - | - | 1.46 |

The oilseed rape production is most extensive in the Pomurska and Podravska region where geographic and agro-climatic conditions are widely suitable. Fragmental landscape reflected in the field size of production areas;

average field size on the regional level is only 1.46 ha. Graphical presentation of oilseed rape production at the regional level (year 2009) is presented in Figure 2.

**Figure 2**: Graphical presentation of oilseed rape production in 12 statistical regions in Slovenia in 2009 (modelling and cartography: Jani Bergant, Agricultural Institute of Slovenia; data from the GERK evidence by Ministry of Agriculture, Forestry and Food; map: Ministry of the Environment and Spatial Planning)

**Slika 2**: Grafična predstavitev pridelave oljne ogrščice v 12ih statističnih regijah Slovenije v 2009 (oblikovanje in cartografija: Jani Bergant, Kmetijski inštitut Slovenije, podatki izvirajo iz GERK evidence na Ministrstvu za kmetijstvo, gozdarstvo in prehrano; podlaga: Ministrstvo za okolje in prostor).

## 3 VARIETAL STRUCTURE OF *B. napus*

The overall data about cultivated varieties in the Slovenian production area in the past was collected through official publications (National List of Varieties -NLV) and internal sources (market catalogues from different seed companies in Slovenia, results from variety tests and other internal registers held at Agricultural Institute of Slovenia).

In the period between 1984 and 1994 when the *B. napus* production in Slovenia was organised, farmers grew varieties with higher yields and lower levels of anti-nutritive substances (erucic acid, glucosinolates) for food and feed. These varieties were the following: the French variety 'Jet neuf', two German varieties, 'Darmor' and 'Tandem', later on the French variety 'Bienvenu' and the two Croatian varieties, 'Danica' and 'Zora', and also the variety 'Gorczanski' or the synonym 'Brilland' (Kocjan-Ačko, 1999). These varieties were also grown until 2000 (Ambrožič-Turk et.al., 1997; Ileršič, 1999; Ileršič and Rogelj, 2000); the first NLV was published in 1997 (Ambrožič-Turk et.al., 1997). In 2001, 'Darmor', 'Tandem', 'Bienvenu', 'Danica', 'Zora' and 'Gorczanski' were inscribed in the NLV (Bogolin et al., 2001). Only varieties 'Darmor', 'Bienvenu', 'Danica', 'Zora' and 'Gorczanski' were registered in the NLV in 2002 (Bogolin et al., 2002). The result of the national *B. napus* breeding programme was the fodder variety 'Starška' released in 1989. In 2003, NLV was not published due to the revision of the previous NLV and adaptation to the new seed legislation.

**Table 3**: The *B. napus* varieties and hybrids in the NLV from 2004 to 2010 (● registered or ○ unregistered in each year)

**Preglednica 3**: Sorte in hibridi *B. napus,* vpisani v Sortno listo od 2004 do 2010 (● registrirana ali ○ neregistrirana v posameznem letu)

| **Varieties of *B. napus*** | **2004 (Bogolin et al., 2004)** | **2005 (Bogolin et al., 2005)** | **2006 (Ileršič et al., 2006)** | **2007 (Ileršič et al., 2007)** | **2008 (Grižon et al., 2008)** | **2009 (Rakovec et al., 2009)** | **2010 (Rakovec et al., 2010)** |
|---|---|---|---|---|---|---|---|
| **'Adder'** | ○ | ● | ● | ○ | ○ | ○ | ○ |
| **'Akela'** | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| **'Alaska'** | ○ | ● | ● | ○ | ○ | ○ | ○ |
| **'Allure'** | ○ | ● | ● | ○ | ○ | ○ | ○ |
| **'Arista'** | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| **'Bienvenu'** | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| **'Brilland'/' Gorczanski** | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| **'Bristol'** | ○ | ● | ● | ● | ● | ● | ○ |
| **'Danica'** | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| **'Daniela'** | ○ | ● | ● | ● | ● | ● | ● |
| **'Darmor'** | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| **'Digger'** | ○ | ● | ● | ○ | ○ | ○ | ○ |
| **'Gabriella'** | ○ | ● | ● | ● | ● | ○ | ○ |
| **'GK Helena'** | ○ | ● | ● | ○ | ○ | ○ | ○ |
| **'Helga'** | ○ | ○ | ● | ● | ● | ● | ● |
| **'Milena'** | ○ | ● | ● | ○ | ○ | ○ | ○ |
| **'Petranova'** | ● | ○ | ○ | ○ | ○ | ○ | ○ |
| **'PR45D01'** | ○ | ○ | ○ | ○ | ○ | ○ | ● |
| **'PR45D03'** | ○ | ○ | ○ | ○ | ○ | ○ | ● |
| **'PR45W04'** | ○ | ● | ● | ● | ● | ● | ● |
| **'PR46W14'** | ○ | ○ | ○ | ○ | ○ | ○ | ● |
| **'PR46W15'** | ○ | ○ | ○ | ○ | ○ | ○ | ● |
| **'PR46W31'** | ○ | ● | ● | ● | ● | ● | ● |
| **'Starška'** | ● | ○ | ● | ● | ● | ● | ● |
| **'Titan'** | ○ | ○ | ○ | ● | ● | ● | ● |
| **'Viking'** | ○ | ○ | ○ | ● | ● | ○ | ○ |
| **'Zenith'** | ○ | ● | ● | ○ | ○ | ○ | ○ |
| **'Zora'** | ● | ○ | ○ | ○ | ○ | ○ | ○ |

Along with the *B. napus* varieties registered in the Slovenian NLV, other commercially important varieties have been and still are cultivated and are inscribed in the Common Catalogue of Varieties of Agricultural Plant Species. From 1984 to 2010 a total of 58 different varieties for food and feed were grown in Slovenia. These varieties were produced as oil varieties (for oil industry) and as spring varieties for animal feed as fresh fodder. The list of all varieties grown in Slovenia and their use is presented in Table 4.

**Table 4**: Varietal structure of *B. napus* in Slovenia from 1984 to 2010 and forms/type of use (Pipan et. al., 2010; Hasan et. al., 2006); W-winter form, S-spring form, OSR-oilseed rape, F-fodder rape

**Preglednica 4**: Sortna struktura *B. napus* v Sloveniji od 1984 do 2010 in oblike/tipi uporabe (Pipan et. al., 2010; Hasan et. al., 2006); W-ozimna oblika, S-jara oblika, OSR-oljna ogrščica, F-krmna ogrščica

| Name of variety/hybrid | Form/type of usage | Name of variety/hybrid | Form/type of usage |
|---|---|---|---|
| **'Adder'** | WOSR | **'Petrol'** | WOSR |
| **'Akela'** | WF | **'PR44W29'** | WOSR |
| **'Alaska'** | WOSR | **'PR45D01'** | WOSR |
| **'Allure'** | WOSR | **'PR45D03'** | WOSR |
| **'Arista'** | SF | **'PR45D05'** | WOSR |
| **'Baldur'** | WOSR | **'PR45W04'** | WOSR |
| **'Baros'** | WOSR | **'PR46W14'** | WOSR |
| **'Bienvenu'** | WOSR | **'PR46W15'** | WOSR |
| **'Brilland'='Gorczanski** | WOSR | **'PR46W24'** | WOSR |
| **'Bristol'** | WOSR | **'PR46W31'** | WOSR |
| **'Danica'** | WOSR | **'Rasmus'** | WOSR |
| **'Daniela'** | WF | **'Remy'** | WOSR |
| **'Darmor'** | WOSR | **'Robust'** | WOSR |
| **'Digger'** | WF | **'Rodeo'** | WOSR |
| **'Express'** | WOSR | **'Rohan'** | WOSR |
| **'Gabriella'** | WOSR | **'Smart'** | WOSR |
| **'GK Helena'** | WOSR | **'Starška'** | WF |
| **'Helga'** | SF | **'Tandem'** | WOSR |
| **'Honk'** | WOSR | **'Tassilo'** | WOSR |
| **'Jet Neuf'** | WOSR | **'Titan'** | WOSR |
| **'Kronos'** | WOSR | **'Triangle'** | WOSR |
| **'Milena'** | WF | **'Viking'** | WOSR |
| **'Mohican'** | WOSR | **'Visby'** | WOSR |
| **'Molino'** | WOSR | **'Viva'** | WF |
| **'Navajo'** | WOSR | **'X08W982 I.'** | WOSR |
| **'NK Nemax'** | WOSR | **'X08W984 I.'** | WOSR |
| **'NS-L-36'** | WOSR | **'Xenon'** | WOSR |
| **'NS-L-39'** | WOSR | **'Zenith'** | WOSR |
| **'Ontario'** | WOSR | **'Zora'** | WOSR |
| **'Petranova'** | SF | **NK Bikovo** | WOSR |

## 4 THE SIGNIFICANCE OF GENE FLOW OF *B. napus*

Gene flow through pollen transfer between different appeared forms of *B. napus* and its sexually compatible relatives which are mostly found as weedy plants has biological, genetic, economical and ecological consequences. An important breakpoint in the farming process was the adoption of national legislation and regulation on co-existence of different cropping systems in the Slovenian production area in 2009 when Slovenia adopted the Act on Co-existence of Genetically Modified Plants with Other Agricultural Plants (Uradni list, 2009).

### Genetic origin of *B. napus*

*B. napus* originated through spontaneous inter-specific hybridisation (followed by polyploidisation) between turnip rape (*B. rapa* L.; genome AA, 2n=20) and cabbage (*B. oleracea* L.; genome CC, 2n=18), resulting in alotetraploid genome comprising the full chromosome complements of its two progenitors (Friedt and Snowdon, 2009). Song and Osborn (1992) suggest that *B. montana* (2n=18) might be closely related to the prototype derived to both cytoplasms of *B. rapa* and *B. oleracea* through analyses of mitochondrial and chloroplast DNA.

The origin of *B. napus* is also described by Triangle of U (Nagaharu, 1935). This theory explains the evolution and the relationships between six oil and vegetable species from the *Brassica* genus. It is about the combination of three genomes from the genus *Brassica* (*B. nigra, B. oleracea, B. rapa*) which are ancestral to other three inter-specific hybrids (*B. carinata, B. juncea, B. napus*) (Figure 3).

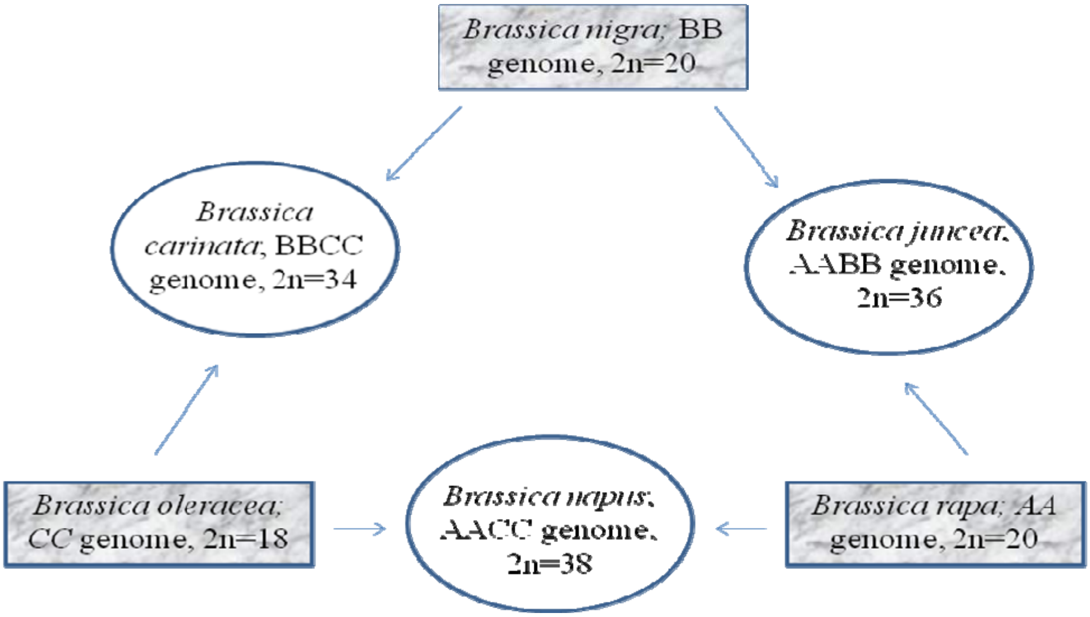

**Figure 3:** Triangle of U-diagram (Nagaharu, 1935)
**Slika 3:** Trikotni U-diagram (Nagaharu, 1935)

It is considered that *B. napus* is a relatively young species originated only a few centuries ago. Spontaneous hybridization between *B. rapa* and *B. oleracea* (from Europe and Asia) occurred by contemporary cultivation of both species in a small geographic area of the Mediterranean region (Friedt and Snowdon, 2009).

*B. napus* is a self fertile and mainly autogamous species with variable degree of cross-pollination. Treu and Emberlin (2000) reported on 5-30% cross-pollination level under field conditions and about 41% out-crossing with the amount differing in relation to the prevailing environmental conditions. its pollen could be dispersed mainly by insects and to a smaller amount also by wind. Both are probably affected by location, weather and plant genotype. The pollen grains are fairly large (32-33µm), heavy, sticky and consequently typical for insect pollination (Simpson et al. 1999; Treu and Emberlin, 2000).

There is no evidence that *B. napus* does exist as a wild plant but it frequently appears as a feral plant outside the cultivation areas. There are a few traits which are typical for wild plants and are also presented in *B. napus* (easy dehiscence of its pods, secondary dormancy of the seeds, annually adapted seed germination) (Pascher et al., 2010).

### Possibilities for cross-pollination with *B. napus*

Pollen dispersal and gene flow between *B. napus* and other sexually compatible plants is possible due to its variable cross-pollination level. In the Slovenian production area *B. napus* from various habitats (cultivated, volunteers, feral populations) and some compatible relatives from *Brassicaceae* family could be found.

#### *Intra-specific hybridization*

Intra-specific hybridization is possible among *B. napus* in field conditions and between different plant forms from various (semi) natural habitats. Pollination relations occur between cultivated *B. napus* (mainly oilseed rape varieties), volunteers (grown from seed losses in previous years inside cultivated areas) and ferals (grown from the soil seed bank outside cultivation areas, mainly along the transportation infrastructure) (Pascher et al., 2006, Pipan et al., 2008). In the case of coexistence of different farming systems which includes genetically modified (GM) oilseed rape production, introduction of transgenes in *B. napus* or related species is possible (Pascher et al., 2010).

Volunteers within cultivation areas originated from seed losses due to the natural shedding and crop disturbance during harvest (inappropriate additional equipment of combine harvesters). Because of unsuitable agro-technical procedures, the lost seeds are accumulated in the soil seed bank and appear when conditions for germination are optimal. Consequently, *B. napus* appears as a volunteer and as a weed in other crops cultivated in the same production areas in the following years (Price et al., 1996). Lutman (1993) reported that seed losses in adverse conditions can exceed 20% of the yield.

The presence of volunteers in Slovenia is concentrated in regions where oilseed rape production is widely extended. The problematic zones within cultivated areas are field margins that are very often not included in cultivation and fields where oilseed rape production is not intensive (leaving off farming) (Pipan et al., 2010).

Feral *B. napus* populations appear outside cultivation areas. Their persistence is typical for pioneer habitats like tracks, roadsides, waste sides, rubble tips, and riverbanks (Privard et al., 2008). The origin of feral *B. napus* populations results from the soil seed banks around transportation infrastructure. The seeds are viably persistent in the soil for at least eight years. Soil seed bank enrichment is mainly possible continually via seed spillage from trucks in transit over the country (Crawley, 2004; Pessel, 2001).

The presence of volunteer and feral *B. napus* populations in oilseed rape production areas can cause problems for varietal purity and yield quality. Food and feed products could consequently contain higher levels of anti-nutritional substances. Coexistence of GM and non-GM oilseed rape production could cause the uncontrolled transfer of transegnes into cultivated non-GM *B. napus* or to other sexually compatible plants (Claessen et al., 2005). The ecological aspect of described relations has an important impact on *B. napus* gene pool and sustainable food and feed production. The persistence of feral *B. napus* population in Slovenia was observed allong all types of roads and in other ruderal habitats (embankments, heaps) (Pipan et al., 2010).

*B. napus* is a model species of particular interest for assessing risks of transgene escape from cultivated fields. Modeling approaches have been shown as essential since they allow exploration of a wide range of input values. Begg et al., (2006) has developed a model on the persistence of existing transgenic event introduced into oilseed rape which explores the effects of demographic and agronomic factors under the influence of spatial processes. Colbach et al. (2001a and 2001b) developed the so-called GENESYS model in which they established how the agricultural practice influenced the gene flow from GM oilseed rape (it possesses an inserted gene for the resistance to herbicide) to volunteers in the following years of production.

*Inter-specific hybridization*

Inter-specific hybridization between *B. napus* is possible with some sexually compatible plants from *Brassicaceae*. The relatives of *B. napus* are cultivated as crops and others that are known as weeds in farming systems and wild flowers outside cultivated areas (Estham and Sweet, 2002). There are many species closely related to *B. napus* such as: *B. rapa, B. juncea, B. oleracea, B. nigra, Hirchfeldia incana, Raphanus raphanistrum, Sinapis arvensis, Diplotaxis erucoides, D. tenuifolia, D. muralis, S. alba, R. sativus* and *Rapistrum rugosum*. (Estham and Sweet, 2002; Pascher et al. 2010; Treu and Emberlin, 2000). These plants could be found inside and outside the cultivated areas (field edges, shelterbelts, road verges, slag heaps, embankments, etc) (Pascher et al., 2010). Scheffler and Dale (1994a) report that the opportunity for inter-specific hybridization is dependent on: physical distance between two species, synchrony of flowering, method of pollen dissemination, parental genotype characteristics and environmental conditions. The highest pollination affinity to *B. napus* has especially *B. rapa* and also *B. oleracea* and *B. juncea*. The hybrids which were obtained between *B. napus* and the wild relative are not always viable. The success of its existence is influenced by: fertility, ability to propagate vegetatively, ability to give viable F2 generation (Scheffler and Dale, 1994a). Successful spontaneous hybridizations (without emasculation or manual pollination) between *B. napus* and *B. rapa, B. juncea, B. nigra, H. incana, R. raphanistrum* have been documented and all of the F2 hybrids and backcross progeny was produced (Scheffler and Dale, 1994b).

Sagers et al. (2010) have recently reported about the problem of uncontrolled gene flow, which describes the presence of the transgene (for herbicide tolerance) in feral populations of *B. napus* among transportation infrastructure in North Dakota. The report especially exposed a high probability of gene transfer between sexually-compatible relatives of *B. napus* outside production areas and global implications of such activities for farmers, consumers and for the environment.

There are some closely related *Brassicaceae* species, which are also represented in the Slovenian production area. According to Jogan (2001) different species are spread around the country: *B. rapa, B. oleracea, B. nigra, Raphanus raphanistrum, R. sativus, Diplotaxis muralis, D. tenuifolia, Sinapis alba, S. arvensis* and *Rapistrum rugosum*. They could be cultivated (*S. alba, B. rapa, B. oleracea*), weedy or wild.

**Pollen dispersal characteristics of *B. napus***

The biennial *B. napus* is grown in Slovenia because of overwintering vernalization demands. It flowers in April to early May, the annual (spring) *B. napus* which is sown and harvested in the same year, flowers one month later than winter forms. Consequently, it is widely believed that the insects have more important role in the cross-pollination of later flowering crops (Estham and Sweet, 2002). Ramsay et al., (1999) reported that bees from the same hive could transfer pollen from 2 up to 4 km in all directions. The theoretically expected pollen transfer by insects is up to 10km, but the distances are influenced by environmental and topographical conditions. The pollen viability under *in vitro* conditions is between 24 hours and 7 days (Mesquida and Renard, 1982) and under natural conditions from 4 up to 5 days (Ranito-Lehtimäki, 1995). *B. napus* is a facultative out-crossing species; in presence of insect pollinators (*Apis mellifera*, *Bombus* sp.) higher proportion of cross-pollination could occur (Snowdon et al., 2007). *B. napus* pollen (8-40% of proteins) is also highly nutritive for bees (Estham and Sweet, 2002).

Inside cultivation areas the usual level of cross-pollination is from 20 to 30 % (Squire et al., 2003). The level of cross-pollination between different varieties with full fertility is up to 0.1 % on the field-to-field scale, while in varieties with incorporated male sterility (bait plants; they produce no pollen on their own and represent the ‘worst case scenario’ on the cross-pollination level) it is higher than 1 % (Squire et al, 2003). Cross-pollination is most prominent on field margins and starts diminishing after 10 m, but, in spite of that, the pollination at greater distances is not excluded. This is more frequent in cases when in the surrounding of donor plant/cultivated crop no other flowering plants are present. The rate of cross-pollination is significantly influenced by proportions between donor and recipient plants (Squire et al., 2003).

Cleistogamy of *B. napus* is a trait of non-opening flowers which could be used to reduce pollen dispersal and it could be beneficially used in combination with other means in a gene flow control strategy. The problem of cleistogamous *B. napus* genotypes is their stability under field conditions because cleistogamous plants do not exist naturally among *B. napus* gene pool (Leflon et al., 2010).

Fragmented landscape and small-sized oilseed rape fields in Slovenia is very heterogeneous. Because of different ecological barriers like landscape structural elements (small woods, hedges, overgrown paths and hills), the pollen transfer by insects and wind to long distances is disturbed. There is no specific pattern of pollen dispersal and out-crossing on short distances (neighboring fields, weedy relatives, ferals, and volunteers) is favorable.

## 5 CONCLUSION

Heterogeneous growing conditions are reflected in the fragmental landscape production on the regional level and small sized fields. In Slovenia, the production of *B. napus* is mostly concentrated in the Pomurska and Podravska region (East Slovenia), where the average field size at the regional level is 1.46 ha.

Diverse list of varieties and hybrids, which are (were) grown in Slovenia is mostly the consequence of agro-political regulations at EU level. As type and form of *B. napus*, mostly the winter oilseed rape is produced in Slovenia. The GM *B. napus* is not yet grown in the EU although national legislations on co-existence of genetically modified plants with other agricultural plants are generally in place; in Slovenia it has been regulated since 2009.

Knowledge about all *B. napus* forms (cultivated, volunteer plants, feral populations) and their relevance in hybridization relations brings important basic information for survey studies of naturally occurred genetic diversity of *B. napus* on the national level. Consequently, it is important to assess reasons, solutions and problems related with persistence of volunteers and ferals which directly and indirectly influence food and feed production. In case of the genetically modified *B. napus* cultivation, there is a possibility of introduction of transgenes to non-GM relatives due to the persistence from cultivation or transport spillage. Growing conditions and production in different cropping systems dictate consistent and intensive *B. napus* (mostly oilseed rape) production which should be economically and environmentally friendly. Choosing right solutions will significantly influence the yield quality or seed purity in sustainable food and feed production and could have impact on the agro-biodiversity.

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# Massive occurrence of *Podagrica fuscicornis* (L.) (Coleoptera, Chrysomelidae) on common marshmallow (*Althaea officinalis* L.)

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## ABSTRACT

In May 2011 we noticed high number of *Podagrica fuscicornis* adults on the leaves of common marshmallow (*Althaea officinalis*) grown on Laboratory Field of Biotechnical Faculty in Ljubljana. In Slovenia, this beetle was already recorded earlier but it did not caused any damage on common marshmallow (*Althaea officinalis*). Due to the feeding of beetles on leaves, 1 to 2 mm large holes appear. By expecting climate changes, already known bionomics of some related species and giving more emphasis on herb production in the future, Chrysomelid *P. fuscicornis* might represent medium sized biological factor in producing common marshmallow.

**Key words:** flea beetles, *Podagrica fuscicornis*, medicinal plants, common marshmallow, *Althaea officinalis*

## IZVLEČEK

### MOČAN POJAV VRSTE *Podagrica fuscicornis* (L.) (Coleoptera, Chrysomelidae) NA NAVADNEM SLEZU (*Althaea officinalis* L.)

V maju 2011 smo na listih navadnega sleza (*Althaea officinalis*) na Laboratorijskem polju Biotehniške fakultete v Ljubljani ugotovili večje število odraslih osebkov bolhača *Podagrica fuscicornis*. Vrsta je bila v Sloveniji že prej zastopana, a ni bila škodljiva pri pridelovanju navadnega sleza (*Althaea officinalis*). Na listih rastlin se kot posledica hranjenja hroščev pojavijo 1-2 mm velike luknjice. Ob pričakovanih podnebnih spremembah, znani bionomiji nekaterih sorodnih vrst in večjem pomenu pridelave zelišč v Sloveniji bi lahko vrsta *P. fuscicornis* v prihodnosti bila srednje pomemben biotični dejavnik pri pridelavi navadnega sleza.

**Ključne besede:** bolhači, *Podagrica fuscicornis*, zdravilne rastline, navadni slez, *Althaea officinalis*

## 1 INTRODUCTION

In Europe herbal plants production is taking place on land area of around 120,000 hectares and utilisation of medicinal plants, flavour and aromatic plants, spices and herbs for special purposes (natural pigments, antioxidants etc.) is becoming an important category of agricultural production. Slovenia is among those countries which do not have any particular tradition in medicinal plant production. In Flora of Slovenia there are 3216 species which are registered as higher plants and almost 12 % are less or more used as medicinal or aromatic plants. According to survey data from 2004 medicinal plants are grown on 20 to 25 hectares in Slovenia (Rode and Knapič, 2006), and afterwards their production extent decreased (Statistični urad RS, 2011).

Flea beetles are members of family leaf beetles (Chrysomelidae) and occur in all vegetation habitats. Palaearctic species occur most frequently in the open, namely in the vicinity of grasslands, forests and water bodies (Gruev and Döberl, 1997; Çilbiroglu and Gok, 2004). Only some of these species cause damage on cultivated or native plants permanently or on occasions.

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In the following paper we want to introduce the species of flea beetles, which is a typical member of subfamily Alticinae, but in contrast to some other related species – flea beetles (*Phyllotreta* spp.) (Brelih *et al*., 2003) or hop flea beetle (*Psylliodes attenuatus* [Koch] (Brelih *et al*., 2003; Rak Cizej and Milevoj, 2007) – not yet presenting special danger in plant production. In 2011 we noticed on common marshmallow a massive occurrence of adult beetles which fed on leaves and flowers and consecutively evoke typical symptoms – spherical and oblong hollows.

## 2 MATERIALS AND METHODS

In May 2011 we noticed during the survey of occurrence of pest organisms on herbal plants on Laboratory Field of Biotechnical Faculty, hollows on leaves of common marshmallow. The hollows were characteristical for representatives of Chrysomelidae family. On leaves (Figures 1 and 2) we found larger number of beetles, which were determined as *Podagrica fuscicornis* (Linnaeus, 1767) according to morphological characters. During the monitoring in July we noticed that the adults feed also on flowers of common marshmallow (Figure 3).

**Figure 1:** Flea beetle *Podagrica fuscicornis* and damage spots made because of its feeding on leaf of common marshmallow (photo by T. Bohinc)

**Figure 2:** Damage on the leaf of common marshmallow due to the feeding of *Podagrica fuscicornis* adults (photo by T. Bohinc)

**Figure 3**: Damage on the flower of common marshmallow due to the feeding of *Podagrica fuscicornis* adults (photo by T. Bohinc)

## 3 PRESENTATION OF THE SPECIES

### 3.1 Description

We describe flea beetles as small bugs with a size from 1.5 do 4 mm. They can jump due to the enlarged backside organ (hind legs) – Maulik's organ. They also have an ability to fly. When plant is heavily attacted by flea beetles, small round holes (up to 1 mm) caused by an individual flea beetle's feeding may coalesce into larger areas of damage (Maceljski, 1999).

Adult beetles of *Podagrica fuscicornis* are 3.0 to 6.0 mm in size. Head and neck shield are coloured red. Front wings (sheath-wings) are dark blue to blue green and rarely are detected metal coloured. Sheath wings contain hollows which are deep, very abundant and spread scarced. Hollows on neck shield are even more compacted. Legs are characteristically yellowish to bright brownish, and upper labium is also yellow (Hubble, 2010).

### 3.2 Systematics

Flea beetles are classified into family leaf beetles (Chrysomelidae) and subfamily Alticinae (Aslan *et al*., 1998). In Slovenia up till now 204 species and 5 subspecies of flea beetles (Brelih *et al*., 2003) were detected, while on the global level more than 7000 species were described (Jolivet, 1988). In Slovenia beside *Podagrica fuscicornis* we are ranging in the same genus also species *P. malvae* (Illiger), *P. menetriesi* (Falderman) and *P. fuscipes* (F.) (Podagrica…, 2011).

### 3.3 Geographical distribution

*Podagrica fuscicornis* belongs to European-Mediterranean species group. The abundance of its populations is very large in the majority of Europe. Flea beetle is not present in Nordic countries. In Asia it occurs in Turkey and western part of Arabian Peninsula. It can be found also in Tunisia, Morocco and on Canary Islands. The abundance of this pest is from time to time also high in warmer parts of Slovenia (Brelih *et al*., 2003), but till now no clear evidence is noted that it would cause extensive range of damage on its host plants.

### 3.4 Bionomics

Flea beetles are insects with complete metamorphosis. Adult females lay eggs, which hatch into larvae of various shapes. After feeding and molting several times, larvae mature, pupate and later emerge as adult beetles; the length of time it takes to complete the life cycle varies greatly from species to species and is also dependent on weather and other environmental conditions (Rak Cizej *et al*., 2001).

Physical and chemical factors of plant defence many times have influence on the survival ability of larvae. The latter can be also affected by the appearance of natural enemies, unfavourable weather conditions,… (Rak Cizej *et al*., 2001).

Larvae molt three to four times and than pupate. After one to two weeks adults occur. The Chrysomelid has only one generation per year (Cmoluch, 1988). Adults overwinter usually in the soil under the grass sward, where often enough organic matter or nearby host plant are present. During overwintering they avoid exposed surfaces as arable field are (Rak Cizej *et al*., 2001).

### 3.5 Host plants

Very know members of genus *Podagrica* cause most damage on plants from family Malvaceae. *Podagrica fuscicornis* belongs among oligophagus herbivores and it induces damage on plants from genera *Althaea, Malva* and *Lavatera* (Neubauer *et al*., 1974; Cmoluch, 1988; Lecheva *et al*., 1996; Rotrekl, 1996; Brelih *et al*., 2003). Above mentioned species can cause damage also on plants from families Lamiaceae and Urticaceae (Petitpierre, 1985).

#### 3.5.1 Common marshmallow (*Althaea officinalis* L.)

Common marshmallow or iviscus (Figure 4) is classified into family Malvaceae. The plant is perennial and soft and velvety due to a dense covering of stellate hairs and grows 2.2 m in height. Its origin is Europe but it can be found also in North and South America. Its natural habitats are usually moist fields, but it could be found also by the seasides and more and more it is also cultivated (Chevallier, 1996). Growers cultivate it on warmer soils. Common marshmallow is not very demanding for nutrients, it only needs deep soils and with good porosity and no rocks. It likes moisture but not standing water. We can reproduce it by seeds or vegetative with roots. Planting distance depends on which part of the plant we are aiming as a final product (Rode and Knapič, 2006).

**Figure 4**: Plants of common marshmallow (*Althaea officinalis*) at the Laboratory Field of Biotechnical Faculty in Ljubljana in 2011 (photo by T. Bohinc).

Roots of common marshmallow contain 37 % of starch and 11 % of plant mucilage and both represent important part of herbal plant. Important medicinal parts of the plant are also leaves and flowers. Common marshmallow is used to treat infections which affect mucous membrane as its appeasing effect reassures and protects against harmful influences (Chevallier, 1996; Franova *et al*., 2006). It is used particularly in herbal tea mixtures (Franova *et al*., 2006).

## 4 POTENTIAL ECONOMIC IMPORTANCE OF *Podagrica fuscicornis* IN SLOVENIA WITH CONCLUSIONS

Among most important flea beetles pests in Slovenia cabbage flea beetles (*Phyllotreta* spp.) are placed as they can have an influence on reducing the yield of cabbage and other cultivated Brassicas (Brown *et al*., 2004). Also hop can be damaged and this is attribute to hop flea beetle (*Psylliodes attenuatus*). Important host plants of hop flea beetle are common hop plant (*Humulus lupulus* [L.]), common hemp (*Cannabis sativa* ssp. *sativa* var. *sativa* [L.]), and common nettle (*Urtica dioica* [L.]) (Rak Cizej and Milevoj, 2007).

The presence of *P. fuscicornis* in Slovenia for now did not represent larger danger. But in tropical and subtropical regions representative of genus *Podagrica* spp. regulary threaten the plant production from family Malvaceae. In this relation a permanent treath of economical reasonableness of okra production (*Abelmoschus esculentum* [Moench]) in Africa (Echereobia *et al*., 2010) is worth to mention as this species carries also viruses (Triendrébéogo *et al*., 2010).

Different plant species are differently adapted to drought periods. Some herbs originate from arid regions and are basically already adjusted to the shortage of moisture in the soil (Rode and Knapič, 2006). Plants of common marshmallow are described as the one which love marshy environments and are very well present all around Europe (Ross, 2001; Gardner, 2005). Global climate changes could have already in the near by future an extensive influence on greater importance of some pests, also those which harm common marshmallow. And *P. fuscicornis* could spread in the forthcoming time from warmer parts of the Europe to regions which have till now unfavourable climate factors for the occurrence of species.

In our opinion, induced extent of damage made by flee beetle presented in this paper did not influence the productivity of common marshmallow. But the abundance of up till now less known insect species warns us that changes in weather patterns and future climate changes could have influence on bionomics of the plant pests (Fuhrer, 2003; Thomson *et al*., 2010), which was not till know classified as economically important. If expecting the increase in herbal plants production, as well on domestic as broader, we must namely take into consideration also this factor.

## 5 ACKNOWLEDGEMENT

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**Agris category code**: H20, F03

# Impact of fungicides and other preparations for seed treatment and different cultivation techniques on seed contamination of winter wheat (*Triticum aestivum* L. emend. Fiori et Paol.)

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## ABSTRACT

Contamination of cereal crops and their products could be a result of inappropriate cultivation techniques as well as disregarding usual agro-technical measures like the seed treatment with fungicides. The aim of the study was to determine the impact of pre-sowing seed treatment on wheat seed infection and contamination of produced grains from the field trial at the Biotechnical faculty (BF) and contamination of crop samples from Slovene farms with conventional and organic type of production. In our laboratory we performed grain incubation on agar with various disinfectants before sowing the grain on the field. We found that seed treatment with fungicides Maxim 050 FS and Vitavax 200-FF had significantly improved the health status (2% infected grains) compared to untreated processed seed (25% infected grains). The effectiveness of both tested fungicides was significantly better (2% infected grains) compared to the Agrostemin and Fitolife preparations, which are allowed in organic farming (15% infected grains). Compared to the untreated seed, treating the seeds with both fungicides and the Agrostemin preparation reduced visible ear contamination with fusariosis during the time of dough maturity. With incubation of grain from our field experiment with different seed treatments, the positive effect of fungicides on the health status of grain yield was confirmed (27% infected grains), compared to sowing of seed that was not treated with disinfectants (34% infected grains). When compared to the control group a positive effect of wheat seed treatment with disinfectants used in organic farming was determined. Furthermore, seed treatment with fungicides had a greater influence on improving the health status of produced grain, compared to the above mentioned preparations allowed in organic farming (29% infected grains). The effect of seed treatment on the health status of the grain was the greatest when using the Vitavax 200-FF fungicide. The percentage of infected grains in laboratory incubation of produced wheat grain on agar, sampled from eight Slovenian farms, ranged from 1.5 to 19.5%. Contamination of sampled grain from organic production (7% infected grains) was comparable with infection of grain from the conventional farming (8.4% infected grains), where the infection ranged from 1.5% to 19.5%.

**Key words:** crop management, *Triticum aestivum*, seed infection of winter wheat, fungicides and the other preparations for seed treatment, laboratory tests for infection of seed

## IZVLEČEK

### VPLIV FUNGICIDOV IN DRUGIH PRIPRAVKOV ZA RAZKUŽEVANJE SEMENA TER NAČINOV PRIDELAVE NA OKUŽENOST SEMENA OZIMNE PŠENICE (*Triticum aestivum* L. emend. Fiori et Paol.)

Onesnaženja pridelkov in izdelkov žit so lahko posledica slabe pridelovalne prakse, zlasti neupoštevanja običajnih agrotehničnih ukrepov, med katerimi je razkuževanje semena. Namen raziskave je bil ugotoviti vpliv razkuževanja semena na okuženost semena pšenice pred setvijo in pridelka zrnja iz poljskega poskusa Biotehniške fakultete (BF) ter okuženost vzorcev pridelanega zrnja iz konvencionalne in ekološke pridelave. Pri laboratorijski inkubaciji zrnja na agarju pred setvijo poljskega poskusa na BF z različnimi razkužili smo ugotovili, da tretiranje semena s fungicidoma Maxim 050 FS in Vitavax 200-FF (2 % okuženih zrn) pomembno izboljša zdravstveno stanje v primerjavi z nerazkuženim dodelanim semenom (25 % okuženih zrn). Delovanje obeh fungicidnih pripravkov je bilo statistično značilno boljše (2 % okuženih zrn) od pripravkov Agrostemin in Fitolife, ki sta dovoljena v ekološkem kmetijstvu (15 % okuženih zrn). Razkuževanje semena s fungicidoma in pripravkom Agrostemin je v primerjavi z nerazkuženim semenom zmanjšalo vidne okužbe klasov s fuzariozami v času voščene zrelosti. Pri inkubaciji

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pridelka zrnja iz poljskega poskusa z različnimi razkužili smo potrdili pozitiven vpliv tretiranja semena s fungicidoma na zdravstveno stanje pridelanega zrnja (27 % okuženih zrn) v primerjavi s setvijo nerazkuženega semena (34 % okuženih zrn). V primerjavi s kontrolo je bil ugotovljen pozitiven vpliv razkuževanja semena pšenice s pripravkoma, ki ju lahko uporabljajo tudi ekološki kmetje. Razkuževanje s fungicidoma je imelo večji vpliv na boljše zdravstveno stanje pridelanega zrnja kot pripravka dovoljena v ekološkem kmetijstvu (29 % okuženih zrn). Vpliv razkuževanja na zdravstveno stanje pridelanega zrnja je bil največji pri fungicidu Vitavax 200-FF. Pri laboratorijski inkubaciji pridelka zrnja pšenice na agarju z osmih slovenskih kmetij smo ugotovili, da je bila okuženost vzorcev pridelanega zrnja iz ekološke pridelave (7 % okuženih zrn) na ravni vzorcev iz konvencionalne pridelave (8,4 % okuženih zrn), pri katerih pa je bil razpon okuženosti od 1,5 % do 19,5 %.

**Ključne besede**: načini pridelave, *Triticum aestivum*, okuženost semena za setev in pridelanega zrnja ozimne pšenice, fungicidi in drugi pripravki za razkuževanje semena, laboratorijski testi okuženosti semena

## 1 INTRODUCTION

Contamination with fungal diseases is becoming more and more common, causing a food safety risk for the consumers of cereal products. Numerous fungi contaminating the wheat grains form and secrete secondary metabolites, the so called mycotoxins. These mycotoxins are the cause of mycotoxicosas - diseases than can cause direct poisonings like chronic liver illness and tumour activation. Mycotoxins are formed by the *Aspergillus*, *Penicilium* in *Fusarium* fungal plant pathogens that are frequently present on our cereal grains (Kovač, 2009; Jakovac-Strajn et al., 2010). *Fusarium* fungi can contaminate grains during the growing period and in the ripening phase. Weather conditions, especially during the time of grain ripening have an important influence on fungi development on the wheat crop, since wet conditions importantly increase the possibility of fungal development in that morphological state. (Cowger et al., 2009). Poor agricultural practice especially ignoring the usual agro technical measures can cause higher occurrence of different fungal infections on wheat grains.

Seed treatment is the basic measure to assure an adequate health of crops at emergence and during further growth of plants. Numerous diseases could be transferred to the wheat crops through the use of poorly cleaned and untreated or inappropriately treated seed. They can completely prevent the emergence of plants, reduce overwintering or systemically infected the plants that later become a new focal point for further spreading of the disease. Stinking bunt (*Tilletia tritici* (Bjerk.) Wolff), Dwarf bunt (*Tilletia controversa* Kühn), Septoria leaf blotch (*Septoria tritici* Roberge ex Desmaz), Septoria leaf and glume blotch (*Phaeosphaeria nodorum* (E. Müller) Hedjaroude) and Loose smut (*Ustilago nuda* (Jensen) Rostr. *f. sp. tritici* Schaffnit) (Maček, 1987;) can all be transferred through the seed. Also the agents causing the Pink snow mould (*Monographella nivalis* (Schaffnit) E. Müller) with similar signs as Fusarium fungi just without violet-pink coloration could be transferred through the seed. Different to the fusariosis the fungus causing the Pink snow mould does not secrete mycotoxins (Maček, 1987; Murray et al., 2009).

During the last few years there is an increasing trend of sowing untreated wheat seed in Slovenia; mainly from two reasons. The first reason is the prohibition to treat seed on agricultural holdings which also caused the withdrawal of treatment substances from retail shops (Rules on …, 2009). The second reason is organic farming guidelines that prohibit the use of synthetic chemical substances for the seed disinfection. The rules for organic farming in Slovenia do not allow the use of any substances used exclusively for the seed treatment with a purpose to reduce the hazard of contamination with agents causing fungal diseases transmitted through the seed. (Bavec et al., 2009). Treatment of certified seed is legally obligatory only when disease thresholds for different diseases have been exceeded (Rules on marketing …, 2005).

Other agro technical measures performed before sowing and during the vegetation period can also influence the health condition of the seed. Adequate crop rotation, tillage, fertilizing (mainly with nitrogen), protection with fungicides and other cultivation procedures are among the most efficient measures. In Slovene cultivation practice a relatively narrow crop rotation with a high share of maize and wheat represent an important hazard for the poor health status of wheat seed (Kocjan and Šantavec, 2010; Statistical yearbook, 2008; Tajnšek and Šantavec, 1998).

The goal of this study was to test the impact of fungicides and other seed treatment preparations to the contamination of wheat seed before sowing and contamination of grain crop from the field trials at the Biotechnical faculty (BF). With the analysis of wheat seed samples obtained from eight Slovene wheat producers we examined the contamination of produced grains depending on the wheat cultivation practice (conventional, organic).

## 2 MATERIALS AND METHODS

### 2.1 Contamination of wheat seed from the BF field trial

In the first part of the research we studied the influence of different fungicides and other preparations on contamination of wheat seed with fungal diseases. We used the Ficko winter wheat variety, which was developed in Croatia and registered in Slovenia in 2007.

The following treatments were included in our field trial:

**K -** untreated, certified processed seed,
**A -** certified processed seed that we treated with Agrostemin (concentrate) in the amount of 30 g/ha,
**F -** certified processed seed, that we treated with Fitolife in the amount of 300 g/100 kg of seed,
**M -** certified processed seed, that we treated with Maxim 050 FS fungicide (difenoconazol 2.5% + fludioxonil 2.5%) in the dose of 1.5 l/t,
**V -** certified processed seed treated by the processor with the fungicide Vitavax 200-FF (carboxin 20% + thiram 20%).

Treatments A and F are the two seed treatments allowed in organic farming. Both this preparations are allowed in organic farming (Bavec et al., 2009), but until now they were mostly used for the treatment of plants and not for the treatment of the seed. Agrostemin is a growth promoter acquired from the Common corncockle (*Agrostemma githago* L.). Fitolife is a natural mineral substance with the majority of calcium carbonate ($CaCO_3$) (88%), also including 4% of magnesium carbonate ($MgCO_3$) and 0.3% of iron. With the M treatment we tried to illustrate the most common seed treatment used on the Slovene farms.

One part of the seeds from all five treatments was used for the incubation in the laboratory to determine the presence of the pathogenic fungi. Incubation was performed in four repetitions. The wheat seed was incubated in closed petri dishes filled with sterilised agar; we have placed fifty wheat seeds into each dish. A 5-day incubation was carried out in the growth chamber at permanent light and on 20 to 22 °C. After the incubation period we counted all contaminated seeds. Any seed with visible development of fungal mycelium was considered contaminated.

In the autumn of 2009 we started our field trial by sowing the wheat seed of Ficko variety on the laboratory field at the Biotechnical faculty in block trial with three repetitions. The soil on the laboratory field is medium deep pseudogley (planosol), with the silt loam texture, and meliorated, but still occasionally flooded on the surface at times of heavy rains. It contains 4.5% of organic matter through the depth of arable land and is well supplied with phosphorus and potassium. The previous crop was the seed maize.

The sowing was performed on the October 19th with the plot sowing machine to the 12.5 cm row spacing, the sowing density was 700 sprouting seeds/m$^2$. The size of the basic parcel was 5.6 m$^2$. The 1000-grain weight of the seed was 44 g. The trial was fertilized with KAN (60 kg N /ha) during the tillering period (BBCH 21-23). The crop was not treated with fungicides for the purpose of monitoring the influence of the seed treatment to the incidence of fungal diseases. At the time of dough maturity (BBCH 73-75) we determined the crop density (number of ears/m$^2$); at the same time we counted the ears with visible contamination with the *Fusarium* fungus. We than calculated the share of contaminated ears. All counting was performed using the 50 cm × 50 cm wire frame, twice on all parcels. The trial was harvested with the plot harvester on July 21st. The grain produced on individual plots was incubated in laboratory conditions in two repetitions. We used the same incubation procedure and the same procedure to determine the share of contaminated seeds as for the grains intended for sowing.

### 2.2 Contamination of wheat grains in conventional and organic production

In July 2010 we obtained the samples of wheat grain produced on eight wheat producing farms (six conventional and two organic) in different geographic areas of Slovenia. We questioned the owners of the farms about the previous crop in the rotation, cultivation methods, intensity of fertilization with mineral nitrogen and protection against fungal diseases. The seed samples from eight locations were incubated in laboratory conditions in four repetitions. The incubation procedure and the method for the determination of the share of contaminated seed were the same as the ones used in the trial with the two fungicides and other seed treatment preparations.

With analysis of variance we analysed the data from both parts of the trial using the Statgraphics Plus for Windows and presented the results in the form of graphs.

## 3 RESULTS AND DISCUSSION

### 3.1 Results of wheat seed treatment

After incubation of the certified processed wheat seed, the average 25% of all grains were contaminated with different fungal disease agents (Figure 1). Treatment with the two preparations that are allowed in organic farming (Figure 1, treatments A and F), reduced the seed contamination by 40%. Both preparations equally improved the health state of the wheat seed in the laboratory conditions. Even better effects on the health status of the wheat seed compared to the untreated wheat seed was proved for the treatment with organic fungicides (Figure 1, treatments M and V). The share of contaminated seeds has been reduced by 90%. The positive effect of the Vitavax 200FF fungicide to the general health state of wheat seed was also proved by

Samobor et al. (2008), but they also state in their paper that treatment with fungicide Vitavax 200FF does not influence the reduction of seed contamination with the *Fusarium* fungi.

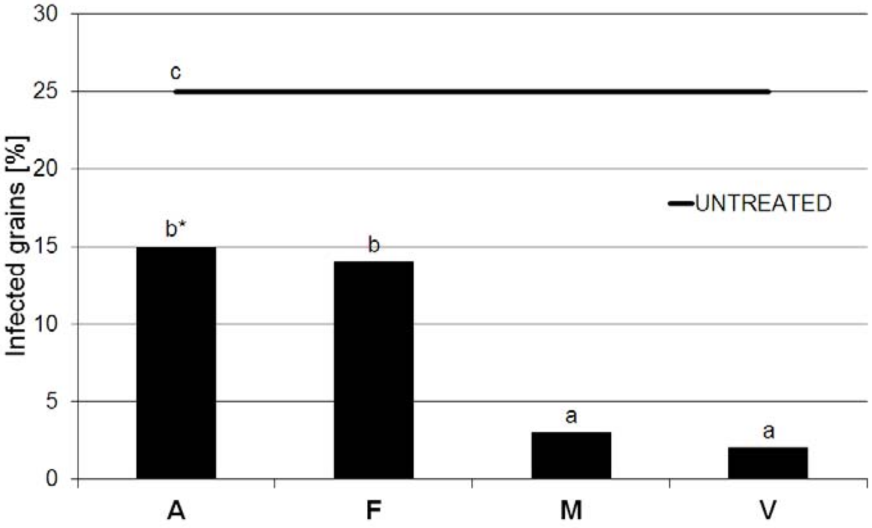

**Figure 1**: Impact of wheat seed treatment on development of contamination in laboratory incubation (agar, 5 days, 20 to 22 °C). Different letters above the columns mark the statistically significant difference between the two treatments (Duncan, p≤0.05).

At the time of dough maturity we also estimated the share of ears with visible contamination with *Fusarium*. All ears with visible contamination of bract at least in one spikelet were considered contaminated. As we expected a higher number of contaminated ears was detected in the control group (K) where we only sow untreated certified wheat seed (Figure 2) and 2% of all ears were contaminated. In the F treatment where we treated the seed with the Fitolife preparation contamination was statistically significantly equal to the contamination in the control group. There was statically significantly less contaminated ears in A, M and V treatments than in the control treatment K (Figure 2).

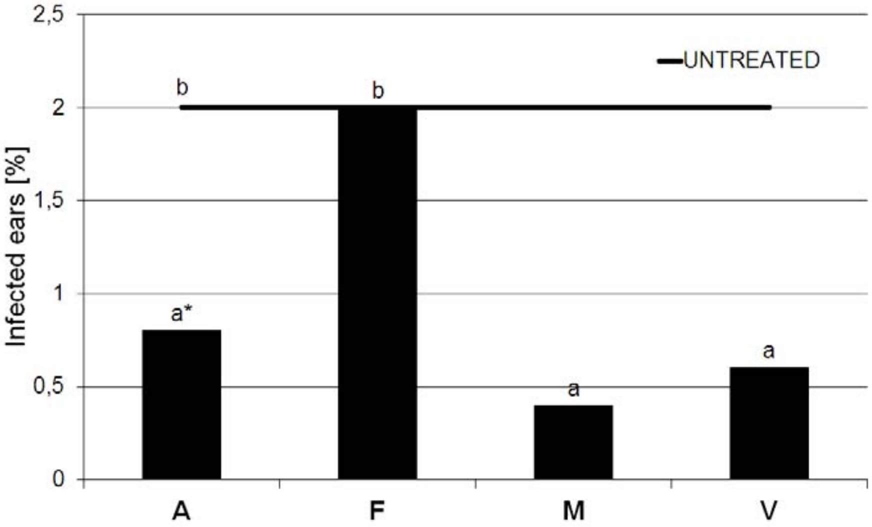

**Figure 2**: Impact of wheat seed treatment on the share of visibly contaminated ears with fusariosis in the dough maturity stage (Ljubljana, laboratory field BF, last decade of June, 2010). Different letters above the columns mark the statistically significant difference between the two treatments (Duncan, p≤0.05).

After the harvest the seed grains produced in the trial were incubated in the same way as before the sowing of the trial. The share of contaminated grains was statistically significantly the highest at the control group

(K), where wheat grains were not treated before sowing (Figure 3). On average one third of incubated grains were contaminated. The health state of the produced grains was the best at the M and V treatments with fungicides (27% of contaminated grains). Higher seed contamination was determined with the use of the preparations Agrostemin (A) and Fitolife (F) (29% contaminated grains), but the average contamination of grains treated in these two ways was still lower as the control group. Samobor et al. (2010) state in their paper that the Fitolife has a lesser effect on health status of produced wheat grain compared to the Vitavax 200-FF fungicide, however they confirmed statistically significant differences in the share of contaminated grains between the two treatments only for the contamination with *Alternaria alternata*. They could not prove the differences between the two preparations for the contaminations with other thirteen tested fungal species. Different results were reported by Zemljič et al. (2008), the share of grain contamination with *Fusarium* sp. fungi was higher when sowing treated seed than for the untreated seed.

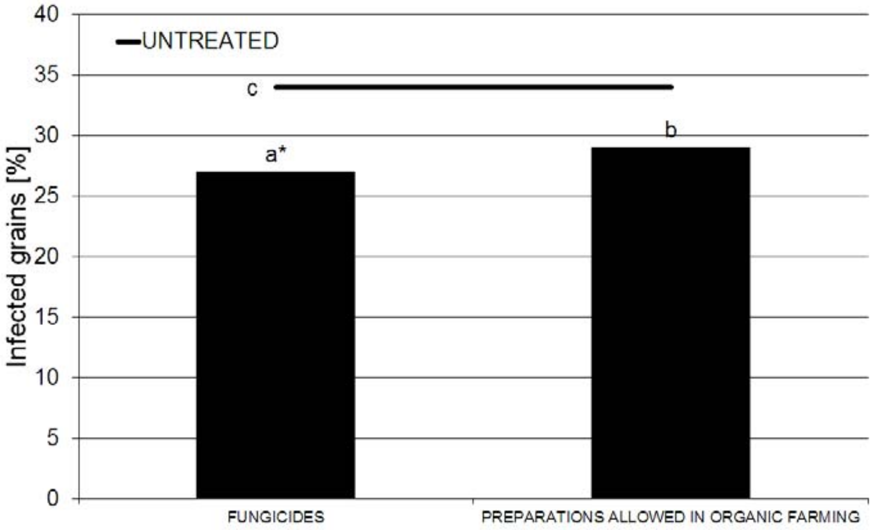

**Figure 3**: Contamination of wheat grain from the field trials with different seed treatments (Ljubljana, laboratory field BF, 2009-2010) after laboratory incubation (agar, 5 days, 20 to 22 °C). Different letters above the columns mark the statistically significant difference between the treatments ($p \leq 0.05$).

We could not confirm statistically significant regression and correlation connection between the share of contaminated ears at the stage of wax maturity and the share of infected grains in the final crop. The explanation why plants with higher infection level do not give higher ratio of infected grain could partly be contributed to the fact that usually grains in contaminated ears are very light, highly damaged or shrivelled, and stay on the field after the crop is harvested by a machine harvester, which can later cause permanent contamination of the wheat crop on this soil, especially in the narrow crop rotation.

### 3.2 Contamination of wheat grains from different types of production – results

Grain samples from two organic farmers and six conventional producers were incubated in the same way as all the seed from the previously presented trial. All data on the location of the farm, type of production, insensitivity of nitrogen fertilization, crop rotation and treatments with fungicides are presented in Table 1. Compared to the conventional production (8.4% contaminated grains) the grain samples from organic farms (7% of infected grains) had in average the similar lever of contamination (Figure 4). Similar findings were confirmed in Czech Republic where the content of DON, which is a consequence of grain contamination with *Fusarium*, fungi, the samples from organic farming were no different from the samples from intensive conventional production (Váňová et al., 2008). The results show that samples obtained from the central part of Slovenia had the highest variability of seed contamination (from 1.5% to 19.5% contaminated grains). This also shows that humid climate increases the incidence of seed contamination. Higher number of wet weather days after the wheat flowering period increases the grain contamination with fungal infections (Cowger et al., 2009). Figure 4 shows that despite consistent protection a small share of corn and grains in the crop rotation do not necessarily bring a good health condition of the crop. Especially corn with conservation tillage as a previous crop increases the risk of a serous wheat gain contamination with *Fusarium*, fungi, which were confirmed by Swiss researchers from 284 wheat samples from 16 cantons (Vogelsang et al., 2009). Zemljič et al. (2008) believe that the higher intensity of nitrogen fertilization is not always in correlation with higher levels of seed contamination. They contribute a high influence to the selection of the varieties (Zemljič et al., 2008; Argyris et al., 2003), although great differences in contamination of different varieties can occur also between years and locations due to more or less favourable conditions for contamination (Hecker et al., 2009). Considering our own research and the results from other researchers we can conclude that the seed contamination is caused by a mix of environmental factors beyond producers influence. It's not surprising that it is possible to grow a healthy wheat crop despite narrow crop rotation with consistent use of relevant agro technical measures and in optimal weather conditions.

**Table 1**: Crop rotation and agro technical measures in wheat production on eight Slovene farms that gave the samples for the laboratory incubation of wheat grain in July 2010 (K – conventional farming, E – organic farming)

| **Geographic area** | **Pomurje and lower Podravje** | | **Savinjska valley with Kozjansko** | | **South Gorenjska with Ljubljana basin** | | | **South-east Dolenjska** |
|---|---|---|---|---|---|---|---|---|
| Farm no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Cultivation type | K | E | K | E | K | K | K | K |
| Pre-crop | maize | buckwheat | maize | maize | potatoes | maize | red clover | potatoes |
| Share of grains and maize in last four years [%] | 100 | 50 | 25 | 50 | 25 | 25 | 25 | 50 |
| Number of fungicide sprayings | 2 | 0 | 1 | 0 | 1 | 1 | 2 | 1 |
| Number of nitrogen fertilizations | 3 | 0 | 2 | 0 | 3 | 3 | 3 | 1 |

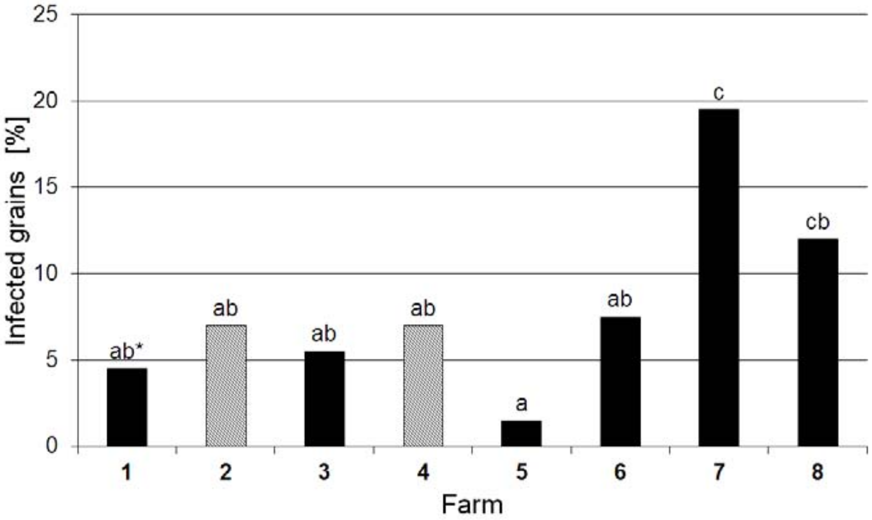

**Figure 4**: Seed contamination of wheat grain from eight Slovene farms at laboratory incubation (agar, 5 days, 20 to 22 °C). Different letters above the columns mark the statistically significant difference between the two treatments (Duncan, p≤0,05) The two lighter columns represent the samples from the organic farms.

## 4 CONCLUSIONS

Treating the wheat seed with fungicides has importantly improved the health status of the seed between sprouting in laboratory conditions. Alternative seed treatment preparations that can be used in organic farming have also improved the health status of sprouting seeds in laboratory conditions, but their effect was not as strong as with fungicides.

Our results prove that the health state of produced grain was best when sowing the seed treated with fungicides, the two preparations allowed in organic farming were slightly less effective but still importantly reduced crop contamination with different fungal diseases within the organic farming guidelines.

We could not establish a connection between different cultivation techniques and plant-health situation. Wheat seed contamination was the same in samples from organic and conventional farms. Protection with fungicides and suitable crop rotation do not assure a healthy crop since the grain health is highly dependent on weather conditions during the ripening period which cannot be influenced by producers.

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# Adventitious presence of GMOs in maize in the view of coexistence

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## ABSTRACT

The introduction of genetically modified (GM) crops in the European Union (EU) demand specific management. The EU recommendations for the management of different agricultural practices are oriented towards the sustainable agriculture in the frame of flexible coexistence. Coexistence may be affected by the adventitious presence of genetically modified organisms (APGM) in non-GM crops along the supply chain. Various biological and environmental parameters as well as technical management have influence on the occurrence and the degree of APGM. These parameters are being used for the development of preventive coexistence measures in individual EU countries. Applicability of the prescribed coexistence measures is critically reviewed, also in the view of possible introduction of GM maize cultivation in Slovenia. From the review, it is concluded that some coexistence measures are not in line with coexistence principles because they demand excessive work and are sometimes difficult to implement in practice. Alternative cooperation of different cropping systems are discussed for potential future implementation. The review is focused on maize, the predominant GM crop cultivated in the EU and an interesting crop for cultivation in Slovenia.

**Key words:** coexistence, GMO, adventitious presence of GMOs, maize

## IZVLEČEK

### NENAMERNA PRISOTNOST GENSKO SPREMENJENE KORUZE IN MOŽNOSTI SOOBSTOJA

Uvajanje gensko spremenjenih (GS) rastlin v pridelavo v Evropski uniji (EU) zahteva posebne ukrepe za zagotavljanje soobstoja različnih sistemov pridelovanja. Evropska priporočila za soobstoj spodbujajo upravljanje različnih pridelovalnih sistemov v smeri trajnostnega kmetijstva. Na soobstoj vpliva naključna, nenamerna prisotnost gensko spremenjenih organizmov (APGM) v gensko nespremenjenih pridelkih in proizvodih vzdolž celotne pridelovalne verige. Na prisotnost in stopnjo APGM vplivajo številni dejavniki, kot so biološki in okoljski ter načini upravljanja. Na podlagi teh dejavnikov so pripravljeni ukrepi za zagotavljanje soobstoja različnih kmetijskih praks. Podrobneje smo pregledali uporabnost posameznih ukrepov v luči možnosti uvedbe pridelovanja gensko spremenjene koruze v Sloveniji. Iz preglednega prispevka ugotavljamo, da nekateri ukrepi niso v skladu s temeljnimi načeli soobstoja, da zahtevajo ogromno dela in so zato v praksi težko izvedljivi. V razpravi smo predstavili alternativne načine upravljanja kmetijskih praks, ki bi jih lahko izvajali v prihodnosti. V prispevku smo se osredotočili na koruzo, ki je prevladujoča gensko spremenjena poljščina v EU in najbolj zanimiva za uvajanje v Sloveniji.

**Ključne besede:** soobstoj, GSO, naključna prisotnost GSO, koruza

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## 1 INTRODUCTION

In the last two decades, in the European Union (EU) cultivation of genetically modified organisms (GMOs) has become one of the cropping systems in addition to conventional and organic crop production. In the EU, two GMO events are approved for cultivation, MON810 maize and Amflora potato. In 2009, MON 810 maize was cultivated on 94.750 ha, which is a slight decrease compared to the previous years due to the moratorium in individual EU countries (GMO Compass, 2010). Although GM maize is produced on less than 1 % of the total maize cultivation area in Europe, GM cultivation presents concerns to the EU consumers since they perceive potential risk for the environment, animal and human health. To ensure consumer's freedom of choice between GM and non-GM products labelling is required in the EU for all products that contain GMOs (Regulation EC No. 1830/2003). Due to possibilities for unintentional and technically unavoidable presence of GMOs (i.e. adventitious presence of genetically modified organisms - APGM), tolerance thresholds are prescribed for food and feed; labelling is required for all the products that exceeds 0.9% of GMOs (Regulation EC No. 1829/2003). However, even lower contractual thresholds are used in practice within the supply chain (Recommendation 2010/C. 200/01). The presence of GM in non-GM crop may cause economic losses to farmers that trade the product as non-GM, loss of the market and legal disputes.

The introduction of GM crops in agriculture poses several questions how to manage the coexistence of different cropping systems. According to the EU Guidelines on general measures for ensuring coexistence (Recommendation EC 2003/556/EC) and subsequent Commission recommendations on guidelines for the development of national co-existence measures (Recommendation 2010/C. 200/01) farmers should be able to cultivate the types of agricultural crops they choose, GM, conventional or organic. Since there are different agricultural production practices and environmental conditions, individual EU countries can define their own national strategies for managing coexistence.

Many field experiments have been carried out in the last decades in order to gather data for the reliable science-based technical measures of coexistence (Henry *et al.*, 2003; Melé 2004; Weekes *et al.*, 2007; Della Porta *et al.*, 2008; Langhof *et al.*, 2010). However, these results are not always considered in the legislation that is currently in place in individual EU countries. Certain technical measures are difficult to be applied in practice due to organisational and logistic problems and difficulties in implementing in diverse environments (e.g. isolation distances). The coexistence of GM and non-GM crops/products downstream the supply chain is proven to be technically manageable, while the monitoring of APGM in the field is far from being resolved (Co-Extra project, 2005-2009).

Slovenia is characterized by fragmented landscape with an average field size of 1.4 ha as well as with specific and diverse environmental conditions, therefore specific co-existence measures need to be implemented. The Act on coexistence of GM plants with other crops is in place since 2009 (ZSGSROKR, 2009). Sub legislation acts were adopted in 2010/2011 (e.g. detailed measures for cultivation of GM maize). However, there has been no cultivation and no field trials with GM crops yet.

The aim of the article is to review the sources and parameters influencing APGM in maize cultivation and to discuss different coexistence measures in the view of implementation in practice. The results of several scientific studies are gathered in order to reveal the gaps and needs of existing coexistence measures. The review is focused on maize, since among the currently approved GM crops; maize is the most interesting for cultivation in Slovenia.

## 2 ADVENTITIOUS PRESENCE OF GMOs

The adventitious presence of genetically modified organisms (APGM) in the environment and within the supply chain is unavoidable due to biological characteristics of plants, open nature of the field and technical management along the supply chain from sowing in the field to harvest, storage and processing. APGM may have negative consequences on the environment, cause problems in the agriculture and have negative economic impact. Transgenes may be introduced to other genotypes of non-GM crop as well as to wild and weedy relatives via gene flow as is the case in traditional agriculture. The introduction of genes from GM crop to wild and/or weedy recipient may introduce novel or enhanced fitness-related traits into ecosystems that consequently increase adaptability to cultivated conditions (e.g. introgression of herbicide tolerance) (Poppy and Wilkinson, 2005). The presence of APGM can have economic consequences, APGM in a non-GM crop may prevent conventional and organic farmers from declaring their products as non-GM.

## 2.1 Sources of APGM

Several sources are recognized that contribute to APGM, i.e. seed impurities, cross-fertilization between GM and non-GM fields, wild relatives and weedy plants, occurrence of volunteer plants and technical management (mixing in the machinery at sowing and harvesting, seed dispersal at transport) (Figure 1). The degree of cross-fertilization depends on biological characteristics of the donor and recipient plant, mode of pollination and seed dispersal, cross compatibility between crops, wild and weedy relatives, the frequency and density of hybridizing genotypes and the environmental conditions (flowering synchronization, presence of pollinators, wind) (Poppy and Wilkinson, 2005).

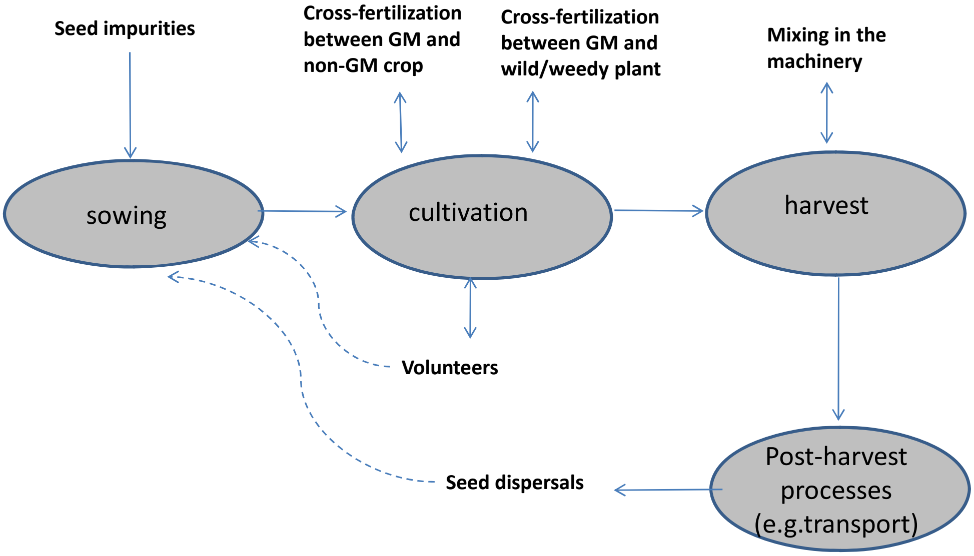

**Figure 1**: Schematic presentation of APGM sources in non-GM crop in the field (the dotted lines indicate the source of APGM in preceding growing seasons)

Most of the APGM in a conventional maize field comes from cross-fertilization with the neighbouring GM maize fields. Cross-fertilization rate declines with the distance from the pollen donor (GM field). In general, cross-fertilization rate is higher in the border rows and decreases with the distance from the donor towards the centre of the recipient field (Messeguer, 2006). Weekes et al. (2007) reported the maximum of 60% cross-fertilization adjacent to the GM crop. Similarly, Byrne and Fromherz (2003) reported cross-fertilization rates up to 46% at the border between the two fields with the declination below 1% within the first 10 m. The analysis of 1174 observation studies with maize in Europe revealed that in 41% of field studies cross-fertilization rate is below 0.9% within first 10 m (Riesgo *et al.*, 2010). The fast decline of cross-fertilization with the distance from the donor was also confirmed in experiments carried out in Slovenia for the situation of small field sizes (GM variety was simulated with a conventional maize variety differing in grain colour from the recipient non-GM variety) (Rostohar *et al.*, 2008).

Although most of the pollen is deposited close to the donor field, long distance hybridization events may occur. There is evidence that cross-fertilization was detected at distances of 650 meters from a known GM source (Henry *et al.*, 2003; Bannert and Stamp, 2007) and that maize pollen can travel up to several kilometres (Brunnet *et al.*, 2011). However, in such cases the pollen competition from other sources may be strong protection against cross-fertilization events thus lowering GMO levels (Bannert and Stamp, 2007).

Maize does not have wild or weedy relatives in Europe; therefore the introduction of transgenic material into the wild is unlikely. Maize kernels do not survive low winter temperatures in Central European climatic conditions (Luna *et al.*, 2001), but may occur as volunteers in warmer climates (e.g. Spain) (Palaudelmas *et al.*, 2009).

The level of APGM that comes from the seed impurities varies due to flowering coincidence of the two varieties and climatic conditions. If the varieties are of different maturity, the level of APGM in the harvest is reduced (Njontie *et al.*, 2011). If assuming the worst case presence of seed impurities (0.5% GM in non-GM seeds), the APGM from the field (cross-fertilization at the field) and post-field processes (mixing at harvest and inpost-harvest processes) should not exceed more than 0.4% (Sanvido *et al.*, 2008). The contribution of APGM due to contamination of the machinery can be minimized by dedicating the machinery exclusively for GM production or by properly following the instruction for cleaning (thorough cleaning between the GM and non-GM sowing and harvesting).

One of the possibilities to reduce transfer from GM to non-GM crop could be the introduction of transgene to organelle instead of to nuclear genome. The chloroplast genetic engineering has been successful in some species (tobacco, soybean etc.), but not yet in maize (Clarke and Daniell, 2011). Another alternative is the induction of cross-incompatibility between maize genotypes, but due to difficulties in breeding, this technique is still in its infancy. Another option could also be to use cytoplasmic male sterility for the production of unviable pollen in maize (Munsch *et al.*, 2007; Weider *et al.*, 2007).

## 2.2 Parameters influencing APGM

From a number of field trials the most important parameters influencing the APGM were identified that were used for the development of preventive coexistence measures and are described in the following sub-sections.

### 2.2.1 Flowering synchrony

The synchrony of flowering between GM and non-GM fields contributes to higher cross-fertilization rates (Westgate *et al.*, 2003, Halsey *et al.*, 2005, Goggi *et al.*, 2006, Angevin *et al.*, 2008). Therefore different planting dates are proposed to minimize the chance of unintended cross-fertilization in the climate where wide sowing interval is manageable. A four to five day shift leads to a 25% reduction of cross-fertilization rate and a six day shift leads to a 50% reduction (Della Porta *et al.*, 2008). Up to 10 days difference in planting dates declines the cross-fertilization rate up to 65% and up to 70% when there is more than 10 days difference (APROSE, 2004).

### 2.2.2 Isolation distances

Statistical analysis of different field trials with maize revealed that an isolation distance of 30 m between GM and non-GM field results in cross-fertilization value below 0.9% at 95% probability (Pla *et al.*, 2006; Sanvido *et al.*, 2008; Devos *et al.*, 2009; Riesgo *et al.*, 2010). Implementation of isolation distance of 40 m is sufficient to keep APGM below 0.9% with 99% probability (Riesgo *et al.*, 2010). For silage maize Sanvido *et al.* (2008) propose to shorten the isolation distance by factor 2.5 due to the dilution of the transgenic part in harvest. The factor 2.5 is used when an average proportion of 40% grain in the completely dry plant tissue is assumed but it depends on the varieties and stages of maturity at harvest. Proposed isolation distances can be reduced when additional measures are applied to minimize the cross-fertilization events.

### 2.2.3 Wind

Different field studies show that cross-fertilization greatly depends on wind conditions (direction and speed). Wind direction has a strong influence on cross fertilization rates at certain distances as shown in Table 1. However, there are also some reports of inconsistent correlation between the wind direction and cross-fertilization rate. The maximum cross-fertilization rate did not always coincide with the area of prevailing wind (Della Porta *et al.*, 2008; Lahghof *et al.* 2008). In such cases, wind peaks in critical days may explain the results (Feil and Schmid, 2002; Halsey *et al.*, 2005). Therefore, the adoption of data from different local climatic condition should be applied carefully.

**Table 1**: Distances at which different cross-fertilization rates are achieved depending on wind direction

| WIND DIRECTION | | REFERENCES |
|---|---|---|
| DOWNWIND | UPWIND | |
| 10 m[a] | 2m[a] | Messeguer *et al*., 2005 |
| 10 m[a] | 2 m[a] | Melé *et al.,* 2004 |
| 10-12 m[b] | 5-7 m [b] | Bénétrix and Bloc, 2003 |
| 54-66m >0.1% | 12-13 m[b] | Langhof *et al*., 2008 |

[a] - below the 0.9% of cross-fertilization rate
[b] - below the 1.0 % of cross-fertilization rate

2.2.4 Size and shape of the donor and recipient field

The cross-fertilization rate in an individual field is the result of interaction between different fields (GM and non-GM) and depends on the position of fields in the landscape (Messeguer, 2003a; Viaud *et al*., 2008). Low donor to receptor ratio leads to lower cross-fertilization rates due to large receptor pollen cloud competing with small incoming cloud (Messeguer, 2003b). Weber and Bringezu (2005) concluded that overall cross-fertilization rate does not exceed 0.9 % if the recipient and the donor fields are of the same size, while Melé (2004) reported the decrease for 50% when the size of the recipient field increased from 0.25 ha to 1 ha. The report of Bannert *et al*. (2008) showed some unexpected results for small fields. For the field situation with the donor/receptor ratio 3.6:1, higher rates of cross-fertilization at the receptor field were expected due to lower ratio to the pollen donor; however, values were almost identical at all distances to the pollen donor. This could be explained with the fact that most of the cross-fertilization events occurred within the first 6 m from the donor, irrespective of the donor size; so as long as the receptor and the donor field are wider than 6 m the ratio of donor to receptor is irrelevant. When up scaling from field to the landscape level, field geometry becomes less crucial while the importance of spatial interaction among the fields is increasing (Viaud *et al*., 2008). The orientation of fields is also very important, since the edge effects may contribute considerably to the average cross-fertilization rates in the whole receptor field. Langhof *et al*. (2008) pointed out that pronounced edge effects contribute disproportionally to the overall GM content of the harvest.

The majority of field trials were performed on adjacent or concentric field layout; however, these results should be carefully transferred to the coexistence measures. First, the pollen from non-GM field reduces the impact of GM pollen due to high competition ability more efficiently than open ground isolation distance (Messeguer *et al*., 2006; Pla *et al*., 2006). Second, the decrease of cross-fertilization events in the first 25 m is more evident in concentric and adjacent field experiments comparing to separate field designs, where such declination is less pronounced (Sanvido *et al*., 2008).

The topography has an impact on downhill increase of cross-fertilization; however over long distances (more than 17.5 m) it does not have a significant impact (Vogler *et al*., 2009).

### 2.3 Prediction of APGM in the field

The estimation of expected cross-fertilization rates are important for the management of GM and non-GM crops along the supply chain and for the prediction of APGM. A simulation gene flow model for maize, MAPOD was developed for the prediction of impurities in the harvest due to cross-fertilization events under real agronomic and environmental conditions (Angevin et al., 2008; Lavigne et al., 2008). It operates with different variables such as landscape patterns, environmental data, agricultural practices and cultivar characteristics. The results are reported as the expected proportion of GM seed in total seed for non-GM pixel (Viaud et al., 2008). A windows software LandSFACTS was developed that simulate crop allocation and crop spatial-temporal arrangements within agricultural landscapes (Messéan et al., 2009). The LandFlow-gene is a generic gene flow modelling platform that operates for maize and rapeseed and predicts the adventitious presence of GM in non-GM fields under different conditions of GM adoption. Within the SIGMEA project a decision support tool called SMAC Advisor was developed to assess the coexistence between GM and conventional maize in a given agricultural environment (Bohanec et al., 2007).

The measurement of all parameters that influence cross-fertilization rates in real situations, such as field geometry and distribution, biology of a plant, environmental parameters, pre- and post- harvest processes, is not realistic in practice, since all data are not always available. For example, data from one meteorological station may not be representative for the whole region if the landscape is fragmented with diverse topology. Instead, a few parameters shall be measured and incorporated in pre-existing databases and used for empirical and simulation modelling.

One of the strategies to minimize the *ex-post* consequences in the supply chain is the estimation of APGM in a non-GM crop pre-harvest. Appropriate sampling methods are needed to accurately predict the APGM in the field (Pla *et al*., 2006, Allnutt *et al*., 2008). The main difficulty for the development of sampling schemes in the field is obtaining a representative sample from a heterogenous matrix, as the field is. Lowering the threshold aggravate reliable prediction with acceptable measurement certainty. The accuracy of estimation of cross-fertilization rates depends on the number of samples taken, which is in direct correlation with the labour and costs. A simple random sampling was found the most feasible for the collectors and accurate in the estimation of true threshold. Šuštar-Vozlič *et al*. (2010) developed a sampling approach for determination of the GMO presence at the field level. It is based on the use of fitting functions to estimate approximate distances where sampling is performed.

## 3 PREVENTIVE MEASURES FOR COEXISTENCE IN MAIZE

Different co-existence measures are implemented in order to minimize the APGM in conventional and organic crops. The objective of reducing the APGM in non-GM products is to minimize the impact on the environment and to prevent the economic loss. Preventive measures for maize in the field include i) the use of certified seeds, ii) scheduling different flowering periods (flowering asynchrony), iii) using isolation distances and barrier zones, and iv) implementing good agricultural practice (crop rotation, optimal soil preparation, adequate cleaning and separate use of machinery). One of the most discussed safety measures for minimizing APGM in the field are isolation distances and barrier zones (Devos *et al*., 2005).

### 3.1 Isolation distances

The isolation distances proposed or imposed by different EU member states for maize range from 15 m in the Sweden to 800 m in Bulgaria (Devos *et al*., 2009; Riesgo *et al*., 2010). To maintain genetic purity in seed producing programs for conventional maize the minimum separation distance of 200 m is prescribed in EU. For the cultivation of pharmaceutical GM maize plants at least 1.6 km is required (Stevens *et al*., 2004).

The proposed isolation distances by national authorities are not always science based but are compromise between science, social and political demands therefore adequate isolation distances remain a subject of controversy among scientist and regulators (Sanvido *et al*., 2008). Isolation distances are sometimes also economically not proportionate and demand great effort for management. As for example, in the case of monoculture in a region with small farms and fragmented landscape (as is the case in Slovenia), fixed isolation distances are not manageable in practice because they are not proportionate to agricultural heterogeneity. The prescribed minimal isolation distance for maize in Slovenia is 600 m, which would be difficult to implement for majority of maize fields. In such cases, large distances may also have negative economic effect because they contribute to domino effect that impose severe burden on GM crop production and are not proportional to the farmer's basic economic incentives (Demont *et al*., 2008). The drastic consequence would also be the abandonment of GM production in a certain area.

### 3.2 Barrier zones

Barrier zones are planted with any plant species that physically limit pollen movement of a donor plant. When the barrier zone is planted with the same but non-GM species as GM donor, it is called buffer zone. There are at least two different options to manage gene flow from GM fields using buffer zones; one is with the exclusion of first few rows in the non-GM fields and the other is planting the non-GM border in the GM field.

In order to maintain the cross-fertilization rate below 0.9% using the buffer zones several facts should be considered:

- with the increased distance between the GM and non-GM field the number of border rows decreases (Gustafson *et al*., 2006);
- the legal threshold (0.9 %) can be obtained by planting buffer zones within first 20 m or even less, depending on samples taken – upwind or downwind (Bannert and Stamp, 2005; Melé 2004, Weber and Bringezu, 2005);
- for the receptor field of 1 ha and the donor field of 4 ha or larger the use of border rows of 20 m in combination with isolation distance results in cross-fertilization rate less than 0.9 % (Messéan *et al*., 2009) and
- separate harvesting of the first 10 m border rows in the receptor adjacent to 1 ha donor are effective to keep the cross-fertilization below the threshold 0.9%, (Gustafson *et al*., 2006).

Instead of using buffer zone a barrier, i.e. non-maize crop could be planted. The study of Langhof *et al.* (2008) included clover-grass as short crop and a sunflower as a tall crop. The comparison of the effectiveness of the two crops showed that the cross-fertilization rates downwind did not differ between the crops. They concluded that the height of a crop is not a sufficient criterion for efficient reduction of gene flow and that the architecture of leaf and other organs is very important criterion. Several authors (Aylor *et al.*, 2003; Goggi *et al.*, 2006) propose buffer zones as more efficient measure for managing coexistence than barriers zones. Barrier, non-maize rows do not compete with the donor pollen, while the maize buffer rows are a source of additional pollen that increases the pollen competition (Wilhelm *et al.*, 2005).

## 4 DISCUSSION

In order to reduce APGM in the non-GM crop, different measures are applied in the supply chain from the field on. In maize, most of the APGM comes from cross-fertilization between GM and non-GM crops, from seed impurities, volunteers or because of technical management. Seed impurities are minimized by the use of certified seeds. The volunteers can be managed by crop rotation and optimal soil preparation after harvest and before sowing. At post-harvest processes, adequate cleaning and separate use of machinery for GM and non-GM crop can reduce mixing. In order to minimize APGM different coexistence measures are used. Combination of different measures is applied in different countries according to farming systems, cropping patterns and environmental conditions in the region (Demont *et al.*, 2010).

The experiences with GMO cultivation in Europe have shown that some of the coexistence measures are rigid and difficult to manage in practice. The results of field trials are not always directly transferable between different environments and fluctuate greatly across years and sites within a region. In order to develop reliable guidelines field trials should be conducted in different environments and repeated in years. Such guidelines should incorporate a decision support system.

The coexistence measures should be regulated at different levels with *ex ante* regulations: i) at the farm level (e.g. the use of isolation distances or barrier zones), ii) at the regional level with respect to heterogeneity of field conditions, managerial expertise, education, market access and pest infestation and iii) at the national level through the policy making and *ex post* liability schemes (Demont and Devos, 2008; Demont *et al.*, 2008; Demont *et al.*, 2010). Due to heterogeneity in farming, legal and social environments, more flexible coexistence should be handled by the lowest authority possible (Devos *et al.*, 2009).

Demont *et al.* (2010) proposed free choice for farmers to use different coexistence measures within four different coordination systems (isolation distances, buffer zones and separate harvest of first few rows). However, it has to be considered that cross-fertilization is not the sole source of APGM in the field, although the main source of APGM comes from cross-fertilization between GM and non-GM plant through gene flow.

Another alternative to manage the coexistence is to establish wide-regions declared as GMO or GMO-free. These areas could then be managed based on private contracts of individual farmers instead of EU or national administrative regulations and recommendations. Furtan *et al.* (2007) proposed coexistence of three different cropping systems (conventional, GM and organic) within a region with the formation of a private landscape clubs. In such cooperation, organic farmers form a club, GM producers stay outside of the club and the conventional producers stay in the buffer zone and are compensated for their loss. Organic farmers are paying for the costs for functioning of the club from the premium prices of organic products and compensate farmers in the buffer zone. However, this model of coexistence may not be possible in all situations due to many institutional and logistic problems.

Food industries and consumers demand lower acceptable threshold than 0.9%, as low as 0.1%. When demanding lower thresholds we have to consider the opposite effect on coexistence; this could lead to banning of the GMO production in certain regions (Devos *et al.* 2009). Apart from the coordination of different coexistence measures at the farm and along the supply chain level, the coexistence raises a number of issues at the economic and social level that also need to be carefully addressed. In the future, new management strategies will be needed since novel GM crops are merging with other traits than for food and feed.

From the scientific review, we revealed gaps of existing rules and measures (e.g. large isolation distances) that can be avoided in the future management of GMO in Slovenia and find the possibilities and opportunities for managing different cropping systems for sustainable agriculture.

## 5 ACKNOWLEDGEMENTS

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# Validation of the method for the determination of some wine volatile compounds

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## ABSTRACT

Wine aroma is influenced by a number of volatile compounds. This article describes the validation of the method for 26 volatile compounds found in wine. Volatile compounds were determined with discontinuous liquid-liquid extraction and GC-MS detection. It was determined, that the method is linear with square correlation coefficient ranging from 0.961 to 0.999. Limits of quantitative determination range from 0.52 µg/L to 14.8 µg/L. Recoveries range from 71.1% to 105.7% except for two compounds with lower recoveries. Measurement uncertainty ranges from 5.0% to 28.9%. According to the validation, the method is suitable for the determination of at least 24 volatile compounds common to wine. A practical method application was presented on Zelen wine variety from two different production procedures.

**Key words:** wine, aroma, volatile compounds, GC-MS

## IZVLEČEK

### VALIDACIJA METODE ZA DOLOČANJE NEKATERIH HLAPNIH SPOJIN V VINU

Na aromo vina vplivajo številne hlapne spojine. Ta članek opisuje validacijo metode za 26 hlapnih spojin, ki jih najdemo v vinu. Hlapne spojine so bile določene z diskontinuirano ekstrakcijo tekoče-tekoče in GC-MS detekcijo. Določili smo, da je metoda linearna, z razponom kvadrata korelacijskega koeficienta od 0,961 do 0,999. Meje kvantitativne določitve imajo razpon od 0,52 µg/L do 14,8 µg/L. Izkoristki imajo razpon od 71,1% do 105,7%, razen za dve spojini, katerih izkoristek je nižji. Merilna negotovost ima razpon od 5,0% do 28,9%. Z ozirom na validacijo lahko potrdimo primernost metode za določanje vsaj 24 hlapnih spojin značilnih za vino. Praktični prikaz uporabe metode smo predstavili na vinih sorte Zelen iz dveh različnih postopkov pridelave.

**Ključne besede:** vino, aroma, hlapne spojine, GC-MS

Prispevek je del doktorske disertacije z naslovom "Vpliv maceracije na aromatične značilnosti primorskih belih vin", mentorica prof. dr. Tatjana Košmerl

## 1 INTRODUCTION

Wine aroma, a very important sensory parameter is produced by a complex balance of several volatiles. More than 800 volatile compounds such as alcohols, esters, phenols, monoterpenes, norisoprenoides, lactones, aldehydes and ketones have been identified in wine (Selli *et al.*, 2004; Tamborra *et al.*, 2004).

The wine aroma is complex due to a large number of compounds present and their different chemical nature with a wide range of polarity, volatility, solubility and pH values. Therefore the sample preparation and particularly the extraction and concentration of volatile compound are an important factor in their determination (Cabredo Pinillos *et al.*, 2004).

Appropriate extraction of wine volatile compounds must be performed before their detection. Exceptionally so called major wine volatile compounds present in mg/l, like acetaldehyde, ethyl acetate, methanol and higher alcohols are detected directly without previous extraction where samples are only diluted and deacidificated prior to analysis (Peinado *et al.*, 2004; Lukić *et al.*, 2008).

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Extraction of minor volatile compounds, present in wine in µg/L, is done today mostly in three different ways. The first is discontinuous or continuous liquid-liquid extraction (LLE) of wine with organic solvent. Both discontinuous and continuous liquid-liquid extractions are suitable to measure volatiles, but to perform a second one special apparatus must be provided and main disadvantages, like time consuming process and large volumes of solvents, are not avoided (Cabredo Pinillos *et al.*, 2004). As solvents mainly dichloromethane (Selli *et al.*, 2003) or mixture of pentane: dichloromethane = 60:40 (Pérez-Coello *et al.*, 2003; Izquierdo *et al.*, 2008) are used. The second approach is solid phase extraction (SPE) using Sep Pack $C_{18}$ cartridges (Tamborra *et al.*, 2004) or LiChrolut EN resins (Loscos *et al.*, 2010; Sáenz-Navajas *et al.*, 2010). The third approach is Solid Phase Micro Extraction (SPME) with different fibers used: carbowax-divinylbenzene (Lambropoulos and Roussis *et al.*, 2007; Antalick *et al.*, 2010), polydimethylsiloxane (Nasi *et al.*, 2008; Antalick *et al.*, 2010), polydimethylsiloxane/ divinylbenzene (Nasi *et al.*, 2008; Antalick *et al.*, 2010), carboxen/polydimethylsiloxane (Nasi *et al.*, 2008; Antalick *et al.*, 2010) or divinylbenzene/carboxen/ polydimethylsiloxane (Nasi *et al.*, 2008; Antalick *et al.*, 2010).

Detection of volatile compounds is performed by gas chromatograph (GC) coupled with Flame Ionisation Detector (FID) (Pérez-Coello *et al.*, 2003; Selli *et al.*, 2003; Selli *et al.*, 2004; Tamborra *et al.*, 2004; Selli *et al.*, 2006; Loscos *et al.*, 2010) or mass spectrometer (MS) (Pérez-Coello *et al.*, 2003; Selli *et al.*, 2003; Selli *et al.*, 2004; Tamborra *et al.*, 2004; Selli *et al.*, 2006; Lambropoulos and Roussis *et al.*, 2007; Izquierdo Cañas *et al.*, 2008; Nasi *et al.*, 2008; Rodriguez-Bencomo *et al.*, 2008; Loscos *et al.*, 2010; Antalick *et al.*, 2010; Sáenz-Navajas *et al.*, 2010). Quantification can be done with both detectors, while unequivocal identification only by MS.

On Agricultural institute of Slovenia we decided to introduce discontinuous liquid-liquid extraction method with dichloromethane, chosen as the most effective organic solvent for this type of extraction (Cabredo Pinillos *et al.*, 2004). The extraction was performed with intention to determine 26 minor volatile compounds with possible sensorial effect in wines (Schneider *et al.*, 1998; Selli *et al.*, 2003; Selli *et al.*, 2006; Lukić *et al.*, 2008). Liquid-liquid extraction is actually the oldest but still the reference technique for the extraction of volatile compounds in wine (Ortega *et al.*, 2002). 3-octanol and 4-nonanol were used as internal standards because of their high recovery (Cabredo Pinillos *et al.*, 2004; Selli *et al.*, 2006). By this procedure we achieved concentration factor 100. To enable qualitative and quantitative evaluation at the same time, MS was used for detection. After introduction, method was validated. Method was finally applied to real wine samples (variety Zelen) deriving from an experiment, where two different winemaking procedures were confronted.

## 2 MATERIALS AND METHODS

### 2.1 Materials

*Chemicals:*
Dichloromethane (Sigma-Aldrich) and ethanol absolute (Merck) with HPLC grade were used like solvents in our experiment, together with ultrapure water from the Milli-Q system. Similarly only the volatile compounds (Merck, Sigma-Aldrich, Fluka, SAFC) with the highest available purity on market (minimum of 95 %) were used with the exception of 4-vinylphenol (SAFC) only sold like 10 % solution.

*Preparation of solutions:*
Stock solutions in pure dichloromethane of individual volatiles were prepared in 50 ml volumetric flasks with concentrations ranging from 1.8 – 2.5 g/L. From 26 stock solutions one mix solution of all 26 volatiles was prepared in 200 mL volumetric flask. All other solutions used to determine linearity, limits of detection and limits of quantification were prepared from this mix solution with proper dilutions.

Internal standards 3-octanol and 4-nonanol for those dichloromethane solutions were prepared in 100 mL volumetric flask with dissolving them in quantity of 1.1 – 1.2 g/L in dichloromethane. They were added using 0.05 mL Hamilton syringe to 10 mL of dichloromethane solutions and mixed before determination.

*Preparation of model wine solution:*
First a mix stock solution of all volatiles in 100 % pure ethanol was prepared, with individual volatiles concentrations in range of 0.8 – 1.2 g/L. Stock solution was adequately diluted to model solution (mix) using 12 %vol ethanol in water to concentrations similar to ones determined in wines in average, to 0.04 – 0.07 mg/L, in 3000 ml volumetric flask. The pH was then adjusted to pH 3.2 with tartaric acid addition. Model wine solution was finally dispensed in twenty 125 mL flasks and they were stored in dark at 7 ºC before extraction.

Internal standards 3-octanol and 4-nonanol used in our model wine solution were prepared in 100 mL volumetric flask with dissolving them in quantity of 0.04 – 0.06 g in ethanol absolute. They were added using 0.05 mL Hamilton syringe to model wine solution only during extraction process as described below.

### 2.2 Procedure

*Liquid-liquid extraction of volatile compounds:*
100 mL of model wine solution was transferred into 250 ml Erlenmeyer flask and cooled to 0 ºC in an ice bath under nitrogen. 29 µg of 3-octanol and 23 µg of 4-nonanol were added as internal standards using 0.05 mL Hamilton syringe from corresponding ethanol solutions. Dichloromethane (40 mL) was added and the mixture was stirred at 350 min$^{-1}$ for 20 minutes (Moio et al., 1995). Then the mixture was centrifuged at 5°C (RFC = 8500, 10 minutes) and organic phase was recovered. The aqueous phase was re-extracted twice in the same way described above. Finally organic phases were combined and dried over sodium sulphate. They were concentrated to a final volume of 1 mL with Vigreaux distillation column and nitrogen gas flow prior to GC-MS analysis (Schneider et al., 1998).

The same procedure was used for the extraction of wine samples (Moio et al., 1995; Schneider et al., 1998; Selli et al., 2006).

### 2.3 Determination

Chromatographic conditions of GC (HP 6890)-MS (HP 5973) system:

| | |
|---|---|
| Liner | Agilent 5062-3587 |
| Injector temperature | 200 ºC |
| Injection type | Pulsed Splitless |
| Precolumn | 2 m * 0.25 mm |
| Column | Varian, CP-WAX 57CB, 50 m x 0.25 mm ID |
| Temperature gradient | 40 ºC; 12 min<br>5 ºC/min; from 40 ºC to 200 ºC<br>200 ºC; 20 min |
| Ion source temperature | 230 ºC |
| Auxiliary temperature | 200 ºC |
| Detector temperature | 150 ºC |
| Carrier gas | Helium 6.0; constant flow 1.0 ml/min |
| Injection volume | 1 µl |
| Detection | Selective Ion Monitoring (**T**, Q1, Q2, Q3):<br>1,6-Heptadien-4-ol (**71**,43)<br>1-Hexanol (**56**, 43, 55, 69)<br>2-Phenylethyl acetate (**104**, 43, 91)<br>3-Octanol (**59**, 83, 101)<br>4-Ethylguaiacol (2-Methoxy-4-ethylphenol )(**137**, 152)<br>4-Nonanol (**55**, 73, 83, 101)<br>4-Vinylguaiacol (2-Methoxy-4-vinylphenol) (**150**, 135, 107, 77)<br>4-Vinylphenol (**120**, 91)<br>Benzaldehyde (**77**, 105, 106)<br>Benzyl alcohol (**79**, 108, 107)<br>*cis*-3-hexen-1-ol (**67**, 41, 82)<br>Diethyl succinate (**101**, 129)<br>Ethyl butyrate (Ethyl butanoate)(**71**, 43, 88)<br>Ethyl cinnamate (**131**, 103, 176)<br>Ethyl decanoate (Ethyl caprate) (**88**, 101, 155)<br>Ethyl dodecanoate (Ethyl laurate) (**88**, 101)<br>Ethyl hexadecanoate (Ethyl palamitate) (**88**, 101)<br>Ethyl hexanoate (**88**, 99)<br>Ethyl lactate (**45**, 75)<br>Ethyl octanoate (Ethyl caprylate) (**88**, 101, 57)<br>Geraniol (**69**, 93, 123)<br>Hexyl acetate (**43**, 56)<br>Isoamyl acetate (**70**, 43, 55)<br>Nerol (**69**, 84, 93)<br>n-Hexaldehyde (Capronaldehyde) (**56**, 44, 57)<br>*trans*-2-hexen-1-ol (**55**, 69, 83)<br>β-Ionone (**177**, 43)<br>γ-Butyrolactone (**42**, 56, 86) |

### 2.4 Aromatic compounds determination in wines from two winemaking procedures - preparation of wine samples

Healthy grapes of Zelen variety (40 kg) were manually harvested in 2008 at the ripeness stage corresponding to wines containing approximately 12 % vol ethanol. Grapes were divided in two equal parts. First half of grapes (Zc = control without skin contact) was immediately destemmed, crushed and pressed up to 150 kPa using a small water press (Lancman VS-A 55, Slovenia). The juice was sulphited with 30 mg/L of sulphur dioxide, left to settle at 6ºC for 12 hours, racked and divided in three glass laboratory fermentor vessels with 1.6 L juice each. The vessels were heated to 17ºC, inoculated with 0.2 g/L of dried *Saccharomyces cerevisiae* (CM, Lallemand), supplemented with 0.2 g/L complex yeast nutrient (Fermaid E, Lallemand) and fermented at 17ºC. After alcoholic fermentations (residual sugars <2.5 g/L) and when most of the lees had settled, the wines were racked, 50 mg/L of sulphur dioxide was added and the wines were stored at 10ºC. The second half of grapes (Zp = freezing of the pomace) was destemmed and crushed. The pomace was equally divided in three plastic vessels, frozen overnight at –20ºC, defrozen at 20ºC and pressed up to 150 kPa. The juice from the individual plastic vessels was sulphited with 30 mg/L of sulphur dioxide, left to settle at 6ºC for 12 hours, racked and poured in 3 glass laboratory fermentor vessels with 1.6 L juice each. The remaining procedure to obtain wines was the same as described previously. In this way, two different types of Zelen wines (Zc, Zp) in three repetitions were obtained.

## 3 RESULTS AND DISCUSSION

### 3.1 Linearity, limits of detection, limits of quantification

Linearity was verified by using the solutions of volatile compounds in dichloromethane (five repetitions for one concentration level, three to eight concentration levels for the calibration curve). Linearity and range were determined by linear regression, using the F test. Linear model is fit and remains linear over the range presented in Table 1. Limits of detection (LD) and limits of quantification (LOQ) were calculated from the calibration curve and are presented in Table 1.

Linearity was verified for wider range also and is presented in Table 2.

Concentration factor for wine samples was due to extraction 100, so realistic linearity range, LDs and LOQs are 100-times lower.

**Table 1**: Linearity, limits of detection, limits of quantification

| | **linearity (mg/L)** | **$R^2$** | **LD (mg/L)** | **LOQ (mg/L)** |
|---|---|---|---|---|
| 1,6-Heptadien-4-ol | 0.12 - 3.35 | 0.999 | 0.062 | 0.206 |
| 1-Hexanol | 0.19 - 3.35 | 0.999 | 0.067 | 0.224 |
| 2-Phenylethyl acetate | 0.0168 - 3.35 | 0.999 | 0.030 | 0.099 |
| 4-Ethylguaiacol | 0.0058 - 3.35 | 0.996 | 0.051 | 0.171 |
| 4-Vinylguaiacol | 0.05 - 3.35 | 0.998 | 0.062 | 0.208 |
| 4-Vinylphenol | 0.05 - 3.35 | 0.998 | 0.061 | 0.203 |
| Benzaldehyde | 0.0111-1.67 | 0.993 | 0.041 | 0.136 |
| Benzylalcohol | 0.0116 - 1.67 | 0.994 | 0.038 | 0.126 |
| *cis*-3-Hexen-1-ol | 1.67 - 10.13 | 0.996 | 0.352 | 1.174 |
| Diethyl succinate | 0.022 - 3.35 | 0.996 | 0.066 | 0.218 |
| Ethyl butyrate | 0.0167 - 1.67 | 0.999 | 0.016 | 0.052 |
| Ethyl cinnamate | 0.05 - 3.35 | 0.998 | 0.056 | 0.186 |
| Ethyl decanoate | 0.05 - 1.67 | 0.995 | 0.056 | 0.186 |
| Ethyl dodecanoate | 0.01 - 3.35 | 0.996 | 0.065 | 0.216 |
| Ethyl hexadecanoate | 0.0139 - 1.67 | 0.995 | 0.034 | 0.115 |
| Ethyl hexanoate | 0.0092 - 3.35 | 0.996 | 0.053 | 0.176 |
| Ethyl lactate | 0.0099 - 1.67 | 0.991 | 0.044 | 0.148 |
| Ethyl octanoate | 0.1 - 9.51 | 0.992 | 0.444 | 1.480 |
| Geraniol | 0.011 - 3.35 | 0.996 | 0.065 | 0.216 |
| Hexyl acetate | 0.0092 - 1.67 | 0.990 | 0.048 | 0.159 |
| Isoamyl acetate | 0.05 - 3.35 | 0.998 | 0.059 | 0.196 |
| Nerol | 0.0058 - 3.35 | 0.996 | 0.056 | 0.187 |
| n-Hexaldehyde | 0.06 - 3.35 | 0.983 | 0.175 | 0.582 |
| *trans*-2-Hexen-1-ol | 0.05 - 3.35 | 0.982 | 0.179 | 0.597 |
| β-Ionone | 0.009 - 3.35 | 0.995 | 0.074 | 0.248 |
| γ-Butyrolactone | 0.024 - 3.35 | 0.997 | 0.055 | 0.183 |

**Table 2**: Linearity, wider range

| | **linearity (mg/L)** | **R$^2$** |
|---|---|---|
| 1,6-Heptadien-4-ol | 0.12 - 11.92 | 0.961 |
| 1-Hexanol | 0.19 - 19.4 | 0.988 |
| 2-Phenylethyl acetate | 0.0168 - 33.53 | 0.995 |
| 4-Ethylguaiacol | 0.0058 - 11.5 | 0.987 |
| 4-Vinylguaiacol | 0.05 - 9.36 | 0.978 |
| 4-Vinylphenol | 0.05 - 9.08 | 0.977 |
| Benzaldehyde | 0.0111-22.18 | 0.994 |
| Benzylalcohol | 0.0116 - 23.14 | 0.994 |
| *cis*-3-Hexen-1-ol | 1.67 - 10.13 | 0.996 |
| Diethyl succinate | 0.022 - 21.62 | 0.993 |
| Ethyl butyrate | 0.0167 - 33.45 | 0.999 |
| Ethyl cinnamate | 0.05 - 10.72 | 0.983 |
| Ethyl decanoate | 0.05 - 9.55 | 0.977 |
| Ethyl dodecanoate | 0.01 - 10.08 | 0.978 |
| Ethyl hexadecanoate | 0.0139 - 27.7 | 0.973 |
| Ethyl hexanoate | 0.0092 - 18.45 | 0.989 |
| Ethyl lactate | 0.0099 - 10.79 | 0.986 |
| Ethyl octanoate | 0.1 - 9.51 | 0.992 |
| Geraniol | 0.011 - 10.8 | 0.985 |
| Hexyl acetate | 0.0092 - 18.31 | 0.989 |
| Isoamyl acetate | 0.05 - 10.79 | 0.983 |
| Nerol | 0.0058 - 11.57 | 0.988 |
| n-Hexaldehyde | 0.06 - 11.31 | 0.998 |
| *trans*-2-Hexen-1-ol | 0.05 - 3.35 | 0.982 |
| β-Ionone | 0.009 - 9.32 | 0.980 |
| γ-Butyrolactone | 0.024 - 24.06 | 0.994 |

### 3.2 Trueness

Trueness was verified by checking the recoveries. Two parallel extracts of model wine solution were prepared each day for ten days and injected once respectively. The average of recoveries was calculated. The results are given in Table 3.

### 3.3 Precision

For the determination of precision (ISO 5725), i.e. repeatability and reproducibility, extracts of model wine solution was analysed (the same as for recovery evaluation). Within the period of 10 days two parallel extracts were prepared each day. Each was injected once. Then standard deviation of repeatability of the level and standard deviation of reproducibility of the level were both calculated. The results are given in Table 4.

**Table 3**: Recoveries for model wine solution

| | **conc. in model wine solution (mg/L)** | **recovery (%)** | **RSD (%)** |
|---|---|---|---|
| 1,6-Heptadien-4-ol | 0.0435 | 84.4 | 3.1 |
| 1-Hexanol | 0.0596 | 98.3 | 4.9 |
| 2-Phenylethyl acetate | 0.0614 | 91.9 | 2.5 |
| 3-Octanol | 0.2930 | 85.9 | 3.1 |
| 4-Ethylguaiacol | 0.0543 | 92.3 | 4.2 |
| 4-Nonanol | 0.2300 | 87.1 | 2.8 |
| 4-Vinylguaiacol | 0.0692 | 95.0 | 8.9 |
| 4-Vinylphenol | 0.0414 | 98.2 | 2.6 |
| Benzaldehyde | 0.0713 | 95.0 | 3.0 |
| Benzylalcohol | 0.0672 | 94.2 | 4.6 |
| *cis*-3-Hexen-1-ol | 0.0566 | 84.0 | 3.0 |
| Diethyl succinate | 0.0498 | 91.3 | 2.7 |
| Ethyl butyrate | 0.0599 | 77.5 | 3.8 |
| Ethyl cinnamate | 0.0697 | 95.1 | 2.9 |
| Ethyl decanoate | 0.0500 | 81.9 | 15.2 |
| Ethyl dodecanoate | 0.0624 | 67.8 | 8.9 |
| Ethyl hexadecanoate | 0.0524 | 27.9 | 9.5 |
| Ethyl hexanoate | 0.0588 | 76.7 | 3.4 |
| Ethyl lactate | 0.0709 | 79.4 | 3.5 |
| Ethyl octanoate | 0.0573 | 71.1 | 7.4 |
| Geraniol | 0.0495 | 105.7 | 3.0 |
| Hexyl acetate | 0.0593 | 80.6 | 4.0 |
| Isoamyl acetate | 0.0604 | 78.0 | 3.8 |
| Nerol | 0.0540 | 96.5 | 2.4 |
| n-Hexaldehyde | 0.0433 | 82.0 | 3.3 |
| *trans*-2-Hexen-1-ol | 0.0548 | 102.4 | 8.2 |
| β-Ionone | 0.0564 | 89.2 | 2.6 |
| γ-Butyrolactone | 0.0609 | 88.1 | 3.1 |

**Table 4**: Standard deviation of repeatability and reproducibility of the method, in mg/L

| | **conc. in model wine solution (mg/L)** | **means of the levels (mg/L)** | **standard deviation of repeatability (*sr*)** | **standard deviation of reproducibility (*sR*)** |
|---|---|---|---|---|
| 1,6-Heptadien-4-ol | 0.0435 | 0.0367 | 0.0007 | 0.0011 |
| 1-Hexanol | 0.0596 | 0.0583 | 0.0011 | 0.0029 |
| 2-Phenylethyl acetate | 0.0614 | 0.0563 | 0.0012 | 0.0014 |
| 4-Ethylguaiacol | 0.0543 | 0.0501 | 0.0020 | 0.0024 |
| 4-Vinylguaiacol | 0.0692 | 0.0659 | 0.0055 | 0.0056 |
| 4-Vinylphenol | 0.0414 | 0.0406 | 0.0009 | 0.0010 |
| Benzaldehyde | 0.0713 | 0.0676 | 0.0013 | 0.0021 |
| Benzylalcohol | 0.0672 | 0.0632 | 0.0014 | 0.0028 |
| *cis*-3-Hexen-1-ol | 0.0566 | 0.0475 | 0.0010 | 0.0014 |
| Diethyl succinate | 0.0498 | 0.0455 | 0.0012 | 0.0012 |
| Ethyl butyrate | 0.0599 | 0.0463 | 0.0010 | 0.0018 |
| Ethyl cinnamate | 0.0697 | 0.0662 | 0.0014 | 0.0019 |
| Ethyl decanoate | 0.0500 | 0.0404 | 0.0010 | 0.0064 |
| Ethyl dodecanoate | 0.0624 | 0.0419 | 0.0008 | 0.0039 |
| Ethyl hexadecanoate | 0.0524 | 0.0147 | 0.0012 | 0.0014 |
| Ethyl hexanoate | 0.0588 | 0.0449 | 0.0010 | 0.0016 |
| Ethyl lactate | 0.0709 | 0.0562 | 0.0015 | 0.0020 |
| Ethyl octanoate | 0.0573 | 0.0405 | 0.0009 | 0.0031 |
| Geraniol | 0.0495 | 0.0524 | 0.0014 | 0.0015 |
| Hexyl acetate | 0.0593 | 0.0476 | 0.0011 | 0.0019 |
| Isoamyl acetate | 0.0604 | 0.0470 | 0.0012 | 0.0017 |
| Nerol | 0.0540 | 0.0521 | 0.0012 | 0.0012 |
| n-Hexaldehyde | 0.0433 | 0.0354 | 0.0007 | 0.0012 |
| *trans*-2-Hexen-1-ol | 0.0548 | 0.0559 | 0.0044 | 0.0050 |
| β-Ionone | 0.0564 | 0.0503 | 0.0010 | 0.0013 |
| γ-Butyrolactone | 0.0609 | 0.0536 | 0.0012 | 0.0016 |

### 3.4 Uncertainty of repeatability and uncertainty of reproducibility

Uncertainty of repeatability and uncertainty of reproducibility were calculated by multiplying standard deviation of repeatability and standard deviation of reproducibility by Student's *t* factor for 9 degrees of freedom and 95% confidence level ($t_{95;9} = 2.262$).

$U_r = t_{95;9} \times s_r$ ; $U_R = t_{95;9} \times s_R$

The results are presented in Table 5.

**Table 5**: Uncertainty of repeatability and reproducibility of the method, in mg/L

| | **conc. in model wine solution (mg/L)** | **uncertainty of repeatability ($U_r$)** | **uncertainty of reproducibility ($U_R$)** |
|---|---|---|---|
| 1,6-Heptadien-4-ol | 0.0435 | 0.0016 | 0.0026 |
| 1-Hexanol | 0.0596 | 0.0025 | 0.0066 |
| 2-Phenylethyl acetate | 0.0614 | 0.0026 | 0.0031 |
| 4-Ethylguaiacol | 0.0543 | 0.0046 | 0.0055 |
| 4-Vinylguaiacol | 0.0692 | 0.0124 | 0.0127 |
| 4-Vinylphenol | 0.0414 | 0.0021 | 0.0023 |
| Benzaldehyde | 0.0713 | 0.0029 | 0.0047 |
| Benzylalcohol | 0.0672 | 0.0031 | 0.0064 |
| *cis*-3-Hexen-1-ol | 0.0566 | 0.0022 | 0.0032 |
| Diethyl succinate | 0.0498 | 0.0026 | 0.0027 |
| Ethyl butyrate | 0.0599 | 0.0022 | 0.0040 |
| Ethyl cinnamate | 0.0697 | 0.0031 | 0.0042 |
| Ethyl decanoate | 0.0500 | 0.0023 | 0.0144 |
| Ethyl dodecanoate | 0.0624 | 0.0017 | 0.0088 |
| Ethyl hexadecanoate | 0.0524 | 0.0027 | 0.0031 |
| Ethyl hexanoate | 0.0588 | 0.0023 | 0.0036 |
| Ethyl lactate | 0.0709 | 0.0034 | 0.0044 |
| Ethyl octanoate | 0.0573 | 0.0021 | 0.0071 |
| Geraniol | 0.0495 | 0.0031 | 0.0034 |
| Hexyl acetate | 0.0593 | 0.0025 | 0.0044 |
| Isoamyl acetate | 0.0604 | 0.0028 | 0.0040 |
| Nerol | 0.0540 | 0.0027 | 0.0028 |
| n-Hexaldehyde | 0.0433 | 0.0016 | 0.0026 |
| trans-2-Hexen-1-ol | 0.0548 | 0.0100 | 0.0113 |
| β-Ionone | 0.0564 | 0.0022 | 0.0029 |
| γ-Butyrolactone | 0.0609 | 0.0028 | 0.0036 |

### 3.5 Aromatic compounds determination in wines from two winemaking procedures

To determine volatile compound in real wine samples, the method proposed in this article was applied and results are presented in Table 6. Results are in correlation with previously observed aromatics content in wines and differences due to two winemaking procedures are comparable to other skin contact procedures (Moio et al., 1995; Ortega et al., 2002; Selli et al., 2003; Selli et al., 2006; Rodriguez-Bencomo et al., 2008).

**Table 6** : Concentrations of individual aromatic compounds in the Zelen wines produced by two different procedures (Zc - control without skin contact, Zp - freezing of pomace), in µg/L.

| | **Zc wine** | **Zp wine** |
|---|---|---|
| 1,6-Heptadien-4-ol | $17 \pm 1^b$ | $12 \pm 1^a$ |
| 1-Hexanol | $1349 \pm 67^b$ | $1118 \pm 18^a$ |
| 2-Phenylethyl acetate | $466 \pm 49^b$ | $253 \pm 12^a$ |
| 4-Ethylguaiacol | - | - |
| 4-Vinylguaiacol | $516 \pm 8^a$ | $908 \pm 53^b$ |
| 4-Vinylphenol | $117 \pm 5^a$ | $354 \pm 23^b$ |
| Benzaldehyde | $2 \pm 0^a$ | $9 \pm 2^b$ |
| Benzylalcohol | $20 \pm 3^a$ | $90 \pm 9^b$ |
| *cis*-3-Hexen-1-ol | $21 \pm 1^b$ | $18 \pm 0^a$ |
| Diethyl succinate | $112 \pm 16^a$ | $129 \pm 8^a$ |
| Ethyl butyrate | $366 \pm 10^b$ | $217 \pm 11^a$ |
| Ethyl cinnamate | - | - |
| Ethyl decanoate | $593 \pm 6^b$ | $443 \pm 9^a$ |
| Ethyl dodecanoate* | $37 \pm 2^a$ | $38 \pm 4^a$ |
| Ethyl hexadecanoate* | $6 \pm 1^a$ | $8 \pm 1^a$ |
| Ethyl hexanoate | $570 \pm 16^b$ | $409 \pm 0^a$ |
| Ethyl lactate | $4276 \pm 475^a$ | $6350 \pm 328^b$ |
| Ethyl octanoate | $1239 \pm 56^b$ | $933 \pm 25^a$ |
| Geraniol | - | - |
| Hexyl acetate | $275 \pm 40^b$ | $46 \pm 5^a$ |
| Isoamyl acetate | $3260 \pm 423^b$ | $1291 \pm 97^a$ |
| Nerol | - | - |
| n-Hexaldehyde | $4 \pm 1^b$ | $-^a$ |
| *trans*-2-Hexen-1-ol | - | - |
| β-Ionone | - | - |
| γ-Butyrolactone | $2501 \pm 60^a$ | $2569 \pm 62^a$ |

Values are the mean value ± error at 95 % confidence level (n = 3).
Significant differences between procedures are indicated a, b at $p \leq 0.05$.
\- = not detected
* = volatile compounds with low recoveries

## 4 CONCLUSIONS

According to the validation, the method is suitable for the determination of at least 24 volatile compounds in wine (the ones with recoveries >70%). The system is linear with $R^2$ higher than 0.96. Limits of detection range from 0.16 µg/L for ethyl butyrate to 4.44 µg/L for ethyl octanoate. Limits of quantitative determination range from 0.52 µg/L for ethyl butyrate to 14.8 µg/L for ethyl octanoate. Recoveries range from 71.1% (ethyl octanoate) to 105.7% (geraniol), except for ethyl dodecanoate (67.8%) and ethyl hexadecanoate (27.9%). Uncertainty of reproducibility ranges from 5.0% for 2-phenylethyl acetate to 28.9% for ethyl decanoate. A practical application was checked and presented for Zelen wines from two different winemaking procedures.

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# Analiza padavin na širšem območju Triglavskega narodnega parka za obdobje 1961-2009

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## IZVLEČEK

Za 18 meteoroloških postaj na širšem območju Triglavskega narodnega parka smo za obdobje 1961-2009 analizirali letne višine padavin, število dni z dnevno višino padavin nad 5, 10, 20, 50, 100 in 150 mm ter število dvodnevnih nalivov z višino padavin nad 50, 100 in 150 mm. Za snežno odejo smo analizirali število dni po letnih časih in za snežno sezono ter trende v številu dni s snežno odejo. Največ padavin je imela v letnem povprečju Žaga pri Bovcu (2972 mm), najmanj Rateče (1532 mm). Medletna variabilnost višine padavin je na vseh postajah velika, v izrazito mokrih letih je višina padavin več kot dvakratna višina tiste v najbolj sušnih letih. Povprečne letne višine padavin, izračunane za dekade, se statistično značilno razlikujejo. Na večini postaj je bila najbolj mokra dekada 1961-1969, najbolj sušni dekadi pa 1980-1989 in 2000-2009. Na vseh postajah se pojavljajo močni enodnevni in dvodnevni nalivi, variabilnost števila izjemnih padavinskih dogodkov se povečuje z višino padavin. Največje število dni z višino padavin >5 mm ima Vogel (90), >10 mm in >20 mm Kneške Ravne (69 oz. 44). Dvodnevnih nalivov >50, >100 in >150 mm je največ na Žagi, najmanj pa v Ratečah. Na vseh postajah lahko vsako leto pričakujemo vsaj en dan, ko višina padavin presega 150 mm. Dolžina snežne sezone se v zadnjih letih zmanjšuje. Trend v številu dni s snežno odejo je na vseh postajah negativen. Velikost statistično značilnih sprememb je od -4,3 dni/10 let na Žagi do -14,9 dni/10 let v Stari Fužini.

**Ključne besede**: padavine, nalivi, snežna odeja, trendi, Triglavski narodni park

## ABSTRACT

### PRECIPITATION ANALYSIS OF THE WIDER AREA OF THE TRIGLAV NATIONAL PARK FOR THE PERIOD 1961-2009

The annual rainfall series were analyzed for the 18 meteorological stations in the wider area of the Triglav National Park for the period 1961-2009: the amount of annual rainfall, the number of days with 24-h rainfall totals exceeding 5, 10, 20, 50, 100, 150 mm and the number of 48-h rainfall totals exceeding 50, 100 and 150 mm. For snow cover, duration and the trend of days with snow cover were analyzed. The maximum annual precipitation over the entire period has been in Žaga by Bovec (2972 mm) and the minimum in Rateće (1532 mm). High interannual rainfall variability was noticed during period observed, more than twice as much rain has fallen in the wettest periods as in the driest periods. There were some significant differences between the decadal precipitation amounts. At most stations, the wettest period was 1961-1969 and the driest were 1980-1989 and 2000-2009. Heavy 24-h and 48-h rainfalls were measured at all stations, variability in the number of extreme rainfall events has increased with the amount of rainfall. The maximum number of days with day-rainfall above 5 mm was measured in Vogel (90), and above 10 mm (69) and 20 mm (44) in Kneške Ravne. Žaga had the highest number with rainfall above 50, 100 and 150 mm and Rateče the lowest one. The snow season has shortened in recent years, negative trends were observed at all stations in the number of days with snow cover. The significant trend rates ranged from -4.3 days/decade in Žaga to -14.9 days/decade in Stara Fužina.

**Key words**: precipitation, heavy rain, snow cover, trends, Triglav national park

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## 1 UVOD

V Sloveniji je za padavinski režim odločilnega pomena relief, ki močno vpliva na prostorsko porazdelitev padavin s tem, ko pogojuje ob pobočjih vzponske tokove. Slovenija dobi največ padavin z jugozahodnimi vetrovi ob gorskih pregradah kot so Trnovski gozd, Snežnik, Julijske Alpe in Savinjske Alpe (Hočevar in Petkovšek, 1995). Največ, nad 3000 mm padavin, pade v severozahodnem delu Slovenije na območju Žage in Kobarida. Višina padavin se proti vzhodu zmanjšuje, na skrajnem severovzhodu Slovenije znaša samo še 800 mm, osrednja Slovenija pa ima letno 1300-1400 mm padavin. V alpskem prostoru je izrazit padavinski maksimum jeseni. Poleg povprečnih vrednosti so pomembni tudi skrajni vremenski dogodki, ki so sestavni del naravnega podnebja. Zaradi izrazite spremenljivosti in zelo redkega pojavljanja skrajnih vremenskih in podnebnih pojavov je težko oceniti dolgotrajne trende, saj dolžina obdobja med dvema pojavoma izjemnih dogodkov na določenem območju lahko traja več let. Največja dnevna višina padavin, celo nad 400 mm, je bila zabeležena na območju Posočja (ARSO, 2010). V toplem delu leta so pogosti močni nalivi, v katerih lahko pade več kot 100 mm padavin v eni uri (Komac in Zorn, 2009; Mikoš in sod., 2006). Poleg obilnih dnevnih in večdnevnih padavin povzročajo vodno erozijo tudi nalivi, ki trajajo od nekaj minut do nekaj ur (Ceglar in sod., 2008; Komac, 2005). Ekstremne hidrološke razmere, ki so se v Sloveniji pojavile leta 2007 (Rusjan in sod., 2007), 2009 in 2010, potrjujejo scenarij o vplivu klimatskih sprememb na vodni krog (ARSO, 2010). Največ škode so povzročile padavine leta 2009 v severnem in zahodnem delu Slovenije (Polajnar, 2009). Slovenijo je v dneh od 16. do 19. septembra 2010 prizadelo močno deževje, ko je v 48 urah v povprečju padlo 170-180 mm padavin, kar je največja višina padavin v takem časovnem obdobju v zadnjih 60 letih (ARSO, 2010). Redno spremljanje in analiza ekstremnih dogodkov sta pomembna za ugotavljanje sprememb klime, saj le-te vplivajo tudi na spremembe v pogostosti in intenziteti ekstremnih dogodkov (Damm in Terhorst, 2010).

Za nastanek snežne odeje (SO) so potrebne padavine v trdi obliki. Trajanje in debelina SO pomembno vplivata na vodne zaloge. Število dni s SO je izrazito povezano s temperaturo zraka in višino padavin. Temperatura zraka z nadmorsko višino pada, višina padavin pa v povprečju z nadmorsko višino narašča, zato je trajanje SO močno povezano z nadmorsko višino. Snežne razmere vplivajo na mobilnost prebivalstva, zato so podatki o snežnih razmerah pomembni v vsakdanjem življenju, pa tudi v številnih vejah gospodarstva: v prometu, komunali, gradbeništvu, turizmu in kmetijstvu. Po ocenah so alpska smučišča pod nadmorsko višino 1200-1300 m manj perspektivna za bodoče investicije v smučarsko infrastrukturo (Žiberna, 1991), številne raziskave za območje Alp pa so potrdile skrajševanje snežne sezone po letu 1980 (Latersner in Schneebeli, 2003; Schöner in sod., 2009; Valt in Cianfarra, 2010). Spremembe v višini padavin pozimi in trajanju snežne sezone so zanimive tudi z vidika vodne bilance in erozije, saj meteorna voda v tekoči obliki v času izven vegetacijske dobe predstavlja večjo možnost za nastajanje novih erozijskih žarišč (ARSO, 2010; Zorn in Komac, 2005).

Triglavski narodni park (TNP) se razprostira na severozahodu Slovenije, na jugovzhodnem delu alpskega masiva, obsega pa 880 km$^2$, kar je 4 % površine Slovenije. Vodno bogastvo parka predstavljajo številni kraški izviri, potoki, reke, podzemne vode in ledeniška jezera. Gorski grebeni med rekama Savo in Sočo zarisujejo hidrološko mejo med Črnim in Jadranskim povodjem. Padavinsko povprečje znaša nad 1500 mm, letno pa je na tem območju 120-146 padavinskih dni. Podatki o vodnih zalogah in kriosferi (snegu in ledu) so pomembni za različna področja: kmetijsko pridelavo, oskrbo z vodo, varovanje okolja, turizem, promet, energetiko itd. Iz dosedanjih analiz padavin na območju Alp je razvidno, da so obilne padavine, ki se pojavljajo v povprečju enkrat na mesec, v zadnjih sto letih narasle (Kajfež-Bogataj in sod., 2010). Ugotovljeno je bilo, da je povečanje padavin značilno za jesen in zimo. Simulacije nakazujejo progresivno rast števila in intenzitete padavin in s tem posebno opazne učinke na pogostost obilnih padavin (ARSO, 2010). Meja za območja, na katerih zapade vedno dovolj snega za obstoj smučarskega turizma, se dviguje na večje nadmorske višine. To ima za posledico neposredno gospodarsko škodo, po drugi strani pa okoljske probleme, saj zaradi zmanjšanja števila snežnih dni poskušajo razširiti smučarski turizem v višje predele.

Analizirali smo padavinski režim na širšem območju TNP za osemnajst postaj za obdobje 1961-2009, obravnavali smo povprečno višino letnih padavin ter število dni z dnevno in dvodnevno vsoto padavin nad 5 mm, 10 mm, 20 mm, 50 mm, 100 mm in 150 mm. Za isto obdobje smo preučili povprečno število dni s SO in trende po letnih časih. Dobljeni rezultati so pomembna osnova za strokovno utemeljene strategije blaženja in prilagajanja na podnebne spremembe ter prispevek k razvijanju vsebin informacijskih središč, ohranjanju kakovosti prostora, kulturne krajine, varstvu narave, izobraževanju in obveščanju.

## 2 MATERIAL IN METODE

Meteorološke podatke smo pridobili iz arhiva Agencije republike Slovenije za okolje, ARSO (Arhiv..., 2010), za obdobje 1961-2009. Analizirali smo višino letnih padavin, število dni z dnevno in dvodnevno vsoto padavin >5 mm, >10 mm, >20 mm, >50 mm, >100 mm, >150 mm, število dni s SO ter trende števila dni s SO za izbrane postaje na širšem območju TNP. Obravnavane meteorološke postaje smo izbrali tako, da je čim bolj pokrito celotno območje TNP (Preglednica 1, Slika 1).

**Preglednica 1**: Obravnavane postaje, njihove nadmorske višine in geografske koordinate (Klimatografija…, 2000).

**Table 1:** Analayzed stations with altitude and geographical coordinates (Klimatografija..., 2000).

| kraj | oznaka postaje | nadmorska višina [m] | zemljepisna širina [φ] | zemljepisna dolžina [λ] |
|---|---|---|---|---|
| Livek | LI | 695 | 46° 12' | 13° 36' |
| Kobarid | KO | 263 | 46° 14' | 13° 34' |
| Planina pod Golico | PG | 948 | 46° 27' | 14° 03' |
| Stara Fužina | SF | 547 | 46° 17' | 13° 53' |
| Vogel | VO | 1800 | 46° 20' | 13° 45' |
| Kredarica | KR | 2514 | 46° 22' | 13° 50' |
| Rateče | RA | 864 | 46° 29' | 13° 42' |
| Zgornja Sorica | ZS | 860 | 46° 13' | 14° 01' |
| Javorniški Rovt | JR | 962 | 46° 27' | 14° 05' |
| Zgornja Radovna | ZR | 750 | 46° 25' | 13° 56' |
| Gorjuše | GO | 940 | 46° 18' | 14° 00' |
| Bohinjska Bistrica | BB | 507 | 46° 16' | 13° 57' |
| Kranjska Gora | KG | 804 | 46° 29' | 13° 47' |
| Log pod Mangartom | LM | 650 | 46° 24' | 13° 36' |
| Trenta | TR | 622 | 46° 22' | 13° 45' |
| Soča | SČ | 487 | 46° 20' | 13° 40' |
| Žaga | ŽA | 419 | 46° 18' | 13° 28' |
| Kneške Ravne | KN | 752 | 46° 12' | 13° 49' |

**Slika 1:** Lokacije obravnavanih postaj z mejo Triglavskega narodnega parka (Medved-Cvikl, 2010).

**Figure 1:** Locations of the analyzed stations with Triglav national park border (Medved-Cvikl, 2010).

Statistične analize meteoroloških podatkov smo izvedli s programoma Statgraphics 5.0 (STSC, Rockwille, USA) in MS Office Excel 2003. Med merami sredine smo prikazali aritmetično sredino, med merami variabilnosti pa variacijski razpon (VR), standardni odklon (SD) ter koeficient variabilnosti (KV). Osnovno dolgoročno tendenco števila dni s SO smo prikazali z linearnim trendom, ki smo ga izrazili v številu dni na dekado (10 let). Negativni trend pomeni, da se vrednost obravnavane spremenljivke v obravnavanem obdobju v zadnjem obdobju zmanjšuje glede na začetno obdobje, pozitiven trend pa pomeni povečanje vrednosti obravnavane spremenljivke glede na začetno obdobje.

## 3 REZULTATI Z DISKUSIJO

### 3.1 Povprečne letne višine padavin

V preglednici 2 so podane povprečne letne višine padavin za obravnavane postaje za obdobje 1961-2009. Povprečna višina letnih padavin je največja na območju Žage (2972 mm), najmanjša pa v Ratečah (1532 mm). Absolutno največjo letno višino padavin v obravnavanem obdobju so izmerili na postaji Žaga pri Bovcu (4042 mm), sledita ji Soča (3759 mm) in Livek (3680 mm), absolutno najmanjšo letno višino padavin med obravnavanimi postajami pa imajo Rateče (1129 mm). Postaja Žaga pri Bovcu, kjer merijo padavine vsak dan, ima uradno največje dolgoletno povprečje letne višine padavin v Sloveniji, na postajah Bogatinsko sedlo in Zadnji Vogel, kjer merijo le letno višino padavin, je bilo v enem letu izmerjeno tudi že več kot 5000 mm padavin. Na padavinski postaji Učja, tik ob italijansko-slovenski meji, so v letu 1960 zabeležili kar 6103 mm padavin (ARSO, 2010). Spremenljivost višine padavin med leti smo prikazali s SD in KV. Najmanjši KV imata postaji Zgornja Sorica in Stara Fužina (13 %), največjega pa Livek (19,9 %).

**Preglednica 2:** Povprečna (pov), maksimalna (max) in minimalna (min) višina letnih padavin v mm, standardna deviacija (SD) in koeficient variabilnosti (KV) za obdobje 1961-2009 za meteorološke postaje na širšem območju TNP.

**Table 2:** The average (pov), maximum (max) and minimum (min) yearly precipitation amount in mm, standard deviation (SD) and coefficient of variability (KV) for the period 1961-2009 for meteorological stations of the wider area of TNP.

| | **ZS** | **JR** | **PG** | **ZR** | **GO** | **BB** | **SF** | **KR** | **KG** |
|---|---|---|---|---|---|---|---|---|---|
| **pov** | 2065 | 1954 | 1735 | 1830 | 1967 | 2112 | 2298 | 2005 | 1648 |
| **max** | 2737 | 2696 | 2572 | 2588 | 2953 | 2876 | 3058 | 2799 | 2472 |
| **min** | 1420 | 1497 | 1260 | 1322 | 1383 | 1446 | 1644 | 1239 | 1242 |
| **SD** | 268,7 | 273,2 | 276,8 | 317,5 | 309,3 | 344,3 | 306,3 | 306,2 | 246,5 |
| **KV** | 13,0 | 14,0 | 15,9 | 17,4 | 15,7 | 16,3 | 13,0 | 15,3 | 15,0 |
| | **RA** | **LM** | **TR** | **SČ** | **ŽA** | **LI** | **KO** | **KN** | **VO** |
| **pov** | 1532 | 2400 | 2149 | 2421 | 2972 | 2476 | 2623 | 2795 | 2691 |
| **max** | 2290 | 3470 | 3065 | 3759 | 4042 | 3680 | 3639 | 3791 | 3576 |
| **min** | 1129 | 1486 | 1479 | 1665 | 1828 | 1497 | 1596 | 1582 | 1908 |
| **SD** | 233,0 | 410,3 | 343,4 | 425,1 | 498,0 | 492,9 | 440,2 | 449,4 | 511,7 |
| **KV** | 15,2 | 17,1 | 16,0 | 17,6 | 16,8 | 19,9 | 16,8 | 16,1 | 19,0 |

Poleg povprečnih letnih višin padavin za celotno obdobje 1961-2009 smo analizirali tudi povprečne letne višine padavin za vse obravnavane postaje (razen postaje Vogel, za katero so podatki meritev od l. 1982 dalje) po desetletjih: 1961-1969, 1970-1979, 1980-1989, 1990-1999 in 2000-2009. Najbolj mokro obdobje je bilo na večini (13) postaj 1961-1969, višina padavin v tem obdobju je statistično značilno večja od višine padavin v kasnejših obdobjih (Duncanov test, $p<0,05$). Dekadi 1980-1989 in 2000-2009 sta med obravnavanimi obdobji najbolj sušni na vseh postajah, z izjemo Kredarice, vendar razlike glede na ostale dekade niso statistično značilne.

### 3.2 Analiza dnevnih padavinskih dogodkov

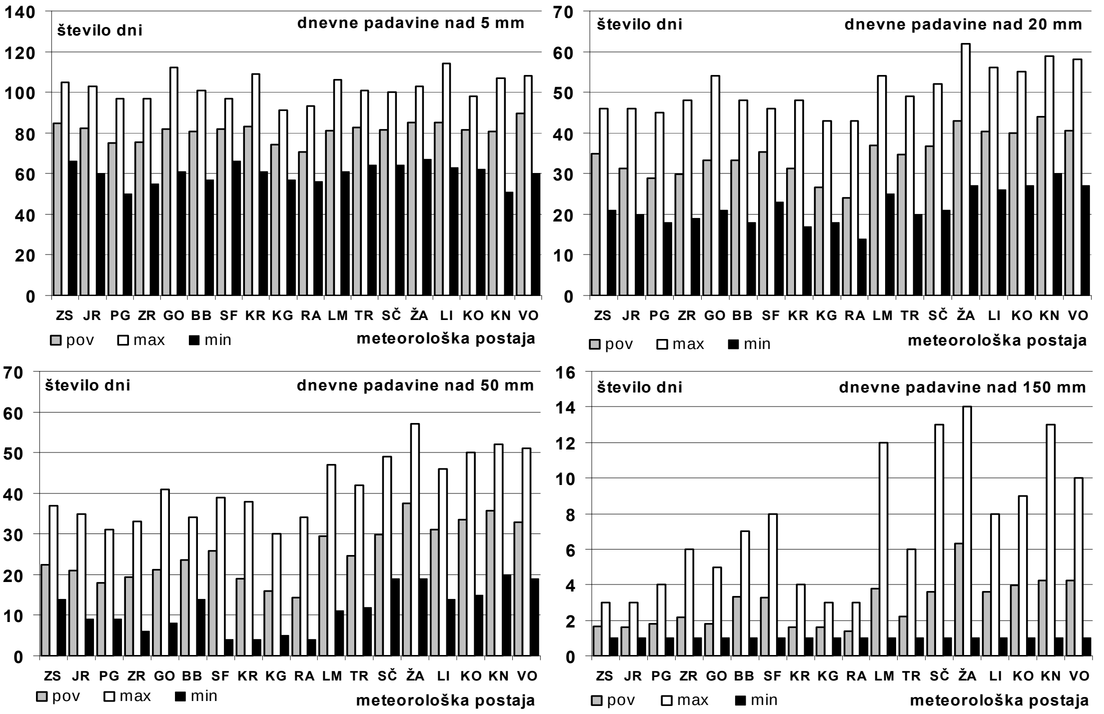
Za dnevne padavine smo analizirali šest različnih padavinskih dogodkov in sicer število dni, ko je dnevna višina padavin presegla 5, 10, 20, 50, 100 ali 150 mm, primeri za 5, 20, 50 in 150 mm so prikazani na sliki 2. V povprečju je letno med 70 (Rateče) in 90 dni (Vogel), ko dnevna vsota padavin doseže >5 mm. KV je majhen, kar pomeni, da se število dni z dnevno višino padavin >5 mm med leti ne spreminja veliko, vrednosti KV pa znašajo od 9,3 % (Stara Fužina) do 15,9 % (Kneške Ravne). Število dni z višino padavin >10 mm znaša od 47 do 70, pri čemer imajo Kneške Ravne v povprečju največ padavinskih dni >10 mm (69,2), najmanj pa Rateče (47,1 dni). Variabilnost med leti se z intenzivnostjo padavinskih dogodkov povečuje; KV števila padavinskih dni >10 mm ima razpon med 11,4 % in 17,2 %. Absolutno največje letno število takšnih padavinskih dni znaša kar 91 na postaji Kneške Ravne, najmanj pa 33 v Ratečah. Rateče imajo prav tako najmanjše povprečno število dni s padavinami >20 mm (24) in Kneške Ravne največ takih dni (44). Na postaji Žaga znaša absolutni ekstrem kar 62 padavinskih dni >20 mm v enem letu, minimalno število pa je zabeleženo za postajo Rateče. KV za povprečno število padavinskih dni >20 mm znaša od 15 % do 22,1 %.

V Ratečah lahko v povprečnem letu pričakujemo 14 dni, ko dnevna višina padavin preseže 50 mm, medtem ko lahko tako intenzivne dnevne padavine pričakujemo več kot tridesetkrat letno na postajah Žaga (38), Kneške

Ravne (36), Kobarid (34), Vogel (33) in Livek (31). V posameznih letih se dnevni nalivi >50 mm pojavijo zelo redko, absolutni minimum, samo štiri takšne nalive letno, imajo postaje Stara Fužina, Kredarica in Rateče. Postaja Žaga ima največje letno število nalivov >50 mm in sicer kar 57. Medletna variabilnost je zelo visoka in znaša do 40,7 % v Ratečah.

Na vseh obravnavanih postajah lahko vsako leto pričakujemo vsaj en dan, ko bo višina padavin presegla 100 mm, v Kobaridu in na Voglu tri takšne dneve, vsaj štiri pa na postaji Žaga. Le-ta ima tudi največje povprečno letno število padavinskih dni >100 mm (14,4) ter absolutni maksimum 23 takšnih padavinskih dogodkov v enem letu. Sicer pa znaša povprečno število letnih nalivov >100 mm od 2 do 3 v Kranjski Gori in Ratečah do preko 10 v Kobaridu (11,1), Kneških Ravnah (11,1 dni), Voglu (10,7 dni) in Žagi (14,4). Število dni z višino padavin >100 mm je zelo variabilno, KV pa znaša od 31,7 % do 59 %.

Tako kot dnevni nalivi >100 mm se tudi nalivi >150 mm v povprečju pojavijo vsaj enkrat letno na vseh obravnavanih postajah. Takšni padavinski dogodki so v povprečju zelo redki, na večini postaj dva do trije letno, štirje na postajah Log pod Mangartom, Soča, Livek, Kobarid, Kneške Ravne in Vogel, na postaji Žaga pa je letno v povprečju 6 do 7 dni z intenziteto padavin >150 mm. Postaja Žaga ima tudi absoluten letni maksimum v število nalivov >150 mm, ki znaša 14. Zaradi redkega pojavljanja tako intenzivnih padavin je medletna variabilnost zelo visoka, vrednosti KV znašajo od 43 % do 71 % .



**Slika 2:** Povprečno (pov), maksimalno (max) in minimalno (min) letno število dni z dnevno višino padavin nad 5, 20, 50 in 150 mm za obdobje 1961-2009.

**Figure 2:** The average (pov), maximum (max) and minimum (min) number of days per year with daily precipitation exceeded 5, 20, 50 and 150 mm for the period 1961-2009.

### 3.3 Analiza dvodnevnih nalivov

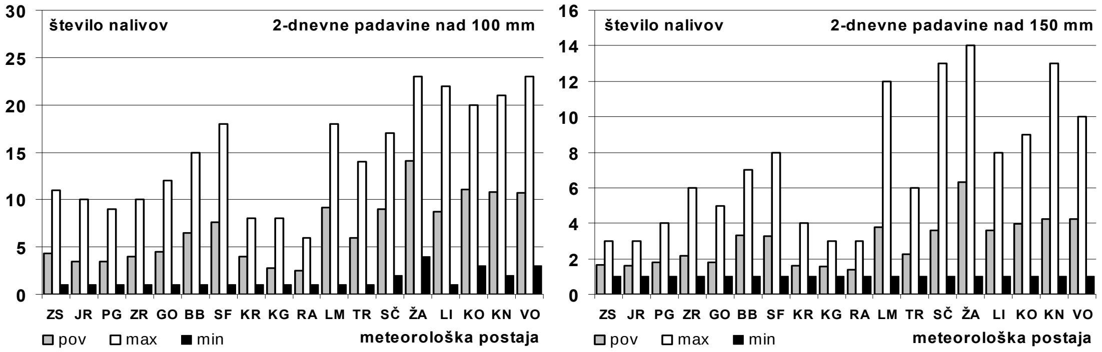
Poleg dnevnih padavin smo analizirali tudi 2-dnevne nalive, ko je višina padavin v 48 urah presegla 50, 100 ali 150 mm. Zaradi načina meritev padavin (vsak dan ob 7h zjutraj zabeležijo višino padavin za zadnjih 24 ur) so pogosto presekani dogodki z močnimi padavinami in razdeljeni na dve dnevni meritvi, tako da jih težko zaznamo kot ekstremni dogodek. Z analizo 2-dnevnih padavin torej povečamo verjetnost, da zaznamo večje število takšnih nalivov. V preglednici 3 je prikazano letno število dvodnevnih nalivov z višino padavin >50 mm s pripadajočimi SD. V letnem povprečju ima največje število dvodnevnih nalivov >50 mm Žaga (37,5), nad 30 takšnih padavinskih dogodkov imajo tudi Kneške Ravne (35,7), Kobarid (33,5) in Livek (31,1); najmanjše povprečno število nalivov >50 mm pa imajo Rateče (13,8). Izračunani KV kažejo, da je variabilnost v številu nalivov >50 mm med leti zelo velika (od 20,8 % do 37,3 %), tako je bilo npr. na postaji Vogel, kjer je KV največji, največje letno število nalivov >50 mm kar

51, najmanjše število pa samo 4. Po pričakovanjih je število dvodnevnih nalivov >100 mm (Slika 3) bistveno manjše kot število nalivov >50 mm. Največ dvodnevnih nalivov >100 mm ima Žaga (14,1), sledita Kobarid (11,1) in Kneške Ravne (10,8), najmanj nalivov >100 mm pa je v Ratečah, kjer se v povprečju pojavijo letno le 2 do 3 takšni padavinski dogodki. Maksimalno število nalivov >100 mm v posameznem letu znaša 23 na postajah Žaga in Vogel, medtem ko je v letu z minimalno vrednostjo bil zabeležen le 1 tak padavinski dogodek. Število dvodnevnih nalivov >100 mm med leti niha bolj kot število nalivov nad >50 mm; KV znaša od 32,6 % do 67 %. Še večja variabilnost med leti je značilna za število nalivov >150 mm (Slika 3). Občasno se v zahodnih predelih Slovenije pojavljajo še močnejši nalivi kot obravnavani v naši raziskavi. Največja 2-dnevna višina padavin, izmerjena s klasičnim dežemerom, znaša za Slovenijo 584 mm za Bovec (november 1960), največja dnevna pa 363 mm za isto postajo in obdobje. Dejanske vrednosti so lahko še večje, tako je samodejna agrometeorološka postaja Zadlog nad Idrijo do 8. ure 18. septembra 2010 zabeležila 377 mm v 24 urah in do 2. ure 19. septembra 620 mm v 48 urah (ARSO, 2010). Ekstremne padavine so eden izmed sprožilnih dejavnikov, ki vplivajo na pojavljanje plazov (Komac, 2005), poleg tega močni kratkotrajni nalivi povzročajo erozijo tal, uničujoče hudourniške vode, poplave, škodo v turizmu, infrastrukturi, kmetijstvu in drugih dejavnostih (Polajnar, 2009). Zaradi vsega naštetega je pomembno poznati verjetnost pojavljanja močnih nalivov, imeti učinkovit sistem za pravočasno opozarjanje pred tovrstnimi nevarnostmi ter pripravljene ukrepe za zaščito ljudi in njihovega premoženja, če pride do ekstremnih padavin (Starec, 2002). Regionalni scenariji bodočih podnebnih sprememb napovedujejo intenzivnejše padavine v spremenjenem padavinskem režimu, pogostejše in intenzivnejše ekstremne vremenske dogodke kot so suše, poplave ter neurja z močnimi vetrovi (Ceglar in sod., 2008).

**Preglednica 3:** Povprečno (pov), maksimalno (max) in minimalno (min) letno število dvodnevnih nalivov z višino padavin nad 50 mm za obdobje 1961-2009, SD-standardna deviacija, KV-koeficient variabilnosti.

**Table 3:** The average (pov), maximum (max) and minimum (min) number of events with 48-h rainfall totals exceeded 50 mm for the period 1961-2009, SD-standard deviation, KV-coefficient of variability.

| | **ZS** | **JR** | **PG** | **ZR** | **GO** | **BB** | **SF** | **KR** | **KG** |
|---|---|---|---|---|---|---|---|---|---|
| **pov** | 22,5 | 20,9 | 18,0 | 19,3 | 21,1 | 23,6 | 25,8 | 19,0 | 15,9 |
| **max** | 37 | 35 | 31 | 33 | 41 | 34 | 39 | 38 | 30 |
| **min** | 14 | 9 | 9 | 6 | 8 | 14 | 4 | 4 | 4 |
| **SD** | 5,1 | 5,9 | 5,0 | 5,6 | 6,3 | 5,5 | 6,4 | 6,5 | 4,8 |
| **KV** | 22,8 | 28,4 | 27,9 | 28,8 | 29,7 | 23,4 | 24,8 | 34,0 | 30,3 |
| | **RA** | **LM** | **TR** | **SČ** | **ŽA** | **LI** | **KO** | **KN** | **VO** |
| **pov** | 13,8 | 29,5 | 24,6 | 29,8 | 37,5 | 31,1 | 33,5 | 35,7 | 29,3 |
| **max** | 30 | 47 | 42 | 49 | 57 | 46 | 50 | 52 | 51 |
| **min** | 4 | 11 | 12 | 19 | 19 | 14 | 15 | 20 | 4 |
| **SD** | 4,9 | 7,1 | 6,7 | 7,4 | 7,8 | 8,8 | 7,3 | 7,8 | 10,9 |
| **KV** | 35,4 | 23,9 | 27,1 | 25,0 | 20,8 | 28,2 | 21,7 | 21,9 | 37,3 |



**Slika 3:** Povprečno (pov), maksimalno (max) in minimalno (min) letno število dvodnevnih nalivov z višino padavin nad 100 in 150 mm za obdobje 1961-2009.

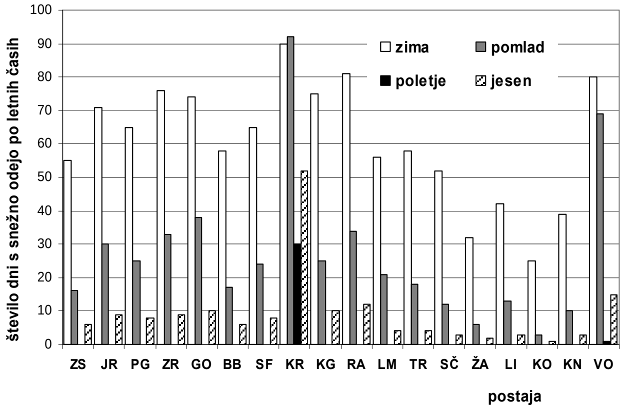
**Figure 3:** The average (pov), maximum (max) and minimum (min) number of events with 48-h rainfall totals exceeded 100 and 150 mm for the period 1961-2009.

### 3.4 Analiza trajanja snežne odeje

V preglednici 4 so podatki o povprečnem, maksimalnem in minimalnem letnem številu dni s SO, na sliki 4 pa smo prikazali število dni s SO po letnih časih. Pri tem smo upoštevali meteorološke letne čase, pri katerih traja zima od 1.12. do 28.2., pomlad od 1.3. do 31.5., poletje od 1.6. do 31.8., jesen od 1.9. do 30.11., snežna sezona pa od 1.7. tekočega leta do 30.6 naslednjega leta. Obravnavali smo samo število dni s SO, ne pa tudi višine SO, ki pomembno vpliva na vodnatost rek in potokov v spomladanskem času, ko se topi sneg. Glede na nadmorsko višino obravnavanih postaj je razumljivo, da ima najdlje trajajočo snežno sezono naša najvišja meteorološka opazovalnica Kredarica, kjer se sneg v povprečju obdrži 264 dni, SO pa se razmeroma dolgo obdrži tudi na Voglu in v Ratečah (166 dni). Najkrajšo snežno sezono ima Kobarid (30 dni), kjer se že pozna vpliv sredozemskega podnebja oziroma prodorov toplega morskega zraka, ki se po dolini Soče dviga proti gorskim območjem vse do Trente in vpliva na višje temperature zimskih mesecev. Med leti so razlike v trajanju SO izrazito velike, v posameznih letih SO praktično ni, v drugih pa traja zelo dolgo; tako je bilo npr. v Kranjski Gori l. 1989 samo 10 dni s SO, l. 1969 pa kar 154. Pozimi je število dni s SO od 25 (Kobarid) do vseh 90 na Kredarici (Slika 4). Na vseh postajah lahko tudi v pomladnih mesecih pričakujemo še nad 10 dni s SO, razen na postajah Žaga, kjer je spomladi SO v povprečju le 6 dni, ter v Kobaridu (3 dni). Na Kredarici se SO obdrži tudi del poletja (30 dni), Vogel pa ima v povprečju poleti le en dan s SO (Slika 4).

**Preglednica 4:** Povprečno (pov), maksimalno (max) in minimalno (min) letno število dni s snežno odejo za obdobje 1961-2009, SD-standardna deviacija, KV-koeficient variabilnosti.
**Table 4:** Average (pov), maximum (max) and minimum (min) numbers of days with snow cover per year for the period 1961-2009, SD-standard deviation, KV-coefficient of variability.

| | **ZS** | **JR** | **PG** | **ZR** | **GO** | **BB** | **SF** | **KR** | **KG** |
|---|---|---|---|---|---|---|---|---|---|
| **pov** | 81 | 110 | 98 | 118 | 123 | 82 | 98 | 264 | 110 |
| **max** | 137 | 160 | 160 | 169 | 169 | 152 | 153 | 309 | 154 |
| **min** | 10 | 12 | 8 | 31 | 29 | 9 | 18 | 235 | 10 |
| **SD** | 32,5 | 32,8 | 38,8 | 31,2 | 30,9 | 37,9 | 36,7 | 16,3 | 29,5 |
| **KV** | 40,2 | 29,9 | 39,7 | 26,4 | 25,2 | 46,4 | 37,6 | 6,2 | 26,7 |
| | **RA** | **LM** | **TR** | **SČ** | **ŽA** | **LI** | **KO** | **KN** | **VO** |
| **pov** | 166 | 81 | 81 | 67 | 40 | 60 | 30 | 52 | 166 |
| **max** | 219 | 139 | 142 | 125 | 92 | 115 | 79 | 120 | 219 |
| **min** | 65 | 5 | 22 | 3 | 2 | 3 | 9 | 3 | 65 |
| **SD** | 13,8 | 37,8 | 33,2 | 32,9 | 23,0 | 29,1 | 20,3 | 25,1 | 31,8 |
| **KV** | 19,2 | 46,7 | 41,0 | 49,1 | 57,2 | 49,0 | 68,4 | 48,2 | 19,2 |



**Slika 4:** Povprečno število dni s snežno odejo po letnih časih za obdobje 1961-2009.
**Figure 4:** The average number of days with snow cover by seasons for the period 1961-2009.

### 3.5 Trendi števila dni s snežno odejo

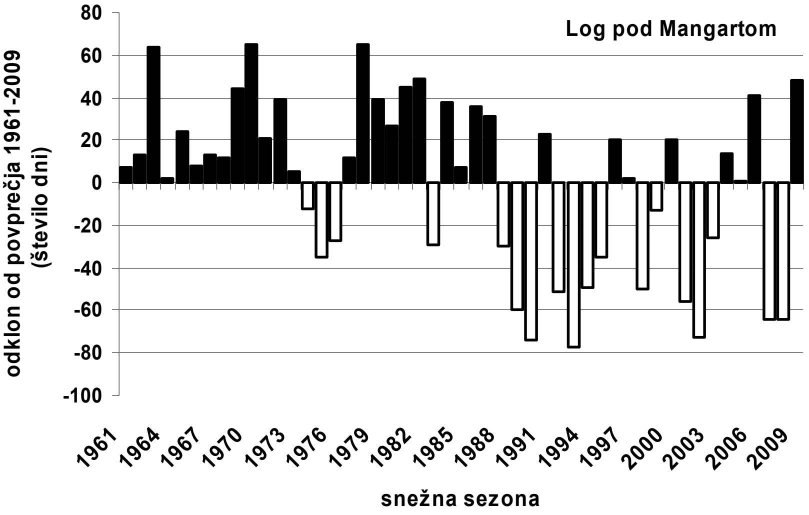
V preglednici 5 so prikazani trendi števila dni s SO, to so dnevi, ko so ob 7.uri zjutraj tla pokrita s snegom. Na vseh obravnavanih postajah je za obdobje 1961-2009 trend števila dni s SO za celotno snežno sezono negativen, na večini postaj (13) je trend statistično značilen. Velikost spremembe je od -4,3 dni/10 let na postaji Žaga do -14,9 dni/10 let na postaji Stara Fužina, kar kaže na to, da so spremembe v trajanju snežne sezone že tako velike, da vplivajo na vodne zaloge, pretoke rek spomladi, zimski turizem in trajanje vegetacijske sezone.

Na sliki 5 smo za Log pod Mangartom prikazali odklone števila dni s SO od dolgoletnega povprečja, ki za to postajo znaša 81 dni na leto. Trend je močno statistično značilen (p<0,01) in znaša –11,9 dni/10 let. V obdobju 1961-1987 prevladujejo leta s številom dni s SO nad dolgoletnim povprečjem, po letu 1987 pa leta, ko je bilo število dni s SO pod dolgoletnim povprečjem. Rezultati naše analize se ujemajo z rezultati raziskave Žagarjeve in sod. (2006), ki so z neparametričnim preizkusom Mann-Whitney-Pettit določili prelomne točke trendov in ugotovili, da se je naraščanje temperatur zraka, krajše trajanje snežne odeje in zgodnejši nastop fenofaz v Sloveniji začelo približno po letu 1987. Skrajšanje snežne sezone so potrdili tudi za območje avstrijskih (Schöner in sod., 2009), italijanskih (Valt in Cianfarra, 2010) in švicarskih Alp (Latersner in Schneebeli, 2003), povsod pa so spremembe izrazitejše po letu 1980. Glede na izračunane trende ter scenarije podnebnih sprememb lahko sklepamo, da bodo zime v prihodnje toplejše in trajanje snežne sezone krajše (Cegnar, 2003; Kajfež-Bogataj in sod., 2004, 2010). Posledice so že vidne v zimskem turizmu, naraščajo potrebe po umetnem zasneževanju, kjer pa so smučišča v zavarovanem območju, je to možno le brez kemikalij ali mikroorganizmov za utrjevanje snega, ki bi škodovali okolju. Umetno zasneževanje povzroči večje stroške obratovanja smučišč, večjo porabo energije, večje pritiske na vodne vire in vodni krog, kar ogroža gospodarjenje z vodo na teh območjih. Snežna sezona močno vpliva tudi na vegetacijsko dobo, zato so podatki o snežnih razmerah pomembni tudi v kmetijstvu. Krajšanje trajanja SO odeje lahko vpliva na zgodnejše viške pretokov na vodotokih, ki so odvisni od taljenja snega, dolgoročno pa lahko pričakujemo tudi spremembo rečnih režimov.

**Preglednica 5:** Trend spreminjanja števila dni s snežno odejo za obdobje 1961-2009.
**Table 5:** The trend of number of days with snow cover for the period 1961-2009.

| **Postaja** | **Zima** | **Pomlad** | **Jesen** | **Snežna sezona** | **Velikost spremembe za snežno sezono (število dni/10 let)** |
|---|---|---|---|---|---|
| ZS | -*** | -** | - | -*** | -11,6 |
| JR | -** | -*** | - | -*** | -11,8 |
| PG | + | - | - | - | -1,4 |
| ZR | - | -** | - | -** | -7,2 |
| GO | - | - | - | - | -4,8 |
| BB | -** | - | - | -*** | -11,9 |
| SF | -*** | -** | - | -*** | -14,9 |
| KR | - | 0 | + | - | -0,6 |
| KG | -* | -** | - | -** | -8,0 |
| RA | - | -** | - | -** | -9,7 |
| LM | -*** | -** | - | -*** | -11,9 |
| TR | -** | -** | - | -** | -8,7 |
| SČ | -** | - | - | -** | -8,3 |
| ŽA | -* | - | + | -* | -4,3 |
| LI | -* | - | - | -* | -5,5 |
| KO | - | - | + | - | -3,1 |
| KN | -** | - | - | -** | -6,6 |
| VO | + | - | - | - | -4,6 |

Stopnja tveganja: * p 0,10 / ** p 0,05 / *** p 0,01 /
Smer trenda : negativen trend (-): manj sneženih dni / pozitiven trend (+): več sneženih dni
Significance: * p 0.10 / ** p 0.05 / *** p 0.01 /
Trend sign: negative trend (-): less snow days / positive trend (+): more snow days



**Slika 5:** Odkloni števila dni s snežno odejo od dolgoletnega povprečja 1961-2009, ki znaša 81 dni na snežno sezono v Logu pod Mangartom.

**Figure 5:** Deviations (days) from long-term average number of days with snow cover for the period 1961-2009, which is 81 days per snow season in Log pod Mangartom.

## 4 ZAKLJUČKI

Razgiban teren in nadmorska višina močno vplivata na prostorsko porazdelitev padavin v Sloveniji. Poleg povprečnih vrednosti padavin in njihove spremenljivosti so pomembni tudi ekstremni padavinski dogodki, ki so sestavni del naravnega podnebja. Za 18 meteoroloških postaj na območju TNP smo za obdobje 1961-2009 analizirali letne višine padavin, število nalivov ter trende v številu dni s snežno odejo. Med postajami na širšem območju TNP obstajajo velike razlike v povprečni letni višini padavin. Največ padavin ima v obravnavanem obdobju Žaga pri Bovcu (2972 mm), najmanj pa Rateče (1532 mm). Razponi med maksimalno in minimalno višino padavin so na vseh postajah veliki, v izrazito mokrih letih je letna višina padavin več kot dvakratna višina tiste v najbolj sušnih letih. Povprečne letne višine padavin, izračunane za desetletna obdobja, se statistično značilno razlikujejo. Najbolj mokro desetletje je bilo 1961-1969, najbolj sušni desetletji pa 1980-1989 in 2000-2009.

Na vseh postajah se pojavljajo močni enodnevni in dvodnevni nalivi, število takšnih padavinskih dogodkov se med postajami in leti bistveno razlikuje. Največje število dni z višino padavin >5 mm ima Vogel (90 dni), >10mm in >20mm Kneške Ravne (69 dni oz. 44 dni). Dvodnevnih nalivov >50, >100 in >150 mm je največ na Žagi, najmanj pa v Ratečah. Na vseh obravnavanih postajah lahko vsako leto pričakujemo vsaj en dan, ko višina padavin presega 150 mm. Z večjo intenziteto nalivov se povečuje tudi variabilnost njihovega števila. Variabilnost je največja pri višini padavin >150 mm, najmanjša pa pri višini padavin >5 mm. Ekstremne padavine vplivajo na pojavljanje plazov, povzročajo erozijo tal, uničujoče hudourniške vode, poplave, škodo v turizmu, infrastrukturi, kmetijstvu in drugih dejavnostih. Zaradi vsega naštetega je pomembno poznati verjetnost pojavljanja močnih nalivov, imeti učinkovit sistem za pravočasno opozarjanje pred tovrstnimi nevarnostmi ter pripravljene ukrepe za zaščito ljudi in njihovega premoženja, če pride do ekstremnih padavin.

Trajanje SO se med obravnavanimi postajami in leti bistveno razlikuje. Na Kredarici, ki je najvišje stoječa slovenska meteorološka postaja, je v povprečju 264 dni s SO, najkrajša snežna sezona med obravnavanimi postajami pa je v Kneških Ravnah (52 dni) in Kobaridu (30 dni). Na vseh analiziranih postajah je opažen negativen trend v številu dni s SO tekom snežne sezone, kar vpliva na turizem, vodni režim in kmetijstvo. Redno spremljanje in analiza padavinskih dogodkov sta pomembna za ugotavljanje sprememb podnebja, napovedi v bodoče ter potrebne prilagoditve na spremenjen padavinski režim na različnih področjih človekovega delovanja.

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# Parametrični in neparametrični pristopi za odkrivanje trenda v časovnih vrstah

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## IZVLEČEK

Eno od najpogosteje uporabljenih orodij za odkrivanje sprememb v časovnih vrstah je analiza trenda. Obstaja veliko parametričnih in neparametričnih testov za odkrivanje značilnih trendov v časovnih vrstah. Slednji se pogosteje uporabljajo zaradi manjšega števila predpostavk potrebnih za njihovo izvedbo. Najpogosteje uporabljen test za odkrivanje značilnih trendov je Mann-Kendallov test, ki še vedno zahteva, da so vzorčni podatki neodvisni. Za odstranitev vpliva serialne korelacije v Mann-Kendallovem testu so bili vpeljani različni popravki in metode pred-beljenja. V tem članku je pregled najpogosteje uporabljenih pristopov za odkrivanje trenda v časovnih vrstah ob prisotnosti serialne korelacije ali brez nje. Na koncu so te metode uporabljene še na realnih podatkih.

**Ključne besede:** analiza trenda, metoda najmanjših kvadratov, Mann-Kendallov test, korelacijski koeficient, avtokorelacija, pred-beljenje.

## ABSTRACT

### PARAMETRIC AND NONPARAMETRIC APROACH FOR TREND DETECTION IN TIME SERIES

One of the most commonly used tools for detecting changes in time series is trend analysis. A number of parametric and nonparametric tests exist to detect the significance of trends in time series. The latter have been widely used mainly because of fewer number of assumptions needed in their implementation. The most often used test for detecting significant trends is Mann-Kendall test, that still requires sample data to be serially independent. To eliminate the effect of serial correlation on the Man-Kendall test different correction and pre-whitening methods have been introduced. This paper reviews the most commonly used approaches for trend detection in time series with or without presence of serial correlation. At the end these methods are applied to real datasets.

**Keywords:** trend analysis, least square method, Mann-Kendall test, correlation coefficient, autocorrelation, pre-whitening.

## 1 UVOD

V zadnjih letih se zaradi negativnih učinkov toplogrednih plinov na okolje veliko znanstvenikov ukvarja s časovnimi vrstami hidrometeoroloških spremenljivk. Za odkrivanje sprememb v časovni vrsti je pogosto uporabljena analiza trenda. V mnogih raziskavah so bili za odkrivanje trenda uporabljeni parametrični in neparametrični testi. Napisanih je bilo tudi več preglednih znanstvenih člankov o metodah za odkrivanje trenda v hidroloških podatkih (npr. Esterby, 1996; Kundzewicz in Robson, 2004; Khaliq in sod., 2009). Med parametričnimi pristopi je pogosto uporabljena ocena trenda po metodi najmanjših kvadratov. Parametrični testi imajo večjo moč od neparametričnih, vendar v mnogih primerih klimatski podatki ne izpolnjujejo predpostavk, ki jih zahtevajo (Kundzewicz in Robson, 2004). Alternativni pristop v takih primerih predstavljajo neparametrični testi. Eden izmed njih je Spearmanov test korelacije rangov za

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odkrivanje monotonega trenda (Lettenmaier, 1976), ki pa ima enako moč kot njemu soroden Mann Kendallov test (Yue in sod., 2002a).

Mann-Kendallov test (MK-test) za iskanje monotonega trenda (Mann, 1945; Kendall, 1975) temelji na rangih vrednosti opazovane spremenljivke v dani časovni vrsti in je zato neodvisen od porazdelitve. V primeru, da v časovni vrsti obstaja pozitivna avtokorelacija, nam MK-test lahko pokaže statistično značilen trend tudi v primeru, ko ta ne obstaja (Cox in Stuart, 1955), kar z drugi besedami pomeni, da ni robusten na avtokorelacijo. Zato so različni avtorji razvili več metod, ki upoštevajo vpliv avtokorelacije na trend.

Za zmanjšanje vpliva avtokorelacije na trend je bilo vpeljano pred-beljenje (von Storch, 1995; Kulkarni in von Storch, 1995). S tem postopkom najprej odstranimo avtokorelacijo iz časovne vrste in nato uporabimo MK-test. Yue in sod. (2002b) ter Yue in Wang (2002) so odkrili, da s pred-beljenjem odstranimo tudi del trenda, kar zmanjša njegovo statistično značilnost. Postopek pred-beljenja so v članku (Yue in sod., 2002b) izboljšali s tako imenovano TFPW metodo (v angl. trend-free pre-whitening). Yue in Wang (2002) sta predlagala, da se MK-test uporabi na originalnih podatkih, kadar sta velikost vzorca in trend dovolj velika.

Hammed in Rao (1998) ter Yue in sod. (2002b) so pokazali, da prisotnost avtokorelacije v časovni vrsti ne spremeni niti oblike porazdelitve (ostane normalna) niti povprečja testne statistike MK-testa, spremeni pa njeno varianco (pozitivna avtokorelacija jo poveča in obratno), kar je imelo za posledico nove pristope, ki so temeljili na korekciji variance Mann-Kendallove statistike glede na avtokorelacijo. Hammed in Rao (1998) ter Yue in Wang (2004) so popravili varianco MK-testa. Narejene so bile tudi prilagoditve za test Spearmanovega korelacijskega koeficienta in za parametrične teste za odkrivanje trenda glede na avtokorelacijo (Wilks, 1995).

Za časovne vrste s sezonsko komponento so Hirsch in sod. (1982) razvili sezonski Mann-Kendallov test (SMK-test), kasneje sta ga Hirsch in Slack (1984) razširila za serialno odvisne časovne vrste. Test je v literaturi znan kot modificiran sezonski Mann-Kendallov test (MSMK-test).

## 2 PREGLED PARAMETRIČNIH IN NEPARAMETRIČNIH METOD

### METODA NAJMANJŠIH KVADRATOV

Za odkrivanje in ocenjevanje linearnega trenda v nizu podatkov $y_1, y_2, \dots, y_n$, izmerjenih v določenih časovnih točkah $x_1, x_2, \dots, x_n$, se uporablja enostavni linearni model

$$y_i = a + bx_i + e_i, \qquad i = 1 \dots n, \tag{1}$$

kjer sta $a$ in $b$ regresijska koeficienta, $e_i$ pa so ostanki, ki zadoščajo predpostavki, da so neodvisni in normalno porazdeljeni z aritmetično sredino $0$ ter konstantno varianco $\sigma^2$. Normalno porazdelitev ostankov lahko testiramo s Kolmogorov-Smirnovim testom.

Z metodo najmanjših kvadratov dobimo nepristranski oceni za koeficienta $a$ in $b$:

$$\hat{b}_i = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sum_{i=1}^{n}(x_i - \bar{x})^2} \tag{2}$$

s standardnim odklonom $s(\hat{b}_i) = \frac{s_e}{\sqrt{\sum_{i=1}^{n}(x_i-\bar{x})^2}}$

in

$$\hat{a}_i = \bar{y} - \hat{b}_i\bar{x} \tag{3}$$

s standardnim odklonom $s(\hat{a}_i) = s_e\sqrt{\frac{\sum_{i=1}^{n}x_i^2}{n\sum_{i=1}^{n}(x_i-\bar{x})^2}}$,

kjer je

$$s_e^{\,2} = \frac{1}{n-2}\sum_{i=1}^{n} e_i^2 \tag{4}$$

nepristranska ocena za varianco ostankov, $\bar{y}$ aritmetična sredina vrednosti $y_i$ in $\bar{x}$ aritmetična sredina vrednosti $x_i$. Ničelno hipotezo, da trend ni značilen ($b = 0$), proti alternativni hipotezi, da je trend značilen ($b \neq 0$), lahko testiramo s statistiko

$$t_b = \frac{\hat{b}_i}{s(\hat{b}_i)}, \tag{5}$$

ki se porazdeljuje po Studentovi $t$ porazdelitvi z $n-2$ prostostnimi stopnjami. Podobno hipotezo lahko testiramo tudi za koeficient $a$.

Časovne vrste le redko izpolnjujejo predpostavko o normalni porazdelitvi in neodvisnosti ostankov, ki jo opisana metoda zahteva. Metoda najmanjših kvadratov je občutljiva tudi na ekstremne vrednosti (osamelce), ki so v časovnih vrstah pogosto prisotne. V takih primerih dobimo s to metodo nerealne rezultate, zato je za oceno linearnega trenda bolje uporabiti neparametrične metode.

## PEARSONOV KORELACIJSKI KOEFICIENT

Pearsonov korelacijski koeficient se izračuna po obrazcu

$$r = \frac{\sum_{t=1}^{n}(x_t - \bar{x})(y_t - \bar{y})}{\sqrt{\sum_{t=1}^{n}(x_t - \bar{x})^2 \sum_{t=1}^{n}(y_t - \bar{y})^2}}. \qquad (6)$$

Če so podatki dvorazsežno normalno porazdeljeni, potem ničelno hipotezo, da ni linearnega trenda (korelacijski koeficient je enak $0$), testiramo s statistiko

$$t = \frac{r\sqrt{n-2}}{1-r^2}, \qquad (7)$$

ki se porazdeljuje po Studentovi $t$-porazdelitvi z $n-2$ prostostnimi stopnjami.

## AVTOKORELACIJA

Avtokorelacija $k$-tega reda je korelacija med nizoma podatkov $y_1, y_2, \dots, y_{n-k}$ in $y_{1+k}, y_{2+k}, \dots, y_n$, ki so merjeni v enakih časovnih razmikih. Avtokorelacijski koeficient $k$-tega reda se zato izračuna kot Pearsonov korelacijski koeficient zgornjih nizov. Zaradi zanemarljivih razlik pri velikem številu podatkov se za izračun uporablja enostavnejši obrazec:

$$r_k = \frac{\sum_{t=1}^{n-k}(y_t - \bar{y})(y_{t+k} - \bar{y})}{\sum_{t=1}^{n}(y_t - \bar{y})^2}. \qquad (8)$$

Za neodvisne in normalno porazdeljene nize podatkov so koeficienti $r_k$ približno normalno porazdeljeni s povprečjem $-\frac{1}{n}$ in varianco $\frac{(n-2)^2}{n^2(n-1)}$ (Anderson, 1942; Kendall in Stuart, 1968). Interval zaupanja za $r_1$ se potem izračuna kot

$$-\frac{1}{n} - z_{\alpha/2}\frac{n-2}{n\sqrt{n-1}} \le r_1 \le -\frac{1}{n} + z_{\alpha/2}\frac{n-2}{n\sqrt{n-1}}. \qquad (9)$$

Če je absolutna vrednost $r_1$ večja od $-\frac{1}{n} + z_{\alpha/2}\frac{n-2}{n\sqrt{n-1}}$, potem je avtokorelacijski koeficient prvega reda statistično značilno različen od $0$ pri stopnji značilnosti $\alpha$ (pri $\alpha = 0{,}05$ je $z_{\alpha/2} = 1{,}96$).

Podobno lahko testiramo tudi avtokorelacijska koeficienta 2. in 3. reda (Kendall in Stuart, 1968).

Poleg omenjenega lahko za testiranje značilnosti avtokorelacije 1. reda uporabimo tudi Durbin-Watsonov test (Durbin in Watson, 1950; 1951), ki pa ne da odločitve v vseh primerih. Svetovna meteorološka organizacija (WMO) priporoča za testiranje statistične značilnosti avtokorelacijskega koeficienta prvega reda interval zaupanja (9).

## PRILAGOJENA PARAMETRIČNA TESTA GLEDE NA AVTOKORELACIJO

Ob prisotnosti avtokorelacije v časovni vrsti zgoraj predstavljena parametrična testa za odkrivanje trenda ne dajeta realnih rezultatov. Vpliv pozitivne avtokorelacije 1. reda pri naraščajočem trendu lahko zmanjšamo z uporabo efektivne velikosti vzorca. Efektivna velikost vzorca v tem primeru temelji na predpostavki, da $n$ serialno koreliranih obravnavanj vsebuje isto informacijo kot manjše število $n^*$ nekoreliranih. Bartlett (1935) ter Mitchell in sod. (1966) so za efektivno velikost vzorca uporabili enostaven izračun

$$n^* = n\frac{1-r_1}{1+r_1}. \qquad (10)$$

Zamenjava velikosti vzorca $n$ z $n^*$ v formuli (4) nam da prilagojeno oceno za varianco ostankov $(s_e^2)^*$, s tem dobimo prilagojeno oceno za standardni odklon ocene naklona $s(\hat{b}_1)^*$ in posledično prilagojeno testno statistiko $t_b^*$ iz enačbe (5), ki se sedaj porazdeljuje po Studentovi $t$-porazdelitvi z $n^*-2$ prostostnimi stopnjami.

Podobno lahko ob prisotnosti avtokorelacije 1. reda zamenjamo velikost vzorca $n$ z $n^*$ v testni statistiki $t$ za testiranje Pearsonovega korelacijskega koeficienta iz enačbe (7). Tudi tako dobljena statistika $t^*$ se porazdeljuje po Studentovi $t$-porazdelitvi z $n^*-2$ prostostnimi stopnjami.

## SENOV NAKLON

Senov naklon (v literaturi tudi Theil-Senova cenilka, Kendallova robustna metoda) je neparametrična cenilka, s katero lahko ocenimo koeficient $b$ v linearnem modelu (1) (Theil 1950, Sen 1968). Senov naklon se izračuna kot mediana naklonov

$$\hat{b}_s = Mediana\left\{\frac{y_j - y_i}{j-i} \,\middle|\, 1 \le i < j \le n\right\}. \qquad (11)$$

Koeficient $a$ lahko potem ocenimo kot

$$\hat{a}_s = Mediana\{y_i - \hat{b}_s x_i | i = 1, \dots, n\}. \quad (12)$$

## SPERMANOV KOEFICIENT KORELACIJE RANGOV

Spermanov koeficient korelacije rangov se izračuna po obrazcu

$$\rho = 1 - \frac{6 \sum_{i=1}^{n} d_i^2}{n(n^2 - 1)}, \quad (13)$$

v katerem $d_i$ označuje razlike med rangi istoležnih členov obeh spremenljivk in je $n$ dolžina časovne vrste. V primeru, ko so v podatkih prisotne vezane skupine (vezana skupina je nabor podatkov, ki imajo enake vrednosti), Spearmanov korelacijski koeficient izračunamo kot Personov korelacijski koeficient, le da v (6) namesto merjenih vrednosti za obe spremenljivki vstavimo pripadajoče range.

Ničelno hipotezo, da ni monotonega trenda, testiramo s testno statistiko

$$t = \frac{\rho}{\sqrt{\frac{n - 2}{1 - \rho^2}}}, \quad (14)$$

ki se porazdeljuje po Studentovi $t$-porazdelitvi z $n - 2$ prostostnimi stopnjami. Ker test temelji na rangih, je neodvisen od porazdelitve podatkov, podobno kot Mann-Kendallov test.

## MANN-KENDALLOV TEST

Kendallov korelacijski koeficient $\tau_b$ se za ekvidistančno časovno vrsto izračuna po formuli

$$\tau_b = \frac{S}{\sqrt{(n_0 - n_1) n_0}}, \quad (15)$$

pri čemer je

$$n_0 = \frac{n(n-1)}{2}, \qquad n_1 = \sum_{i=1}^{m} \frac{t_i(t_i - 1)}{2} \quad (16)$$

in

$$S = \sum_{k=1}^{n-1} \sum_{j=k+1}^{n} sgn(y_j - y_k), \quad (17)$$

kjer je $m$ število vezanih skupin. Število enot v $i$-ti skupini je označeno s $t_i$, funkcija *signum* pa je definirana

$$sgn(y_j - y_k) = \begin{cases} 1; & y_j - y_k > 0 \\ 0; & y_j - y_k = 0. \\ -1; & y_j - y_k < 0 \end{cases} \quad (18)$$

Mann-Kendallov test (Mann, 1945; Kendall, 1975) za ugotavljanje monotonega trenda, ki ni občutljiv na osamelce, temelji na testni statistiki $S$. Pozitivna (negativna) vrednost testne statistike $S$ označuje naraščajoč (padajoč) trend. Ob predpostavki, da so ostanki neodvisni, je za $n \geq 8$ statistika $S$ približno normalno porazdeljena s povprečjem $0$ in varianco

$$Var(S) = \frac{1}{18}\left( n(n-1)(2n+5) - \sum_{i=1}^{m} t_i(t_i - 1)(2t_i + 5) \right). \quad (19)$$

Standardizirana testna statistika $z$, ki se porazdeljuje po standardizirani normalni porazdelitvi $N(0,1)$, se izračuna kot

$$z = \begin{cases} \frac{S - 1}{\sqrt{Var(S)}}; & S > 0 \\ 0; & S = 0. \\ \frac{S + 1}{\sqrt{Var(S)}}; & S < 0 \end{cases} \quad (20)$$

Ničelno hipotezo, da trenda ni (korelacijski koeficient je$0$), zavrnemo, če je absolutna vrednost statistike $z$ večja od $z_{\alpha/2}$ .

Prisotnost serialne korelacije (avtokorelacije) poveča možnost za napako prve vrste pri testiranju značilnosti trenda in to neodvisno od velikosti vzorca (von Storch, 1995). To je posledica dejstva, da varianca testne statistike MK-testa narašča z velikostjo serialne korelacije, kar so z Monte Carlo simulacijami pokazali Yue in sod. (2002b).

Da bi odpravili vpliv avtokorelacije, so Hammed in Rao (1998) ter Yue in Wang (2004) predlagali popravke za varianco Mann-Kendallove testne statistike na osnovi efektivne velikosti vzorca. Poleg tega pristopa v literaturi pogosteje zasledimo boljši pristop, to je pred-beljenje.

## PRED-BELJENJE

Kulkarni in von Storch (1995) ter tudi von Storch (1995) sta predlagala postopek imenovan pred-beljenje (v angl. pre-whitening ali krajše PW). Bistvo te metode je, da se odstrani avtokorelacijo 1. reda iz časovne vrste $(y_1, y_2, \dots, y_n)$, kar da transformirano časovno vrsto dolžine $(n-1)$

$$(y_2 - r_1 y_1, y_3 - r_1 y_2, \dots, y_n - r_1 y_{n-1}). \qquad (21)$$

Nato se uporabi MK-test na transformiranih podatkih, ki so sedaj neodvisni. Če avtokorelacija 1. reda ni statistično značilna, potem se MK-test naredi na originalnih podatkih.

Različni avtorji so raziskovali vpliv trenda na oceno avtokorelacije in vpliv avtokorelacije na oceno trenda (Zhang in sod., 2001; Yue in sod., 2002b; Bayazit in Önöz, 2007; Hamed, 2008, 2009), kar je vodilo do novih postopkov pred-beljenja. V literaturi se poleg zgornjega postopka najpogosteje uporablja še TFPW metoda.

PRED-BELJENJE Z ODSTRANITVIJO TRENDA

Metoda pred-beljenje z odstranitvijo trenda (Yue in sod., 2002b), v angl. znana pod imenom trend-free pre-whitening (TFPW), vključuje štiri korake.

1. Trend ocenimo s Senovim naklonom $(\hat{b}_s)$ in ga odstranimo iz podatkov, da dobimo časovno vrsto $(y'_1, y'_2, \dots, y'_n)$, kjer je

$$y_t' = y_t - \hat{b}_s x_t;\ i \in \{1,2,\dots,n\}. \qquad (22)$$

2. Ocenimo avtokorelacijo z avtokorelacijskim koeficientom 1. reda $(r_1)$ na časovni vrsti $(y'_1, y'_2, \dots, y'_n)$ in jo odstranimo, da dobimo časovno vrsto $(y''_2, y''_3, \dots, y''_n)$, kjer je

$$y_t'' = y_t' - r_1 y_{t-1}';\ i \in \{2,3,\dots,n\}. \qquad (23)$$

3. Trend iz 1. koraka spojimo s časovno vrsto $(y''_2, y''_3, \dots, y''_n)$, da dobimo časovno vrsto $(y'''_2, y'''_3, \dots, y'''_n)$, kjer je

$$y_t''' = y_t'' + \hat{b}_s x_t;\ i \in \{2,3,\dots,n\}. \qquad (24)$$

4. Uporabimo MK-test na časovni vrsti $(y'''_2, y'''_3, \dots, y'''_n)$.

Pripomnimo še, da se v primeru, ko avtokorelacija ni statistično značilna, uporabi MK-test na originalni časovni vrsti (isto v primeru PW postopka).

## 3 PRIMER

Opisane metode so uporabljene na primeru povprečnih letnih temperatur zraka za meteorološke postaje Kredarica, Murska Sobota, Novo mesto in Ljubljana za obdobji 1961 – 2010 in 1986 – 2010. Podatki so dostopni na spletni strani ARSO, obdelani so bili s statističnim programskim paketom *R*, rezultati pa so predstavljeni v preglednicah (Preglednica 1 in Preglednica 2). V stolpcu avtokorelacija je ocenjen avtokorelacijski koeficient 1. reda $(r_1)$ po enačbi (8). Nato je izračunan $95\ \%$ interval zaupanja po formuli (9), s črko z je označena značilna avtokorelacija in s črko n neznačilna. V stolpcu parametrični pristop je izračunan trend $(b_l)$ po metodi najmanjših kvadratov (2), testiran je s testno statistiko $t_b$ iz enačbe (5) in testno statistiko $t^*$ opisano v prejšnjem poglavju. Statistična značilnost obeh testov je podana v stolpcih $t\ sig.$ in $t^* sig.$ V stolpcu neparametrični pristop je izračunan Senov naklon $b_s$ po enačbi (11). Sledijo izračuni statističnih značilnosti testnih statistik za Spearmanov korelacijski koeficient $(\rho\ sig.)$ iz enačbe (14), M-K test $(\tau_b sig.)$ iz enačbe (20) in rezultati dobljeni s postopkoma PW $(PW\ sig.)$ ter TFPW $(TFPW\ sig.)$.

V celotnem obdobju 1961 – 2010 (Preglednica 1) je koeficient avtokorelacije $1$. reda najnižji na Kredarici. Vsi avtokorelacijski koeficienti so statistično značilni. Na vseh opazovanih postajah opazimo pozitiven trend, ki je najmanjši na Kredarici, kjer se je temperatura vsakih $10$ let v povprečju dvignila za $0{,}29$°C. Največji trend opazimo v Novem mestu, kjer se je temperatura vsakih 10 let v povprečju dvignila za $0{,}46$°C. Klasični $t$-test nam da močno statistično značilne rezultate $(sig. = 0{,}000)$, medtem ko test, ki upošteva vpliv avtokorelacije, nekoliko zmanjša statistično značilnost $(t^*\ sig.)$. Kljub avtokorelaciji so trendi na vseh opazovanih postajah statistično značilni pri stopnji značilnosti $0{,}05$. Senov naklon $(b_s)$ je primerljiv z naklonom izračunanim po metodi najmanjših kvadratov $(b_l)$. Tako test Spearmanovega korelacijskega koeficienta $(\rho\ sig.)$ kot MK-test $(\tau_b\ sig.)$ ne upoštevata vpliva avtokorelacije in dajeta zato močno statistično značilne rezultate, primerljive s parametričnim pristopom ($t\ sig.$). PW pristop upošteva vpliv avtokorelacije, s tem se zmanjša statistična značilnost. Kljub upoštevanju vpliva avtokorelacije pa so še vedno vsi rezultati statistično značilni pri stopnji značilnosti $0{,}05$, podobno kot smo že opazili pri parametričnemu popravku za avtokorelacijo ($t^*\ sig.$). TFPW pristop tudi upošteva vpliv avtokorelacije, vendar pri tako močnem trendu in velikem številu podatkov, kot je v našem primeru, razlike niso opazne.

**Preglednica 1**: Analiza trenda z upoštevanjem avtokorelacije 1. reda za časovno vrsto povprečnih letnih temperatur s parametričnim in neparametričnim pristopom za 50 letno obdobje 1961 - 2010 v štirih slovenskih meteoroloških postajah.

**Table 1**: Trend analysis with the respect to 1st order autocorrelation of mean annual temperature time series by parametric and nonparametric approach for 50-year period 1961-2010 in four Slovenian meteorological stations.

| | avtokorelacija | | parametrični pristop | | | neparametrični pristop | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $r_1$ | z/n | $b_t$ | $t$ sig. | $t^*$ sig. | $b_s$ | $\rho$ sig. | $\tau_b$ sig. | PW sig . | TFPW sig. |
| Kredarica | 0,331 | z | 0,029 | 0,000 | 0,002 | 0,032 | 0,000 | 0,000 | 0,003 | 0,000 |
| Murska Sobota | 0,576 | z | 0,039 | 0,000 | 0,011 | 0,042 | 0,000 | 0,000 | 0,015 | 0,000 |
| Novo mesto | 0,636 | z | 0,046 | 0,000 | 0,007 | 0,048 | 0,000 | 0,000 | 0,012 | 0,000 |
| Ljubljana | 0,651 | z | 0,044 | 0,000 | 0,013 | 0,046 | 0,000 | 0,000 | 0,024 | 0,000 |

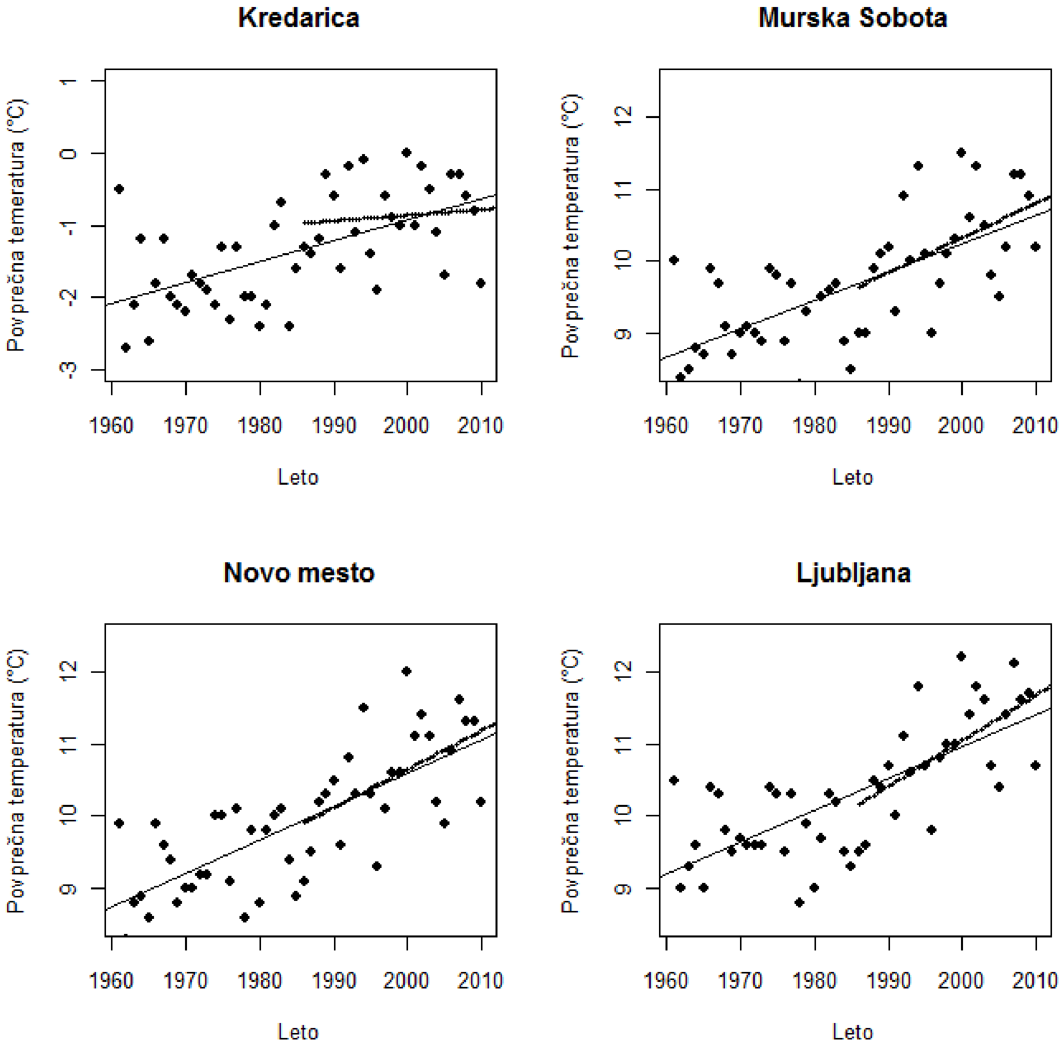
V krajšem časovnem obdobju 1986 – 2010 (Preglednica 2) opazimo, da avtokorelacijski koeficient 1. reda na Kredarici ni statistično značilen (pred-beljenje ni potrebno). Na vseh opazovanih postajah opazimo pozitiven trend, ki na Kredarici ni statistično značilen ($t\ sig = 0{,}632$), na drugih postajah pa je ($t\ sig \leq 0{,}05$). Pri parametričnem testu, ki upošteva vpliv avtokorelacije, je statistična značilnost manjša: mejno statistično značilen trend ($t^*sig \leq 0{,}1$) dobimo le v Novem mestu in Ljubljani. Tako kot pri daljšem časovnem obdobju (Preglednica 1) tudi tu vidimo, da Spearmanov korelacijski koeficient ($\rho\ sig.$) in MK-test ($\tau_b\ sig.$) dajeta primerljive rezultate. V krajšem obdobju pa je bolje vidna razlika med pred-beljenji. Po PW postopku trend ni značilen na nobeni opazovani postaji, pri postopku TFPW pa je statistična značilnost nekoliko manjša kot pri MK-testu.

**Preglednica 2**: Analiza trenda z upoštevanjem avtokorelacije 1. reda za časovno vrsto povprečnih letnih temperatur s parametričnim in neparametričnim pristopom za 25 letno obdobje 1986 - 2010 v štirih slovenskih meteoroloških postajah.

**Table 2**: Trend analysis with the respect to 1st order autocorrelation of mean annual temperature time series by parametric and nonparametric approach for 25-year period 1986-2010 in four Slovenian meteorological stations.

| | avtokorelacija | | parametrični pristop | | | neparametrični pristop | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $r_1$ | z/n | $b_t$ | $t$ sig. | $t^*$ sig. | $b_s$ | $\rho$ sig. | $\tau_b$ sig. | PW sig . | TFPW sig. |
| Kredarica | -0,102 | n | 0,008 | 0,632 | 0,592 | 0,012 | 0,561 | 0,623 | 0,728 | 0,747 |
| Murska Sobota | 0,348 | z | 0,048 | 0,017 | 0,119 | 0,050 | 0,017 | 0,020 | 0,385 | 0,045 |
| Novo mesto | 0,381 | z | 0,053 | 0,008 | 0,096 | 0,059 | 0,016 | 0,013 | 0,333 | 0,045 |
| Ljubljana | 0,431 | z | 0,063 | 0,001 | 0,058 | 0,067 | 0,002 | 0,002 | 0,244 | 0,007 |

Pri primerjavi daljše in krajše časovne vrste opazimo, da je naklon linearnega trenda v zadnjih 25 letih, z izjemo Kredarice, večji kot v celotnem obdobju, kar vidimo tudi iz slike (Slika 1). To je posledica globalnega segrevanja ozračja. Kljub temu pa je statistična značilnost trenda v zadnjih 25 letih manjša, kar je posledica krajšega časovnega obdobja.



**Slika 1**: Linerni trendi za štiri meteorološke postaje v obdobjih 1961 - 2010 in 1986 – 2010.
**Fig. 1**: Linear trends of four meteorological stations in periods 1961 - 2010 and 1986 – 2010

## 4 ZAKLJUČEK

Pri analizi trenda hidrometeoroloških časovnih vrst je najpogosteje uporabljen MK-test. Vpliv avtokorelacije na MK-test je še vedno aktualna tematika. Vrsta popravkov in postopkov pred-beljenja, ki naj bi odpravila vpliv avtokorelacije, daje zelo različne rezultate. Hamed (2009) je s pomočjo simulacij podrobno raziskal, v katerih primerih daje pred-beljenje PW dobre rezultate. Glede na to, da človek vse bolj posega v naravno okolje, in so ekstremni vremenski pojavi vse pogostejši, je razvoj opisanih metod, odkrivanje novih in njihova uporaba izrednega pomena.

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**Agris category code**: H10

# Možnosti biotičnega zatiranja paradižnikovega molja (*Tuta absoluta* Povolny, Lepidoptera, Gelechiidae)

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## IZVLEČEK

Paradižnikov molj (*Tuta absoluta*) velja v svetu za enega najpomembnejših škodljivcev paradižnika, saj se hitro širi s sadikami in predvsem z embalažo. Škodljivec lahko napada vse nadzemske dele rastlin, njegove ličinke težko dosežejo kontaktni insekticidi, ima prilagodljiv razvojni krog ter širok spekter gostiteljev. Zaradi teh lastnosti strokovnjaki menijo, da ga je najustrezneje zatirati z naravnimi sovražniki, saj parazitoidi ali plenilci sami najdejo njihove gostitelje in se razvijajo skupaj z njimi. Številne raziskave kažejo, da je samostojna uporaba insekticidov premalo učinkovita, zato preučujejo različne kombinacije biotičnih pripravkov, biotičnih pripravkov in insekticidov, možnosti zaščite in sanacije zavarovanih prostorov, načrtnega spremljanja in masovnega lovljenja škodljivca. V Evropi v zadnjih letih intenzivno iščejo domorodne naravne sovražnike, ki bi se lahko dovolj hitro prilagodili novemu gostitelju, da bi jih bilo mogoče uporabiti v biotičnem varstvu proti paradižnikovemu molju. Enak cilj smo si zastavili tudi v Sloveniji, čeprav pri nas molj za zdaj še ne povzroča gospodarsko pomembnih poškodb na paradižniku.

**Ključne besede:** biotično varstvo, naravni sovražniki, paradižnikov molj

## ABSTRACT

### BIOLOGICAL CONTROL OF TOMATO LEAF MINER (*Tuta absoluta* Povolny; Lepidoptera, Gelechiidae)

Tomato leaf miner (*Tuta absoluta*) is a major pest on tomatoes worldwide. It can spread very easily with plantings and contaminated packaging, it can attack all aboveground parts of tomato, it is hidden from contact insecticides and has adaptive life-cycle with several secondary host plants. Because of these advantages of the pest, biological control is considered as one of the most efficient methods to control the pest, because natural enemies are active in finding host and they develop with it. According to the researches single products are not as efficient as combinations of biological control agents, biological agents and chemical insecticides, and appropriate phytosanitary measures in greenhouses, monitoring with sex pheromones and mass trapping, if necessary. In Europe, we are looking for indigenous natural enemies that could adapt to the new host and could be therefore used in biological control of tomato leaf miner. We have the same goal in Slovenia, though the damage of *T. absoluta* still does not have economic impact.

**Key words:** biological control, natural enemies, tomato leaf miner

## 1 UVOD

Paradižnikov molj (*Tuta absoluta* Povolny, Lepidoptera, Gelechiidae) je oligofagna žuželka, ki izvira iz Južne Amerike, kjer se ta žuželčja vrsta pojavlja na večini območij, kjer pridelujejo paradižnik (*Lycopersicum esculentum* Mill.). Na takšnih območjih predstavlja enega od najpomembnejših škodljivcev paradižnika na prostem ter v zavarovanih prostorih. Poleg paradižnika so lahko njegovi gostitelji številne druge rastlinske vrste iz družine razhudnikovk (Solanaceae), kot so krompir (*Solanum tuberosum* L.), jajčevec (*Solanum melongena* L.), tobak (*Nicotiana tabacum* L.) in tudi okrasne ter samonikle razhudnikovke (na primer pasje zelišče

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[*Solanum nigrum* L.]). V Evropi so tega škodljivca prvič odkrili na vzhodu Španije leta 2006, nato pa se je v nekaj letih močno razširil, saj so ga do leta 2010 našli že po celem Sredozemlju, na severu Afrike, v Turčiji, pa tudi v severnejših območjih Švice, v Nemčiji, na Nizozemskem, v Veliki Britaniji in številnih drugih državah (Potting, 2010; Varios, 2010). V Sloveniji se je paradižnikov molj prvič pojavil leta 2009, do konca leta 2010 pa se je pojavil že na 55 lokacijah, kjer pa niso zaznali škode (FURS, 2011).

Odrasli metulji paradižnikovega molja merijo v dolžino le 6-7 mm in so sivorjave barve. Samice odlagajo približno 0,4 mm velika jajčeca belo kremaste barve posamično, večinoma na zgornjo ali spodnjo stran lista, lahko pa tudi ob listne žile ali na še zelene plodove. Ena samica lahko odloži tudi do 260 jajčec (Uchoa-Fernandes *et al*., 1995; cit po Desneux *et al*., 2010). Ličinke imajo 4 stopnje, ki se jasno razlikujejo po velikosti in barvi. Po ekloziji se ličinke zavrtajo pod povrhnjico lista in se hranijo z listno sredico ter za seboj puščajo značilne rove (Slika 1). Ličinke delajo rove tudi v steblu paradižnika in s tem povzročajo venenje rastlin ali le vrhnjih delov poganjkov. Ličinka paradižnikovega molja se najraje hrani z listi, lahko pa napada tudi kalčke, cvetove in zelene plodove gostiteljskih rastlin. Ličinka se zavrta tudi v zelene plodove paradižnika in s tem povzroča deformacije plodov, omogoča pa tudi okužbe z glivami in bakterijami, zaradi katerih plodovi gnijejo. Zadnja stopnja ličinke se lahko zabubi v tleh ali pa tudi na listih (Estay, 2000 in Vargas, 1970; cit. po Delbene, 2006). V Latinski Ameriki in Španiji ima lahko paradižnikov molj od 10-12 rodov letno, najhitrejši razvoj pa doseže pri temperaturah nad 25 °C (Desneux *et al*., 2010; Varios, 2010). Tedaj lahko povzroča ogromno škodo predvsem v rastlinjakih, lahko pa tudi na prostem, na različnih pridelovalnih območjih in v različnih pridelovalnih sistemih.

**Slika 1**: Značilni rov paradižnikovega molja z galerijami (sredina lista) in poškodbe listnih zavrtalk iz družine Agromyzidae (ob robu lista) (foto: F.A. Celar).

V Argentini so za zatiranje paradižnikovega molja od začetka pojava uporabljali insekticide, sprva predvsem organske sulfate, katere so nato nadomestili piretroidi in drugi insekticidi, vendar je konec devetdesetih let prejšnjega stoletja škodljivec že pridobil rezistenco na večino le-teh (Lietti *et al*., 2005). Biotično varstvo pred paradižnikovim moljem se nanaša predvsem na plenilce in parazitoide različnih razvojnih stadijev molja, pa tudi na druge entomopatogene oziroma parazitske organizme. Čeprav imajo lahko naravni sovražniki pri integriranem varstva paradižnika pred paradižnikovim moljem pomembno vlogo, pa sodeč po raziskavah, sami niso zmožni ohraniti ali zmanjšati populacije tega škodljivca pod pragom gospodarske škode, ki je opredeljen kot 100 ujetih samcev na feromonsko vabo/dan, 2 samici na rastlino, 26 ličink na rastlino ali pa kot 8 % zmanjšanje listne površine (Monserrat Delgado, 2009; Desneux *et al*., 2010). Zato so izredno pomembni tudi higienski in agrotehnični ukrepi za preprečevanje vnosa in zmanjšanje populacij molja (masovno lovljenje, feromonske vabe, idr.).

V Evropi se raziskave naravnih sovražnikov paradižnikovega molja nanašajo predvsem na pojavljanje in prilagodljivost domorodnih plenilcev in parazitoidov. Predvsem gre za koristne organizme, ki kot plen/gostitelja uporabijo jajčeca ali ličinke (gosenice) molja.

## 2 PREGLED MOŽNOSTI BIOTIČNEGA ZATIRANJA PARADIŽNIKOVEGA MOLJA

### 2.1 Parazitoidi in plenilci jajčec

Med najpomembnejše naravne sovražnike paradižnikovega molja uvrščamo parazitoide jajčec iz družine Trichogrammatidae (Chalcidoidea, Hymenoptera). V Sredozemlju je najbolj obetajoč parazitoid za biotično varstvo pred tem škodljivcem vrsta *Trichogramma achaeae* Nagaraja & Nagarkatti, medtem ko je v Latinski Ameriki najštevilčnejša in učinkovita vrsta *Trichogramma pretiosum* Riley, ki jo tržijo tudi kot biotični agens za varstvo pred različnimi škodljivimi vrstami metuljev. Parazitoidi iz rodu *Trichogramma* so izredno drobne osice (merijo od 0,1-0,5 mm), ki svoja jajčeca odložijo v jajčeca metuljev. So torej endoparaziti jajčec, ki svoj življenjski krog zaključijo v jajčecu gostitelja, katerega ubijejo preden se zabubijo. Iz parazitiranega jajčeca izleti odrasla osica. Navadno imajo vrste iz rodu *Trichogramma* širok spekter gostiteljev, vendar le v redu metuljev. V biotičnem varstvu jih pogosto uporabljajo tudi zaradi lahkega in poceni namnoževanja, saj gostitelja ubijejo že v razvojnem stadiju jajčeca. Posledično ne pride do pojava poškodb na rastlinah, pripravki so dokaj ugodni v primerjavi s kemičnimi pripravki in veliko vrst je navadno tudi domorodnih (Perez in Cadapan, 1986). Poleg družine Trichogrammatidae so v Latinski Ameriki znani tudi parazitoidi jajčec iz družin Encyrtidae in Eupelmidae (Desneux *et al*., 2010), Luna *et al*. (2007) pa poleg vrste *T. pretiosum*, omenjajo tudi vrste *T. fasciatum* (Perkins), *T. rojasi* Nagaraja and Nagarkatti, in *Trichogramatoidea bactrae* Nagaraja.

V Evropi so komercialno dostopne tri vrste jajčnih parazitoidov iz družine Trichogrammatidae, in sicer *Trichogramma brassicae* Bezdenko, *T. achaeae* in *T. evanescens* Westwood za biotično zatiranje škodljivih metuljev. Za najbolj perspektivno evropsko vrsto velja *T. achaeae* v kombinaciji s plenilcem *Nesidiocoris tenuis* Reuter, kjer proizvajalci zagotavljajo več kot 95 % uspešnost zatiranja jajčec paradižnikovega molja 62 dni po izpustu naravnih sovražnikov v zavarovanih prostorih in brez poškodb na plodovih. V istem poskusu je bilo ob samostojnem izpustu plenilca *N. tenuis* uničenih le 33 % jajčec molja in poškodovanih kar 19 % plodov. Kljub dobrim rezultatom pa je vseeno potrebno preventivno čiščenje zavarovanih prostorov in tal, načrtno spremljanje (monitoring) škodljivca in hitro ukrepanje ob prvem pojavu v kombinaciji z izpustom plenilskih stenic iz družine Miridae (*N. tenuis*, *Macrolophus caliginosus* Wagner). Tako je mogoče insekticide uporabiti le ob močnih prerazmnožitvah škodljivca ali ob prepoznem interveniranju z biotičnimi pripravki (Vivan *et al.,* 2002; Kabiri in Vila, 2009; Urbaneja *et al*., 2009).

V primerjavi s parazitoidi paradižnikovega molja, je raziskav o plenilcih tega škodljivca zelo malo, čeprav je ocenjena smrtnost ličink molja zaradi napada plenilcev skoraj 80 %, smrtnost jajčec pa do 5 % (Miranda *et al*., 1998; cit. po Desneux *et al*., 2010). Največ plenilcev jajčec paradižnikovega molja so našli v Braziliji, in sicer 3 vrste polonic (Coccinellidae), eno vrsto strigalic (Labiduridae), 4 vrste plenilskih stenic (Anthocoridae in Geocoridae) in 3 vrste plenilskih resarjev (Aeolothripidae, Phlaeothripidae in Thripidae). Med plenilci jajčec je v Španiji najbolj uspešna vrsta *N. tenuis*, katere bližnji sorodnik je tudi vrsta *M. caliginosus*, ki jo pogosto najdemo tudi pri nas (Škerbot *et al*., 2011).

### 2.2 Parazitoidi in plenilci ličink in drugih razvojnih stadijev

V Južni Ameriki je znanih več kot 20 vrst primarnih parazitoidov paradižnikovega molja in veliko število vrst sekundarnih parazitoidov oziroma hiperparazitoidov. Med njih uvrščamo endoparazitoide zgodnjih larvalnih stopenj (nekatere vrste iz rodu *Apanteles*) in druge endoparazitoide ličink (*Agathis* sp., *Bracon lucileae* Marsh, *Bracon* spp., *Earinus* sp., *Diadegma* sp., *Horismentus* sp., *Orgilus* sp., *Pseudapanteles dignus* (Muesebeck) in *Temelucha* sp.), ektoparazitoide ličink (*Cirrospilus* sp., *Neochrysocharis formosa* (Westwood), *Dineulophus phtorimaeae* De Santis in *Parasierola nigrifemur* (Ashmead), jajčno-larvalne parazitoide (*Chelonus* sp. in *Copidosoma* sp.) ter parazitoide ličink in bub (*Campoplex haywardii* Blanchard). Nekateri omenjeni rodovi iz družin Braconidae, Eulophidae in Ichneumonide so znani tudi v Evropi in tako predstavljajo potencialno zanimive organizme za prilagoditev na novega škodljivca. Predvsem vrsti *Pseudoapanteles dignus* (Braconidae) in *Dineulophus phthorimaeae* (Eulophidae) veljata za najbolj obetavna parazitoida ličink paradižnikovega molja v Argentini, saj sta zelo pogosti vrsti in povzročata parazitiranost tudi do 70 % (Luna, 2007; Desneux, 2010). Loni *et al*. (2011) poročajo tudi o 20 % uspešnosti parazitiranja ličink paradižnikovega molja s parazitoidom *Agathis fuscipennis* Zetterstedt, ki je domorodna evropska vrsta. Pri parazitoidih ličink je pomembno, da samica parazitoida lahko zazna gostitelja pri različni gostoti populacije le-tega, da lahko dnevno najde zadostno število gostiteljev in odloži jajčeca, da so za razvoj ličinke parazitoida ustrezni različni razvojni stadiji gostitelja ter da je njun razvoj bolj ali manj sinhron (Luna, 2007). Najdenih je bilo tudi nekaj vrst parazitoidov bub, katerih stopnja parazitizma Polack (2007, cit. po Desneux, 2010) ocenjuje na prek 30 %. Do sedaj še ni znanih podatkov o parazitoidih odraslih moljev.

Plenilci so lahko veliko bolj prilagodljivi glede na razvojno stopnjo plena kot parazitoidi, pa tudi njihov rang gostiteljev je veliko širši. Tako lahko številne polonice, plenilske stenice, plenilski resarji ter plenilske pršice napadajo svoj plen bodisi v razvojnih stadijih jajčeca, ličinke, bube ali celo odraslega osebka, odvisno od razpoložljivosti plena in razmerja v velikosti škodljivca in njegovega naravnega sovražnika. Največ plenilcev pleni paradižnikovega molja v razvojnem stadiju ličinke. Sem sodijo nekatere vrste pršic in pajkov, med žuželkami pa kar pet rodov brzcev (Carabidae), ena vrsta polonice (Coccinellidae), 8 rodov stenic, številne vrste kožekrilcev iz družine mravelj in pravih os (Formicidae in Vespidae) ter dva rodova tenčičaric (Chrysopidae). Nekateri našteti plenilci se lahko hranijo tudi z bubami in odraslimi osebki paradižnikovega molja (Desneux, 2010). Tudi med plenilci lahko najdemo rodove, ki so zastopani tudi v Evropi.

### 2.3 Entomopatogene glive, bakterije in entomopatogene ogorčice

Čeprav predstavljajo entomopatogene glive in entomopatogene ogorčice pomemben člen v biotičnem varstvu rastlin, pa so doslej le redki avtorji (na primer Batalla-Carrera *et al.,* 2010; Laurici *et al.*, 2010), te organizme uvrstili med učinkovite v varstvu rastlin pred paradižnikovim moljem; najverjetneje ravno zaradi posebne bionomije škodljivca, saj se ta hrani v rastlinah. Delbene Dejeas (2006) je ugotovil, da entomopatogeni glivi *Beauveria* sp. in *Metarhizium* sp. nista učinkoviti za varstvo pred paradižnikovim moljem, vendar so Laurici s sod. (2010) v poskusu z obema rodovoma gliv dokazali, da so nekateri izolati gliv dovolj patogeni za zatiranje molja, poleg tega pa so kompatibilni tudi z nekaterimi insekticidi.

Sodeč po raziskavah drugih avtorjev, je najbolj učinkovit entomopatogen je bakterija *Bacillus thuringiensis* var. *kurstaki*, katere delovanje na omenjenega škodljivca so preizkušali v Braziliji (Giustolin *et al*., 2001; Desneux, 2010) in Španiji (Gonzales-Cabrera *et al.,* 2011; Molla *et al*., 2011). Ugotovili so, da je bil na tretiranih rastlinah obseg poškodb zaradi paradižnikovega molja zmanjšan do 90 % in da so bili za bakterijo dovzetne vse larvalne stopnje molja, najbolj občutljive pa so bile ličinke prve stopnje. Pripravek z bakterijo *B. thuringiensis* var. *kurstaki* bi se lahko uporabljal tudi v kombinaciji z nekaterimi parazitoidi in plenilci paradižnikovega molja, kot je denimo plenilska stenica *N. tenuis*, na katero bakterija nima neposrednega negativnega učinka, vendar pa lahko do negativnih interakcij med organizmoma pride zaradi dodatkov v pripravkih z bakterijo (Molla *et al*., 2011).

## 3 ZAKLJUČKI

Paradižnikov molj lahko napada vse nadzemske dele paradižnika v vseh razvojnih stadijih. Poškodbe povzročajo gosenice molja, ki se hranijo z rastlinskim tkivom ter za seboj puščajo rove, s čimer vplivajo na zmanjšanje fotosintetske aktivnosti rastlin ter omogočajo vstop sekundarnim patogenom. Če so poškodbe povzročene neposredno na plodovih, le-ti niso več tržni oziroma so uporabni le za predelavo. Paradižnikov molj je najpomembnejši škodljivec paradižnika v Južni Ameriki ter tudi v Španiji, kjer poročajo kar o 50-100 % izpadu pridelka (EPPO, 2008).

Škodljivec ima tudi lastnosti, ki otežujejo uspešno kemično varstvo pred njim; ima zelo kratek razvojni krog, zabubi se lahko na rastlinah ali v tleh, ličinke pa so skrite v rastlinah in jih tako kontaktni insekticidi ne dosežejo (Potting, 2010). Kljub temu je obsežen pojav paradižnikovega molja močno povečal uporabo insekticidov, kar lahko izzove številne neželene stranske učinke. Nadaljnja intenzivna uporaba insekticidov lahko vodi do razvoja odpornosti paradižnikovega molja (Desneux, 2010).

Zadovoljiva učinkovitost komercialno dostopnih evropskih biotičnih pripravkov proti paradižnikovemu molju še ni dokazana. Zakrito vrtanje rovov po listih, steblih in plodovih otežuje ali zmanjšuje učinkovitost nekaterih biotičnih pripravkov, ki potrebujejo neposreden stik z gostiteljem (entomopatogene glive in entomopatogene ogorčice). Vprašljiva je tudi učinkovitost dostopnih biotičnih pripravkov na podlagi parazitoida *T. brassicae* oziroma ustreznost gostitelja (Potting, 2010).

Zaradi posebne bionomije, širjenja škodljivca na nova območja in nove gostiteljske rastline iz družine razhudnikovk in tudi metuljnic, bi bili za njegovo zatiranje ustrezni načini izbira odpornih/tolerantnih kultivarjev, biotehniške metode, in ne nazadnje tudi biotično ter kemično varstvo. Najprej je potrebno preprečiti vstop oziroma vnos škodljivca v zavarovane prostore, izogibati se moramo bližnje pridelave alternativnih gostiteljev (paprika, krompir, tobak, kristavec,…) in odstranjevati napadene dele rastlin ter jih uničiti. Potreben je tudi natančno spremljanje škodljivca s feromonskimi vabami in takojšnje posredovanje z vnosom ali ohranjanjem naravnih sovražnikov, po potrebi v kombinaciji z drugimi biotičnimi agensi ali kemičnimi sredstvi, če se škodljivec prerazmnoži (Desneux, 2010). Vnos tujerodnih biotičnih agensov lahko predstavlja tveganje za okolje (van Lenteren *et al.,* 2006), zato si prizadevamo, da bi odkrili domorodne naravne sovražnike paradižnikovega molja in jih uspešno uporabili pri ohranjanju populacije škodljivca pod pragom gospodarske škode.

V Latinski Ameriki že vrsto let za omejevanje škode zaradi paradižnikovega molja uporabljajo biotične pripravke, in sicer v Braziliji so na 2.600 ha njiv vnesli jajčnega parazitoida *T. pretiosum*, prav tako pa so v Čilu v zavarovanih prostorih uporabili vrsto *T. nerudai* Pintereau & Gerding (van Lenteren in Bueno, 2003).

Med biotičnimi agensi, ki so domorodni v Sloveniji (Milevoj, 2011) je bilo doslej v tuji raziskavi (Batalla-Carrera *et al.,* 2010) dokazano zadovoljivo delovanje entomopatogenih ogorčic (Laznik, 2011), zato predstavlja prav ta skupina naravnih sovražnikov eno od možnosti zatiranja paradižnikovega molja. Pri nas pa bi bilo smiselno na tega škodljivca podrobneje preučiti tudi delovanje entomopatogene bakterije *B. thuringiensis* var. *kurstaki* (nahaja se v komercialnem pripravku Delfin), saj je možno, da vsi soji te vrste ne vplivajo enako na smrtnost paradižnikovega molja. Potrebno je omeniti, da so v vseh raziskavah v Latinski Ameriki uporabili lokalne rase bakterije *B. thuringiensis* var. *kurstaki* (Gonzales-Cabrera *et al.,* 2011). Vendar pa velja uporaba entomopatogene bakterije še vedno za eno od najučinkovitejših metod zatiranja paradižnikovega molja in bi jo bilo ustrezno preizkusiti tudi v naših razmerah, ko se bo številčnost škodljivca približala pragu gospodarske škode.

## 4 ZAHVALA

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# LINGRA: model za simulacijo rasti in pridelka travne ruše

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## IZVLEČEK

Razvoj in uporaba agro-meteoroloških modelov sta pomembna za raziskave razvoja travne ruše in tudi za napovedovanje produktivnosti travne ruše ob različnih podnebnih danostih. V delu je predstavljen dinamičen model LINGRA, ki simulira rast travinja na osnovi procesov, ki se dogajajo v rastlini in upošteva, kako na te procese vplivajo okoljske razmere. Podane so glavne enačbe v pod-modelih LINGRA s katerimi računamo prestrezanje svetlobe, učinkovitost izrabe svetlobe, aktivnost vira in ponora, stopnjo razraščanja poganjkov, stopnjo pojavljanja listov, količino vode v tleh in evaporacijo. Model je dokaj enostaven za uporabo in če ga dobro umerimo, je uporaben za različne simulacije vpliva vremenskih in podnebnih razmer na pridelek trave, kar je uporabno pri sprejemanju raznih odločitev ali pri oceni tveganja rastlinske pridelave.

**Ključne besede**: trava, simulacija, model, agrometeorologija, vreme

## ABSTRACT

### LINGRA: GRASS GROWTH AND YIELD SIMULATION MODEL

Grass growth models are valuable tool for grassland management for a decision support application. The grassland growth model LINGRA, which was developed to predict growth and development of perennial rye grass across Europe is presented and some of the main equations are described in this work. Key process simulated in the model are light utilisation, leaf formatin and elongation, tillering and carbon partitioning. Integration time step is one day. LINGRA provides algorithms for the simulatin of grass growth under irrigated and non-irrigated conditins. Model is relatively simple, but it contains the most imprtant features of grass growth. Validation of the model from independent historical European data showed that model predictions are sufficiently accurate to make it a useful aid for on-farm decision-making processes.

**Key words**: Grass, Simulation; modelling; sink-surce, grassland, weather

## 1 UVOD

Po podatkih Statističnega urada RS (SURS, 2010) so leta 2007 v Sloveniji 55 % vse kmetijske zemlje predstavljali trajni travniki in pašniki. Dolgo časa se je naravno travinje večinoma obravnavalo kot omejujoči dejavnik pri razvoju bolj učinkovitih sistemov za vzrejo živine. Danes pa v njem prepoznavamo mnoge koristi za okolje in družbo (Gibon, 2005; Duru in sod., 2009) Travništvo igra po celem svetu glavno vlogo pri trajnostnem razvoju kmetijstva. Produktivnost, trajnost in hranljiva vrednost so pomembne za produkcijo hrane, varovanje tal, ohranjanje naravnega okolja in shranjevanje ogljika. Z vseh vidikov potrebujemo stalne raziskave za razvoj kmetijstva v Sloveniji (Čop in sod., 2009). Boljše razumevanje procesov prilagajanja podnebnim spremembam, kmetijske produkcije, ekologije in uporabe naravnih virov pa bo prispevalo k razvoju primernejših travniških sistemov tako za produkcijo kot tudi za varovanje narave in okolja (Mannetje, 2002).

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V Sloveniji je na področju pridelka travne ruše in vremenskih vplivov sicer že bilo opravljenih mnogo poskusov in obravnav, a predvsem glede učinkov gnojenja (Leskošek, 1998) in števila košenj (Knapič, 1988; Žitek, 1991; Weiss, 1995). Zaenkrat pa ni v uporabi nobenega simulacijskega modela, ki bi služil za spremljanje in napovedovanje komponent rasti ter pridelka travne ruše. Modeli nam pomagajo razumeti kompleksne interakcije med rastjo, hranilno vrednostjo in okoljskimi komponentami (Bonesmo in Belanger, 2002; Kajfež, 2005). Zato v nadaljevanju predstavljamo nizozemski model LINGRA (LINtul GRAssland), ki ga bomo v nadaljnjih raziskavah umerili za slovenske razmere ter uporabili za modeliranje vpliva vremena in podnebja na rast in razvoj travne ruše.

## 2 MODELIRANJE IN RAZVOJ MODELA LINGRA

Razvoj in uporaba modelov sta se pokazala kot velika pomoč pri raziskovanju razvoja travne ruše, na primer pri načrtovanju eksperimentov, testiranju hipotez in odpiranju novih vprašanj (Wolf in van Ittersum, 2009). V tujini sta v zadnjem desetletju razvoj in uporaba modelov za simulacijo rasti travne ruše v okviru spremljanja dejanskega stanja, analiziranja dosedanjih sprememb ter pripravljanja scenarijev za prihodnost postala zelo aktualna. Z modeli za simulacijo rasti opisujemo odvisnost pridelka od okoljskih dejavnikov, kot so sončno obsevanje, temperatura zraka, dostopnost vode in hranil. Zaradi neoptimalnih razmer za rast so modeli lahko precej netočni, zato je še posebej pomembna kalibracija v aktualnih razmerah. V uporabi je več različnih modelov, kot so Hurley Pasture Model, PaSim, LINGRA. Slednji se dobro odziva tako na košnjo kot tudi na pašo. (Dorigo, 2003). LINGRA (Schapendonk in sod., 1998; Rodriguez in sod., 1999; Wolf, 2006) zagotavlja dobro osnovo za napovedovanje produktivnosti travne ruše v zmernih podnebnih pasovih. Ker model delno temelji na splošnih fizioloških principih, ga je mogoče uporabljati v različnih okoljih, vendar pa je pri tem potrebna kalibracija (Shapendonk in sod., 1998).

Mnoge analize preteklih let so v tujini pokazale, da je produktivnost travnikov močno odvisna od kombinacije dostopne vode v tleh, globalnega obsevanja, temperature zraka, dodanega dušika in načina rabe (Trnka in sod., 2006; Riedo in sod., 1997). Pridelki travinja se na medletni skali močno razlikujejo, celo pri standardnih pogojih rabe (Schapendonk in sod., 1998; Trnka in sod., 2005). Medletna variabilnost pridelka travne ruše je na primer v sosednji Avstriji okoli 10-20 %, v posameznih letih (kot je bilo 2003) pa so lahko odstopanja še večja (Schaumberger in sod., 2007). Kot se je izkazalo, je eden glavnih razlogov za upad pridelka suša. Pridelek pomembno določata količina padavin v času od aprila do septembra in količina dostopne vode v tleh. Izrazit je vpliv suše na fenologijo, a njen učinek ni enak v vseh razvojnih fazah (JRC, 2008). Pri mnogih vrstah travinja se lahko kaže pomembno zaostajanje cvetenja zaradi težav s sušnim stresom (Donatelli et al., 1992). Dolžina vegetacijskega obdobja in količina prestreženega obsevanja pa vplivata na produkcijo biomase (Wolf, 2006). Nekatere raziskave segajo tudi v drugo smer in opisujejo učinek nizkih temperatur in snega. Pri tem moramo učinek izredno nizkih temperatur zraka kombinirati s snežno odejo, ki predstavlja izolacijo, in postopnim pripravljanjem rastlin na mraz. Zato je končni učinek zelo težko oceniti (JRC, 2008).

Poleg analiziranja preteklega obdobja se vedno bolj uveljavlja napovedovanje pridelka za tekočo sezono ter simuliranje pridelkov po scenarijih podnebnih sprememb, tudi z uporabo vremenskih simulatorjev. Napovedi na začetku sezone so zelo nezanesljive, vendar pa v kasnejših dneh pridobijo na zanesljivosti. Pri tem raje govorimo o tednu, desetih dneh, kot pa mesecih (Eitzinger in sod., 2007).

Precej študij je bilo v tujini narejenih z modelom LINGRA. Pri primerjalni analizi štirih modelov za simulacijo rasti travinj na dveh lokacijah v Veliki Britaniji in na Irskem na 28-letnem nizu podatkov se je izkazal kot najbolj primeren z vidika prilagodljivosti (Barrett, 2004). Že z enostavnimi in sorazmerno manjšimi spremembami nastavitev namreč lahko dosežemo veliko boljše ujemanje izmerjenih in modeliranih vrednosti. Prav tako je dokaj nezahteven z vidika vhodnih podatkov (Bonesmo in Belanger, 2002). Mnogo je napisanega tudi o oceni zahtevanih parametrov (npr. največja specifična listna površina, optimalna in bazna temperatura, kritični LAI ...) in kalibracijskih območjih (van Oijen in sod., 2005; Lazar in Genovese, 2004; Bonesmo in Belanger, 2002) ter testiranju in validaciji modela (Duru in sod., 2009; Trnka in sod., 2006).

Evropska komisija se na področju kmetijstva naslanja na Sistem za spremljanje rasti poljščin CGMS (Crop Growth Monitoring System), ki ji med drugim zagotavlja objektivno kvantitativno napoved pridelka na regionalni in nacionalni skali. Jedro sistema predstavljata deterministična modela WOFOST (Ceglar, 2011) za poljščine in LINGRA za trave (Gallego in sod., 2007). Za leto 2009 so na ta način določili, da je bila vegetacijska sezona travne ruše karakterizirana s sušnimi pogoji v zimskem času, kar je povzročilo

zgodnjo izčrpanost biomase pašnikov v Južni Evropi in zamujanje prve košnje v večini Evrope (JRC, 2009).
ALTERRA (raziskovalni inštitut Univerze Wageningen na Nizozemskem) je v sodelovanju z raziskovalnim centrom Plant Research International (PRI, prav tako Wageningen) za raziskovalni center Evropske Komisije JRC (Ispra) izbrala WOFOST – model za simulacijo rasti poljščin, ki v kombinaciji z GIS-om in rutino za napoved pridelka tvori CGMS (Crop Growth Monitoring System) (Pogačar in Kajfež-Bogataj, 2009a). Iz modela WOFOST pa izhaja tudi model LINGRA (Bouman in sod., 1996). Celotno skupino modelov so razvili na bivšem centru DLO_Winand STARing Centre (SCDLO) v sodelovanju z bivšim raziskovalnim Inštitutom za agrobiologijo in gnojenje tal (ABDLO), ki sta sedaj oba del ALTERRE. Ime modela LINGRA izvira iz besedne zveze LINTUL GRAssland, saj je bil osnova za razvoj simulator LINTUL (Light INTerception and UtiLisation simulator). Namenjen je bil modeliranju rasti in razvoja trpežne ljulke (*Lolium Perenne*) v državah članicah EU na nivoju potencialne in z vodo omejene produkcije (Lazar in Genovese, 2004), a so jo uporabljali tudi za študije vplivov podnebnih sprememb na rast travne ruše (Rodriguez in sod., 1999). Kasneje je bila prilagojena tudi za simulacije rasti travniškega mačjega repa (*Phleum pratense*). Implementirana je v FORTRAN-ovi kodi. Model LINGRA je kasneje nadgradil Barrett s sodelavci (2005) za potrebe pridelave krme za živinrejo v inačico modela GrazeGro.

Vsi ti modeli sledijo hierarhični razliki med potencialno in omejeno produkcijo in si delijo podobne podmodele za rast poljščin z intercepcijo svetlobe in asimilacijo $CO_2$ kot gonilnima procesoma ter fenološkim razvojem kot procesom, ki kontrolira rast. Precej pa se razlikujejo podmodeli, ki opisujejo vodno bilanco tal in privzem hranil iz tal – tako v pristopu, kot tudi v stopnji natančnosti. Model je posebej primeren za določanje kombiniranega vpliva sprememb $CO_2$, temperature, padavin in sončnega obsevanja na razvoj in rast poljščin ter porabo vode, saj vse pomembne procese simulira ločeno, a upošteva njihovo medsebojno interakcijo (Pogačar in Kajfež-Bogataj, 2009a). Nekatere omejitve modela pa so, da predvideva optimalno količino hranil v tleh in ne upošteva bolezni, škodljivcev ter plevela.

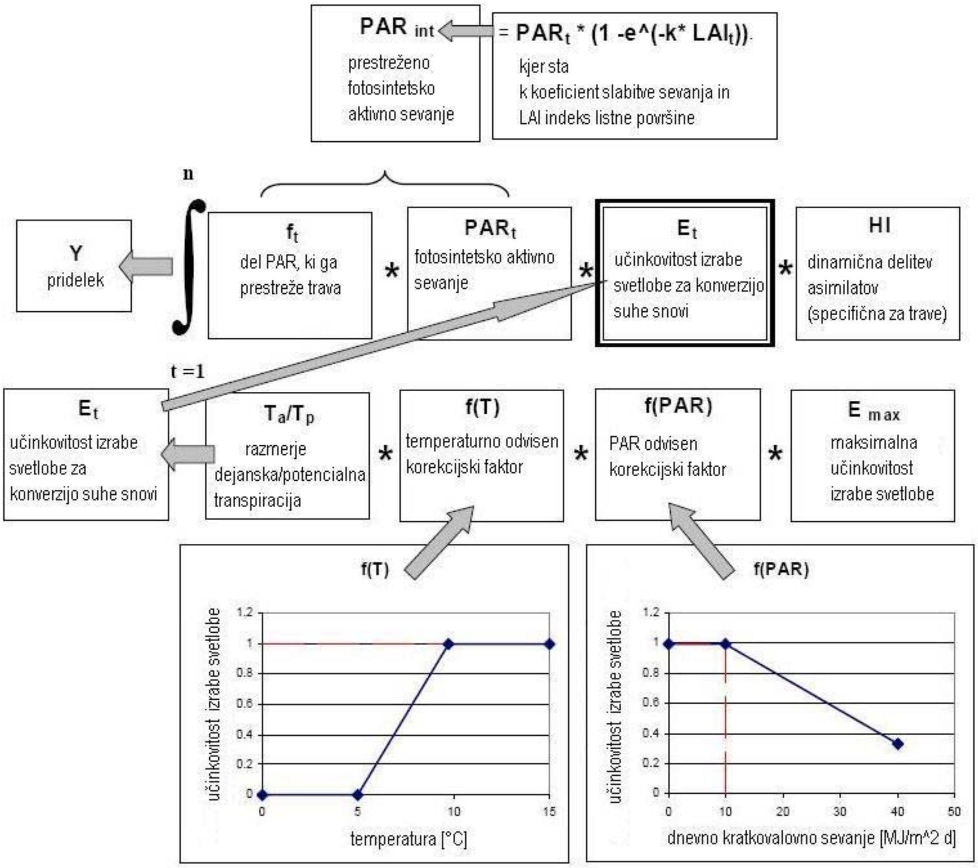
## 3 OSNOVE IZRAČUNOV, KI JIH UPORABLJA LINGRA

### 3.1 OSNOVNI KONCEPT

LINGRA izvira iz modela WOFOST (Pogačar in Kajfež Bogataj, 2009b), ki razlaga rast pridelka na osnovi procesov, ki se dogajajo v rastlini (fotosinteza, dihanje ...), in upošteva, kako na te procese vplivajo okoljske razmere. Pri tem je število procesov omejeno na glavne parametre in le manjši del teh procesov je dinamično simuliranih. S statičnim pristopom pa so obravnavani parametri, ki imajo relativno majhen vpliv na rast ali pa jih slabo poznamo.

Za razliko od poljščin je za travno rušo značilna pogosta defoliacija zaradi košnje ali paše. Ponavljajoča se defoliacija vodi do začasnega pomanjkanja asimilatov, pri čemer je znižana stopnja fotosinteze, saj se morajo najprej tvoriti novi listi, da zagotovijo nadaljevanje produkcije. Formacija novih listov sloni na količini ogljikovih hidratov, shranjenih v listnih zasnovah, to pa povzroča, da se menjata obdobje, ko asimilatov primanjkuje, in obdobje, ko se presežek asimilatov pri visokem indeksu listne površine (LAI) shranjuje. Močan vpliv na opisan proces imajo okoljske razmere in kmetijska praksa. Poraba asimilatov (ponor) je v modelu povezana z rastjo listov, poganjanjem listov in razraščanjem poganjkov (Davies and Thomas, 1983 cv. Schapendonk in sod., 1998), medtem ko je zaloga asimilatov (vir) določena s fotosintezo, ta pa je odvisna od količine svetlobe, ki jo rastlinski pokrov prestreže. Vir je torej odvisen predvsem od sončnega obsevanja, ponor pa od temperature zraka. V modelu LINGRA sta ponor ($\Delta W_d$) in vir ($\Delta W_s$) simulirana semi-neodvisno, kar pomeni, da je vsak dan stopnja rasti rastline določena glede na bolj omejujoč dejavnik (ponor ali vir). Ostale spremenljivke stanja določamo na osnovi stopnje rasti na posamezen dan in niso neodvisno povezane s ponorom ali virom.

Glede na LINTUL so tako inovativni aspekti modela LINGRA ločeni algoritmi za procese, vezane na vir, in procese, vezane na ponor, ter mehanistični, čeprav poenostavljen, pristop k simulaciji morfološkega razvoja trave, ki opisuje naravno zaporedje dogodkov, vključno z defoliacijo zaradi košnje ali paše.

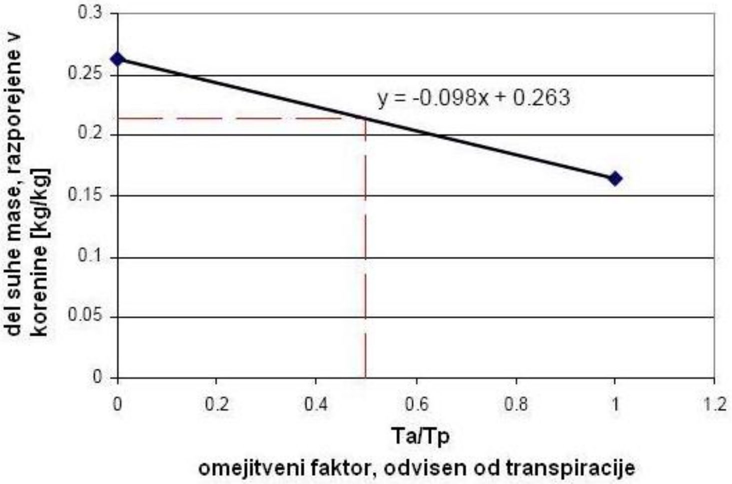


**Slika 1**: Shema izračuna pridelka z modelom LINGRA, s prikazanima izračunoma prestreženega fotosintetskega aktivnega sevanja in učinkovitosti izrabe svetlobe (po Lazar in Genovese, 2004)

**Figure 1**: Overview of the grassland growth model – LINGRA with the calculus of intercepted PAR. The main principle of this concept is that grass growth is proportional to the amount of light intercepted by canopy (modified from Lazar in Genovese, 2004)

Simulacija rasti se v modelu začne, ko 10-dnevno drseče povprečje dnevne temperature zraka preseže temperaturo praga (TMBAS1). O je presežena TMBAS1 se v travni ruši sprožijo fiziološki procesi. Velikost TMBAS1 naj bi bila za severne države 3 °C in za južne pa 5 °C (Schapendonk in sod., 1998). Pridelek je rezultat integracije dnevno nastale suhe snovi, premeščene v žetvene organe. Pri tem je količina novo tvorjene suhe snovi odvisna od prestreženega fotosintetskega aktivnega sevanja (PAR) in učinkovitosti izrabe svetlobe (slika 1). Maksimalna učinkovitost izrabe PAR pri fotosintezi je lahko zmanjšana zaradi vodnega stresa (ocenjen kot razmerje med dejansko in potencialno transpiracijo $T_a/T_p$), temperatur pod bazno vrednostjo in zelo visokih vrednosti PAR. Po košnji je rast listne površine odvisna od števila poganjkov, ki imajo nodij (ta omogoča rast listov). Povprečna širina novih listov je parameter modela (na primer 0,03 m), rast listov pa je opisana kot funkcija temperature.

Porazdelitev novo tvorjenih asimilatov je neodvisna od tega, ali je rast omejena z virom ali ponorom, nanjo pa vpliva vodni stres (slika 2). Viri asimilatov so trenutna fotosinteza in predhodno shranjeni ogljikovi hidrati. Dejanska rast je enaka manjši vrednosti izmed potrebe po asimilatih in njihove zaloge. Ko asimilati, ki nastanejo pri fotosintezi, presežejo potrebe, se razlika shrani kot zaloga ogljikovih hidratov.



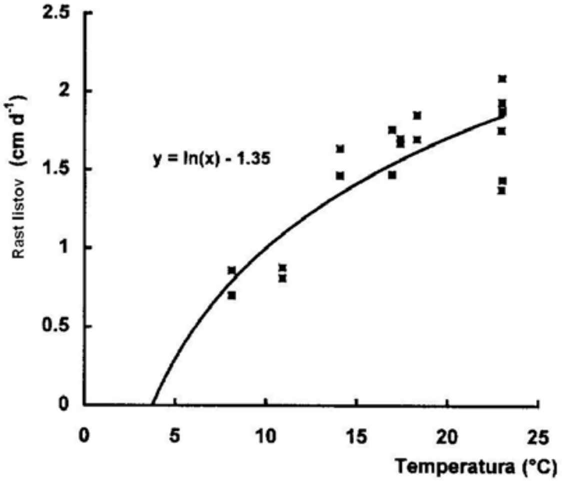
**Slika 2**: Vpliv vodnega stresa na razporeditev asimilatov v korenine (vir: Lazar in Genovese, 2004)
**Figure 2**: The influence of water stress on dry matter allocation to roots (Lazar in Genovese, 2004)

Model LINGRA je torej zgrajen iz podmodelov ali rutin, ki računajo prestrezanje svetlobe, učinkovitost izrabe svetlobe, aktivnost vira in ponora, stopnjo razraščanja poganjkov, stopnjo pojavljanja listov, količino vode v tleh in evaporacijo (zadnji dve enako kot WOFOST).

## 3.2 UČINKOVITOST IZRABE SVETLOBE (VIR), DINAMIKA RASTI POGANJKOV IN LISTOV (PONOR)

Profil svetlobe znotraj rastlinskega pokrova je odvisen od LAI in koeficienta slabitve sevanja (k). Učinkovitost pretvorbe absorbirane svetlobe v asimilacijo ogljika pa je odvisna od intenzitete obsevanja, temperature zraka in dostopnosti vode. Poenostavljeno so učinki temperature zraka opisani kot linearna funkcija, pri čemer je optimalno temperaturo (pri kateri je temperaturni faktor enak 1) potrebno kalibrirati.

Rast listov v prvih petih dneh po košnji je prav tako odvisna od povprečne dnevne temperature zraka. Pri temperaturi 5 °C zrastejo le 0,3 cm/dan, pri 23 °C pa 1,8 cm/dan. Schapendonk in sod. (1998) so eksperimentalno določili logaritemsko povezavo med omenjenima spremenljivkama (slika 3).



**Slika 3**: Povprečje rasti listov v petih dneh po košnji v odvisnosti od povprečne dnevne temperature zraka (vir: Schapendonk in sod., 1998)
**Figure 3**: The influence of air temperature on leaf elongation rate during five days after mowing (Schapendonk et al., 1998)

Tvorba listov na poganjkih je odvisna od temperature tal. Dejanska rast listov je določena s količino dostopnih asimilatov in s stopnjo odmiranja starih listov zaradi samozasenčenja in sušnega stresa. Stopnja razraščanja poganjkov je določena iz deleža brstov, ki so producirali nove poganjke. Največje število poganjkov iz enega brsta je v povprečju 0,69; takoj po košnji pa je to število zmanjšano na 0,335. Ta izkoristek brstov se imenuje razmerje polnjenja (van Loo, 1993). Ko se LAI povečuje, se razmerje polnjenja zmanjšuje zaradi samozasenčenja, saj se pri tem precej zmanjša razmerje med rdečo in infrardečo svetlobo, ki doseže nižje dele lista. Nizka intenziteta svetlobe povzroča remobilizacijo dušika iz listov in s tem hitro staranje. Odmiranje poganjkov in staranje listov sta tako funkciji LAI. Število poganjkov na kvadratni meter je visko v zgodnji pomladi, nato se poleti močno zmanjša in jeseni spet nekoliko poveča. Celoten proces je občutljiv na svetlobo, temperaturo in stresne pogoje.

Stopnja formacije novih listov po košnji je bistvenega pomena za celotno produktivnost, pri čemer je najbolj pomembna prestrežena svetloba v tej fazi. Potrebe po ogljikovih hidratih so v prvih dneh po defoliaciji na splošno večje kot fotosintetski donos (Schnayder in Nelson, 1988), zato rastlina črpa rezervna hranila iz zalog v poganjkih in razrastišču. Kadar imamo pogoje omejene z virom, razvoj listov ni več temperaturno odvisen, temveč je rast listov določena s količino asimilatov, razporejenih v liste, pomnoženo s specifično listno površino (SLA). Skozi sezono se SLA sicer spreminja od 0,03 $m^2/g$ v zgodnji pomladi do 0,018 $m^2/g$ poleti in jeseni, a te fluktuacije v modelu niso upoštevane, uporabljena je povprečna vrednost 0,025 $m^2/g$ (določena v poskusu v Wageningnu).

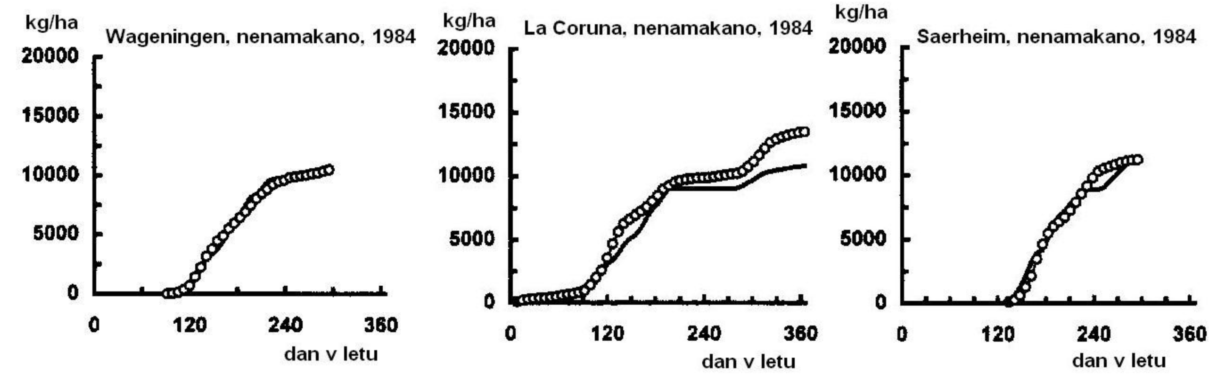
### 3.3 VPLIVI SUŠNEGA STRESA

Rastlini dostopna voda je v modelu funkcija maksimalne globine korenin (40 cm) in količine vode v tleh. Ta je določena kot razlika med padavinami in izgubami vode zaradi perkolacije in evapotranspiracije (po Makkinkovi enačbi z uporabo kratkovalovnega sevanja in temperature zraka). Ob pomanjkanju vode rastlina zapira listne reže, da prepreči izsuševanje. Posledično se zmanjša vnos $CO_2$ iz ozračja, zato pa se zmanjša učinkovitost izrabe absorbirane svetlobe. To opišemo v modelu z razmerjem med dejansko in potencialno transpiracijo kot mero za zmanjšanje stomatalne prevodnosti in učinkovitosti izrabe svetlobe. Poleg tega je posledica suše tudi preferenčna distribucija asimilatov v korenine.

## 4 UMERJANJE IN TESTIRANJE MODELA

Pri umerjanju je primerno uporabljati podatke s tal, kjer naklon ni večji od 5 %, podtalnica globlje od 90 cm in kapaciteta za zadrževanje vode v plasti 0-100 cm med 125 in 175 mm.

Za osnovno kalibracijo modela so Schapendonk in sod. (1998) pridobili standardizirane podatke s poljskih poskusov na 35 lokacijah po Evropi. Poskusi so se izvajali od 1 do 5 let v obdobju 1982-1986 s sortama *Lolium pernne* (trpežna ljuljka) in *Phleum pratense* (travniški mačji rep). Pri tem niso imeli določenih dejanskih lastnosti tal, temveč so uporabljali standardne parametre za srednjo teksturo tal z dobro zadrževalno sposobnostjo. Prav tako je bila privzeta globina koreninjenja 40 cm, simulacije pa so začenjali s polnim talnim vodnim rezervoarjem. Višina košnje je bila 4-5 cm, začela pa se je 1. marca ali takoj ko je skopnel sneg. Stopnja fertilizacije je bila visoka: 600 kg N/ha, 70 kg $P_2O_5$/ha, 280 kg $K_2O$/leto. Za kalibracijo so uporabili podatke s 15 poskusov, ki so dosegli pridelek nad 16 t/ha/leto. Umerjali so LAI po košnji, bazno temperaturo za začetek fotosinteze in razvoja (TMBAS1) in dnevno temperaturo za optimalno učinkovitost izrabe svetlobe LUE (Topt).



**Slika 4**: Izmerjene (°) in napovedane (-) vrednosti pridelka zelinja v Wageningnu (Nizozemska), La Coruni (Španija) in Saerheimu (Norveška) (vir: Schapendonk in sod., 1998)

**Figure 4**: Observed (°) and predicted (-) grass yield at Wageningen – Netherlands , La Coruni - Spain and Saerheimu - Norway (Schapendonk et al., 1998)

Ob simulaciji pridelka suhe snovi je bila normalizirana napaka med napovedanimi in izmerjenimi vrednostmi za celotno Evropo 13-15 % pri potencialni produkciji in 17-21 % pri produkciji v razmerah z omejeno dostopnostjo vode.

Rezultati so zadovoljivi in so pokazali uporabnost modela za celotno Evropo. LINGRA predstavlja dobro osnovo za modeliranje pridelka travne ruše v zmernih podnebnih razmerah. Vendar pa je zaradi določenih poenostavitev in statističnih pristopov nujna kalibracija nekaterih parametrov (Schapendonk in sod., 1998).

Obsežna raziskava je bila z modelom LINGRA narejena tudi v okviru projekta PASK. Pokazalo se je (Wolf, 2006), da pridelek narašča od Wageningna preko Bologne do Seville, kar so pripisali dolžini rastne sezone in količini prestreženega sevanja (RADI), ki se v istem vrstnem redu povečujeta. Pri tem pa povečana RADI ne povzroči tudi večjih zalog, torej omejitve ponora še ne omejujejo rasti (v drugem primeru pa so opazili tudi povečevanje zalog). Večja produkcija biomase pa v povprečju določa višji LAI, s tem pa zaradi samozasenčenja več staranja listov in veliko več odmrle listne mase

Pri testiranju modela ob omejeni dostopnosti vode, so takšno stanje simulirali z nastavljenim razmerjem med dejansko in potencialno transpiracijo na 0,5. Tudi pri stalnem sušnem stresu se pridelek med lokacijami podobno povečuje, vendar celotna produkcija biomase v teh razmerah dosega skromnih 30-35 % optimalne. Rezultat lahko pripišemo 50 % nižji stopnji rasti in počasnejši ponovni rasti listov po košnji. Pridelki dosegajo le 20-40 % optimalne vrednosti. Najslabše rezultate prinaša kombinacija sušnega stresa in nizkih vrednosti sončnega obsevanja. Na vseh treh lokacijah se ne ustvarja nič zalog hranil, kar pomeni, da je stopnja rasti pri omejenem viru precej nižja od stopnje rasti pri omejenem ponoru.

## 5 ZAKLJUĆEK

Model LINGRA je enostavno zastavljen, vendar vsebuje najbolj pomembne značilnosti rasti travne ruše, ne le v povezavi s fotosintezo, temveč tudi glede na morfologijo listov in razraščanje poganjkov (Schapendonk in sod., 1998).

Sedanji modeli pogosto niso dovolj natančni, če jih uporabljamo za lokacije, kjer niso bili razviti. Tudi Hoogenboom in sod. (1999) pravijo, da je potrebno pred uporabo rastlinskega modela za posamezno regijo preveriti njegovo obnašanje pri regijsko značilnih sortah z določenim nizom fenoloških podatkov in podatkov o pridelku. Prav tako je potrebno kalibrirati specifične parametre modela. Pred validacijo modela je torej nujna kalibracija (Alexandrov in sod., 2001). V ta namen potrebujemo pri modelu LINGRA vsaj 10 let podatkov (Wolf, 2006), pri čemer je pomembno, da so slovenski in vemo, kako so bili pridobljeni. Na področju travništva obstaja velika količina podatkov o pridelku travne ruše, ki jih je s sodelavci na Agronomskem oddelku Biotehniške fakultete dolga leta zbiral prof. dr. Leskošek. Del teh podatkov je ohranjenih v diplomskih nalogah njegovih študentov, veliko pa je tudi neobjavljenih, a shranjenih v njegovem osebnem arhivu, kjer jih je uredil, ko jih je vse skupaj želel objaviti v monografiji. Gre za dolgoletne poskuse z več kot 30 let podatki. Tako dolgi nizi podatkov za travno rušo so precej izjemni. Mnogo raziskav v tujini namreč zaradi pomanjkanja podatkov temelji na krajših nizih, saj ima večina poljskih poskusov v osnovi drugačen namen, zato ne zagotavlja celotnega nabora potrebnih vhodnih podatkov (Žalud in sod., 2006).

Umerjen model LINGRA bo uporaben za različne simulacije vremenskih razmer, kot so število dni s snegom, suša ipd. Na ta način bo v prihodnosti moč določiti odvisnost pridelka od različnih razmer v okolju.

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# Analiza fenofaz paš, pri čebelah v Novem mestu

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## IZVLEČEK

Preučevali smo vpliv temperature zraka na izbrane fenološke faze čebel (*Apis mellifera carnica* Pollman) za obdobje 1971-2007 v Novem mestu. Medletna variabilnost nastopa fenofaz (prva paša čebel, paša na robiniji, paša na lipi) je velika, variacijski razmik znaša od 44 do 86 dni. S časovno analizo smo ugotovili, da se kot posledica višjih temperatur zraka ter zgodnejših rastlinskih fenofaz, tudi fenofaze pri čebelah pojavljajo v zadnjem obdobju zgodnejše. Statistično značilen linearen trend za prvo pašo čebel znaša -19 dni/dekado, za pašo na robiniji pa -10 dni/dekado. Fenofaza paša na lipi nima statistično značilnega trenda. Povezanost med fenološkim razvojem in temperaturo zraka smo preučevali s korelacijsko analizo. Vrednosti koeficientov korelacije so med -0,45 in -0,70. Vse tri fenofaze so najbolj korelirane s povprečnimi dvomesečnimi temperaturami marca in aprila. Izdelali smo enostavne regresijske modele za napoved časa nastopa fenofaz na osnovi temperature zraka. Kot pojasnjevalne spremenljivke modela smo vključili povprečne mesečne temperature zraka in termalni čas, potreben za nastop izbrane fenofaze. Akumuliran termalni čas smo izračunali kot vsoto aktivnih temperatur (vsota pozitivnih temperatur od 1.1. dalje) in efektivnih temperatur (temperaturna vsota nad temperaturnim pragom 10 °C od 28.2. dalje). Koeficienti variabilnosti (KV) za izračunan termalni čas znašajo od 15 % do 43 %. Z metodo regresije po korakih smo oblikovali linearne regresijske modele za napoved časa prve paše čebel in paše na robiniji. Z modeli smo lahko pojasnili od 53 % do 91 % variabilnosti v času nastopa fenofaz.

**Ključne besede**: čebele, fenofaze, trendi, termalni čas, regresijski modeli

## ABSTRACT

### THE ANALYSIS OF PASTURE PHENOPHASES FOR BEES IN NOVO MESTO

The influence of air temperature on the chosen bee phenophases (*Apis mellifera carnica* Pollman) was studied in the period 1971-2007 in Novo mesto, Slovenia. The inter-annual phenophase variability (start, first bee pasture, pasture on black locust and pasture on linden) is high - variation interval is from 44 to 86 days. With time analysis it was established that as a result of higher air temperatures and earlier plant phenophases also the bee phenophases appear earlier in the last period. Statistically significant linear trend for the first bee pasture is -19 day/decade, for pasture on black locust it is -10 day/decade. Phenophase pasture on linden has no statistically significant trend. The correlation between phenological development and air temperature was studied with correlation analysis. The correlation coefficient values are between -0.45 and -0.70. All three phenophases are best correlated with average monthly temperatures in March and April. Simple regressions models for the prediction of the start of phenophases on the basis of air temperature were developed. As the models explanatory variables average monthly air temperatures and thermal time needed for an individual phenophase start were included. The accumulated thermal time was calculated as the sums of active temperatures (the sum of positive temperatures from January 1) and effective temperatures (the sum of temperatures above the temperature threshold 10 °C from February 28). The variability coefficients (KV) for the calculated thermal time are between 15 per cent and 43 per cent. With backward selection linear regression models for the prediction of time of the first bee pasture and pasture on black locust were formed. With the models we were able to explain 53 per cent to 91 per cent of variability at the start of phenophases.

**Key words**: bees, phenophases, trends, thermal time, regression models

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Prispevek je del magistrskega dela " Fenološki razvoj izbranih rastlinskih vrst in škodljivih metuljev (Lepidoptera) kot kazalec temperaturnih razmer in njihove spremenljivosti v Sloveniji ", ki ga je pod mentorstvom doc. dr. Zalike Črepinšek napisala Lucija Glorija Jelen.

The manuscript is a part of the M.Sc. Thesis" The phenological development of chosen plants and pest butterflies (Lepidoptera) as the indicator of temperature conditions and their changeability in Slovenia" submitted by Lucija Glorija Jelen (supervisor: Doc. Ph. D. Zalika Črepinšek).

## 1 UVOD

V Sloveniji gojimo avtohtono pasmo čebel 'Kranjsko čebelo' (*Apis mellifera carnica* Pollmann). Čebele oprašujejo prosto rastoče cvetnice ter gojene rastline, pomembne so pri pridelavi sadja in semen. S čebelami pridobivamo tudi med, cvetni prah, vosek, propolis, matični mleček in čebelji strup. Čebele gojimo v panjih, ki jih lahko zlagamo v čebelnjake (Božič, 2010).

Za analizo fenofaz pri čebelah smo se odločili, ker so zanje poleg uporabe pesticidov tudi podnebne spremembe stresni dejavnik, ki lahko dolgoročno vpliva na njihovo izumiranje. Zaradi zgodnejših pomladi in poznejših zim bodo čebele dalj časa dejavne, kar bo skrajšalo zimski odmor čebel. Višje poletne temperature s sušo bodo povzročile dolgotrajnejša brezpašna obdobja. Ob pomanjkanju hrane na travnikih obstaja nevarnost, da bodo čebele cvetni prah nabirale tudi na koruzi, tretirani s fitofarmacevtskimi sredstvi (npr. insekticidi iz razreda neonikotinoidov), ki so za čebele še posebno strupeni. Višje temperature in suše bodo vplivale tudi na medovite rastline, ki bodo zaradi tega manj medile in bodo vse manj zanesljiva paša za čebele, le-te bodo tako izgubile nekatere paše, ostale brez nektarja, cvetnega prahu in vode. Pričakujemo sicer lahko, da bodo zdajšnje medovite rastline nadomestile nekatere nove. Zaradi zvišanja temperature pa bo naše okolje postalo idealno tudi za naselitev nekaterih drugih škodljivcev čebel, ki se hitreje razmnožujejo in širijo v toplejšem podnebju (Auguštin, 2006).

Fenofaze pri čebelah so: prvi izleti, prva paša, prvi roj, paša na robiniji, na lipi, na smreki, na drugih rastlinah (kamor sodi cvetlična paša: regrat, divja češnja, razne vrste vrb ter drugega spomladanskega cvetja) in gozdna paša (jelka ali hoja, smreka, macesen, bor, hrast).

V naši raziskavi smo analizirali podatke za naslednje fenofaze:

**1. prva paša ($Č_{paša}$)**
V prvih pašnih dneh čebele začnejo zrejati zalego (zalega: zaležena jajčeca ali iz njih izležene ličinke, bube, mlade živali (SSKJ, 2005)). Prve pašne dni nektar nima velikega pomena za čebelje družine, saj je v satju dovolj medenih zalog, vendar svež nektar spodbudi čebele k večji nabiralni aktivnosti in povečani vzreji zalege. Pomembnejši je cvetni prah, ki ga čebele nanosijo v očiščeno satje in je glavni vir beljakovin v prehrani ličink in mladih čebel. Brez cvetnega prahu ni pravega spomladanskega razvoja čebel. Beljakovine potrebujejo tudi pašne čebele, ki jih porabijo za obnovitev telesnih celic. Ob pojavu dobrih paš se količina zalege še poveča, ker le-ta oddaja poseben feromon, signalno snov, prisotnost zalege v panju spodbuja pašne čebele k večji aktivnosti, poleg tega čebelam uspe pripraviti prve sveže zaloge medu (Čebelarstvo Bukšek, 2010).

**2. paša na robiniji ($Č_{robin}$)**

- robinija ali akacija (*Robinia pseudoacacia* L.) je znano medovito drevo toplejšega podnebja. Začetek cvetenja je predvsem odvisen od zgodnje ali pozne pomladi. Prvi cvetovi se lahko odprejo že v začetku maja, cvetenje pa se lahko zavleče v junij. Paša na robiniji je dokaj zanesljiva in bogata, v idealnih vremenskih razmerah je lahko tudi rekordna (Božič, 2011a), saj je potencialni donos na ha površin 1000 kg/leto (Medovite lesnate rastline, 2011).

**3. paša na lipi ($Č_{lipa}$)**

- lipa (*Tilia platyphyllos* Scop.) raste v listnatih gozdovih po vsej Sloveniji. Pogosto jo tudi sadijo po parkih in drevoredih, kjer lahko najdemo še nekatere neavtohtone vrste lip. Lipa cveti v juniju, običajno dober teden po cvetenju akacije. Vrsta dobro medi (Božič, 2011b).

## 2 MATERIAL IN METODE

### 2.1 FENOLOŠKI PODATKI

Fenološke podatke za čebele smo pridobili iz arhiva fenoloških podatkov Agencije republike Slovenije za okolje (ARSO), v elektronski obliki za časovno obdobje 1971-2007 (podatki niso kontinuirani, saj v nizu manjkajo podatki za leta: 1974-1980, 1982, 1983, 1998, 2002-2004). Podatki so podani v obliki datumov ter kot zaporedni dan v letu-julijanski dan ($J_d$).

### 2.2 METEOROLOŠKI PODATKI

Meteorološke podatke v elektronski obliki smo pridobili iz arhiva meteoroloških podatkov ARSO za obdobje 1971-2007. Obravnavali smo meteorološko postajo Novo mesto-NM (220 m, 45° 48' S, 15° 11' V), Slovenija. Uporabili smo vrednosti povprečnih dnevnih temperatur zraka, izračunane iz izmerjenih urnih temperatur.

### 2.3 STATISTIČNE METODE OBDELAVE PODATKOV

#### 2.3.1 Deskriptivne (opisne) statistike

Po logični kontroli podatkov, s katero smo izločili lažje vidne napake, smo izvedli še kritično kontrolo - tako smo s statističnimi metodami preverili točnost podatkov. Aritmetična sredina, izražena kot $J_d$, nam prikaže povprečen nastop fenofaze, standardni odklon (SD) pa prikaže odstope od povprečja. Določili smo tudi maksimum-MAX (najpoznejši datum pojavljanja fenofaze) ter minimum-MIN (najzgodnejši datum pojavljanja fenofaze), iz razlike med njima pa variacijski razmik (VR).

### 2.3.2 Trendi - časovna analiza podatkov

Osnovno dolgoročno tendenco razvoja nekega pojava v času predstavimo s trendom. Fenološke trende izrazimo v številu dni na deset let. Negativni trend pomeni, da se fenofaza v zadnjem obdobju pojavlja zgodnejše, pozitiven trend pa, da se pojavlja kasneje glede na začetno obdobje. Običajno izražamo zaupanje v odstotkih, pri naši raziskavi smo uporabili 95 % interval zaupanja. Statistično značilne trende pri p=0,05 pa smo označili z znakom *.

Analiza časovnih vrst mora karakterizirati razvoj obravnavanega pojava v času, pojasniti variabilnost pojava v času na osnovi drugih dejavnikov in omogočiti napovedovanje razvoja pojava (Črepinšek, 2002).

### 2.3.3 Enostavna linearna regresija

Regresija je prilagajanje ustrezne matematične funkcije empiričnim podatkom. Regresijska funkcija je linearna, ko analiziramo vpliv ene (enostavna regresija) neodvisne spremenljivke X (časovna komponenta) na odvisno spremenljivko Y (nastop fenofaze), vrednost ene spremenljivke vpliva na vrednost druge spremenljivke, ne pa tudi obratno.

Napovedi lahko izračunamo le na definicijskem območju neodvisne spremenljivke, izven tega območja pa je zveza lahko drugačna. Namen regresijske analize je proučevanje odvisnosti ene spremenljivke od druge ter napoved vrednosti odvisne spremenljivke na osnovi znane vrednosti neodvisne spremenljivke (Košmelj, 2007). V naši raziskavi je bila odvisna spremenljivka čas nastopa fenofaz, neodvisna spremenljivka pa temperature zraka in termalni čas.

### 2.3.4 Korelacija

Pojem povezanost oz. soodvisnost pomeni, da se vrednosti obeh spremenljivk spreminjajo hkrati. Namen ugotavljanja povezanosti je izračunati ustrezno mero, ki vrednoti jakost povezanosti dveh spremenljivk. Tesnost povezave pri linearni zvezi nam meri koeficient korelacije (r). Pri pozitivni korelaciji vrednosti obeh spremenljivk hkrati naraščata oz. hkrati padata, če pa je korelacija negativna, ob naraščanju vrednosti ene spremenljivke vrednost druge pada. Če korelacije ni, je vrednost koeficienta 0 (Košmelj, 2007). Primer korelacije je lahko povezanost pojava fenofaz dveh različnih rastlin, ki sta močno povezani, lahko sta odvisni od tretje spremenljivke, npr. temperature (Črepinšek, 2002). S pomočjo korelacijskih matrik smo ugotavljali povezanost med časom pojavljanja fenofaz in povprečnimi mesečnimi ($T_{dec}$, $T_{jan}$, $T_{feb}$, $T_{mar}$, $T_{apr}$), povprečnimi dvomesečnimi ($T_{decjan}$, $T_{janfeb}$, $T_{febmar}$, $T_{marapr}$) in povprečnimi tromesečnimi temperaturami zraka ($T_{decfeb}$, $T_{janmar}$). Ker je vsakoletni nastop pomladnih fenofaz v veliki meri temperaturno pogojen, čas pojavljanja fenofaz odraža toplotne razmere v okolju.

## 2.4 ANALIZA FENOLOŠKIH PODATKOV

### 2.4.1 Določitev temperature praga

Za določitev temperature spodnjega praga (TP) smo uporabili metodo najmanjšega koeficienta variabilnosti (KV), ki je najpomembnejša relativna mera variabilnosti, saj omogoča primerjavo variabilnosti različnih spremenljivk (Košmelj, 2007).

### 2.4.2 Termalni čas

Organizmi za prehod od ene do druge točke razvoja potrebujejo določeno količino toplote. Količina toplote, potrebna za celoten razvoj organizma, se ne spreminja, produkt temperature in časa je vedno enak (Zalom in sod., 1983). Razvoj torej temelji na akumulaciji toplotnih enot in ga namesto v koledarskih dneh v fenologiji izražamo s termalnim časom, oziroma vsotami aktivnih ali efektivnih temperatur.

Vsote aktivnih temperatur ($V_{akt}$) dobimo s seštevanjem pozitivnih povprečnih temperatur zraka od datuma, ko je presežena TP 0°C.

$$V_{akt} = \sum_{i=1}^{n} T(i)\,;\ T(i) \geq 0\ ^{o}C \qquad \ldots(1)$$

Vsote efektivnih temperatur ($V_{efk}$) računamo od prestopa višje TP, tako da od povprečne dnevne temperature odštejemo izbrano TP, oziroma biološki temperaturni minimum.

$$V_{eft} = \sum_{i=1}^{n} (T(i) - TP) \qquad \ldots(2)$$

Spodnji TP je definiran s temperaturo zraka, pod katero se rast in razvoj ustavita; zgornji TP pa je temperatura, nad katero se stopnja rasti ali razvoja začne zmanjševati ali se tudi ustavi. Skupna količina toplote, ki povzroči prehod od ene do druge razvojne točke življenjskega cikla, se pogosto računa kot vsota efektivnih temperatur, ki so akumuliran produkt časa in temperature med razvojnima pragoma za vsak dan. Ena efektivna stopinja je dosežena, če imamo en dan (24 ur) s temperaturo, ki je za eno stopinjo višja od temperature praga. Večja kot je razlika med temperaturo zraka in TP, večja je vsota efektivnih temperatur oz. hitrejša je stopnja razvoja organizma. Za izračun akumuliranih toplotnih enot potrebujemo tudi začetni datum, s katerim začnemo šteti termalni čas (starting date ali biofix) (Črepinšek, 2002).

Spodnjo temperaturo praga (TP) in začetni datum (ZD) smo določili s statistično metodo najmanjšega koeficienta variabilnosti (KV) vsote akumuliranih temperatur. Preizkusili smo niz temperatur od 0 ºC do 10 ºC (korak 1 ºC) ter ZD: 1.12, 1.1, 15.1, 31.1, 15.2 in 28.2.

Za izračun termalnega časa smo uporabili metodo pravokotnika, ki je najenostavnejša standardna metoda. Najpogosteje uporabljana enačba po tej metodi je (McMaster in Wilhelm, 1997; Perry in sod., 1997; Liu in sod., 1998):

$$V_{efk} = \sum_{i=1}^{n} \left( \left[ \frac{T_{\max} + T_{\min}}{2} \right] - TP \right) \qquad \ldots(3)$$

če je $\frac{T_{\max} + T_{\min}}{2} < TP$, potem velja $V_{efk} = 0$

pri čemer je $T_{max}$ najvišja temperatura; $T_{min}$ pa najnižja dnevna temperatura.

### 2.5 IZDELAVA FENOKLIMATSKIH MODELOV

Procese rasti in razvoja, njihove interakcije pa tudi interakcije z okoljem lahko opišemo z modelom oz. računalniškim programom (Scharrer in Schmidt, 1998). Z metodo multiple linearne regresije smo v analizo vključili spremenljivke na osnovi predhodne korelacijske analize. Uporabili smo statistični program STATGRAPHICS Plus Version 4.0; metodo backward selection (regresija po korakih nazaj), kjer začnemo z vključitvijo vseh neodvisnih spremenljivk ter postopno odvzemamo posamezne linearne člene in analiziramo spremembo modela glede na predhodnega. V multiplo regresijsko analizo smo vključili povprečne mesečne ($T_{jan}$, $T_{feb}$, $T_{mar}$, $T_{apr}$), dvomesečne ($T_{janfeb}$, $T_{febmar}$, $T_{marapr}$) in trimesečne temperature zraka ($T_{janmar}$, $T_{febapr}$) ter termalni čas ($V_{akt}$, $V_{efk}$). Za modele smo podali poleg pojasnjevalnih spremenljivk ter njihovih regresijskih koeficientov tudi delež pojasnjene variabilnosti in število dni, v koliko naprej lahko z modelom napovemo nastop fenofaze.

Koeficient determinacije ($r^2$) izraža odstotek z modelom pojasnjene variabilnosti odvisne spremenljivke, preostali del variabilnosti odvisne spremenljivke z regresijskim modelom ni pojasnjen (Košmelj, 2007). Delež pojasnjene variabilnosti smo izrazili s prilagojenim koeficientom determinacije ($r_P^2$), ki omogoča realno primerjavo modelov, ki vključujejo različno število pojasnjevalnih spremenljivk (povprečne temperature zraka, termalni čas)

$$r_P^2 = 1\text{-}(1\text{-}r^2)\cdot\left(\frac{n-1}{n-k}\right) \qquad \ldots(4)$$

pri tem je n velikost vzorca, k pa število neodvisnih spremenljivk, izraz n-k pa nam podaja število stopinj prostosti. Razlika med koeficientoma determinacije je največja, kadar imamo majhen vzorec (n) in relativno veliko število neodvisnih spremenljivk (k) (Črepinšek, 2002).

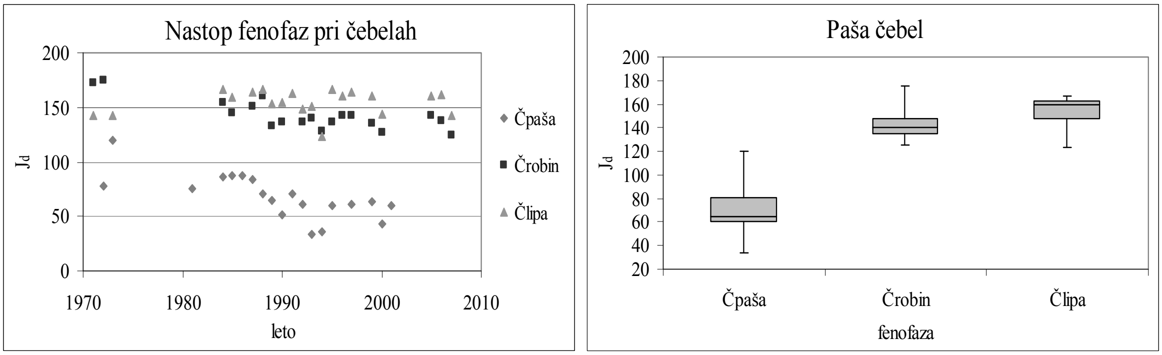
## 3 REZULTATI

### 3.1 ANALIZA PODATKOV

### 3.1.1 Deskriptivne statistike

V obdobju 1971-2007 v NM prva paša čebel povprečno nastopi 68. $J_d$, najzgodnejše nastopi 34. $J_d$, najkasneje 120. $J_d$, VR je 86 dni. Paša na robiniji se povprečno pojavi 143. $J_d$, najzgodnejše 125. $J_d$, najkasneje 175. $J_d$, VR je 50 dni. Paša na lipi se povprečno pojavi 155. $J_d$, najzgodnejše 123. $J_d$, najkasneje 167. $J_d$, VR pa je 44 dni.

Na sliki 1 smo prikazali povprečen nastop fenofaz ter njihov variacijski razmik (graf okvir z ročaji).



**Slika 1**: Povprečen nastop fenofaz pri čebelah, prikazan z okvirjem z ročaji v izbranih časovnih obdobjih
**Figure 1**: The average appearance of phenophases of bees, displayed with the chart Box-and-Whisker Plot in the chosen time periods

### 3.1.2 Časovna analiza pojavljanja fenofaz pri čebelah

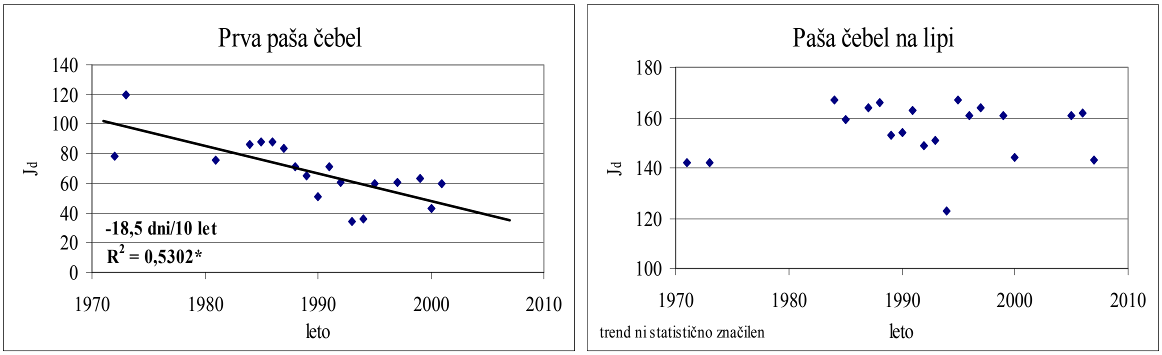
Trend letne temperature zraka v NM je statistično značilno pozitiven. Ravno tako je statistično značilen pozitiven trend za mesece januar, marec, april, maj, junij, julij, avgust in oktober.

**Preglednica 1**: Dolgoletni trendi povprečnih mesečnih in letne temperature zraka v Novem mestu v obdobju 1951-2007; + pozitiven trend, - negativen trend, * statistično značilen trend pri p=0,05
**Table 1**: Long-term trends of average monthly and annual air temperature in Novo mesto for the years 1951-2007; + positive trend, - negative trend, * statistically significant trend at p=0,05

| | Jan | Feb | Mar | Apr | Maj | Jun | Jul | Avg | Sep | Okt | Nov | Dec | Leto |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NM | +* | + | +* | +* | +* | +* | +* | +* | + | +* | + | + | +* |

Zaradi naraščanja temperatur zraka v pomladnih in poletnih mesecih bolj zgodaj nastopijo spomladanske in poletne fenofaze pri rastlinah, posledično pa se zgodnejše pojavljajo tudi fenofaze pri čebelah.

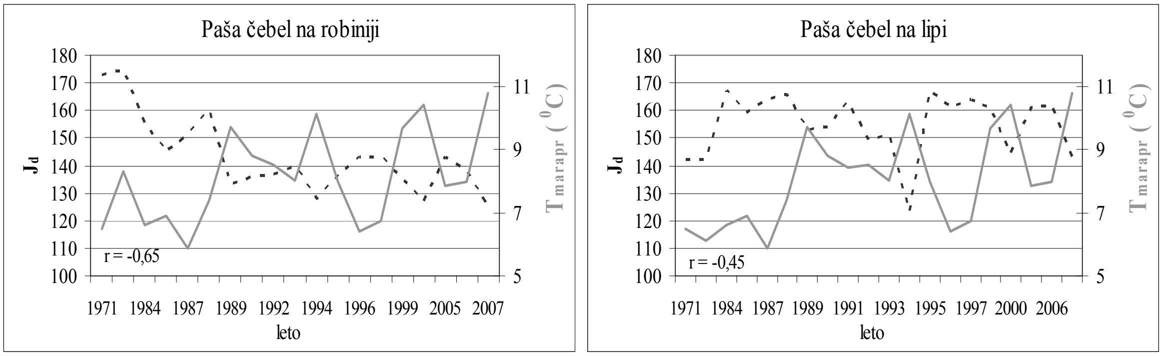
Fenofaza prva paša čebel ima negativen statistično značilen trend (slika 2), ki znaša -18,5 dni/10 let. Fenofaza paša čebel na robiniji ima negativen statistično značilen trend, velikost trenda je -11,4 dni/10 let. Fenofaza paša čebel na lipi nima statično značilnega trenda, predznak je pozitiven (slika 2).



**Slika 2**: Trend prve paše čebel ter trend paše na lipi v obdobju 1971-2007 (p=0,05)
**Figure 2**: The trend of the first bee pasture and the trend of linden tree pasture in the period 1971-2007 (p=0,05)

### 3.1.3 Korelacijska analiza

Korelacije med časom pojava fenofaz ter temperaturami zraka so negativne, vrednosti korelacijskih koeficientov (r) pa so -0,70 pri prvi paši; -0,65 pri paši na robiniji; -0,45 pri paši na lipi. Vse tri fenofaze so najboljše korelirane s $T_{marapr}$. Korelacijo med povprečnimi časom nastopa fenofaz in temperaturami zraka smo prikazali na sliki 3. Nizke povprečne temperature pomenijo kasnejši, visoke pa zgodnejši nastop fenofaz (obratna sorazmernost).



**Slika 3**: Korelacija med povprečnimi temperaturami zraka (v °C) in časom nastopa fenofaz (v julijanskih dneh)
**Figure 3**: The correlation between the average air temperature (in °C) and the time of phenophases appearance (in Julian days)

3.2 IZRAČUN TERMALNEGA ČASA

Vsote aktivnih temperatur (TP=0 °C) smo izračunali pri ZD 1.1., vsote efektivnih temperatur (TP=10 °C) pa smo izračunali pri ZD 28.2.

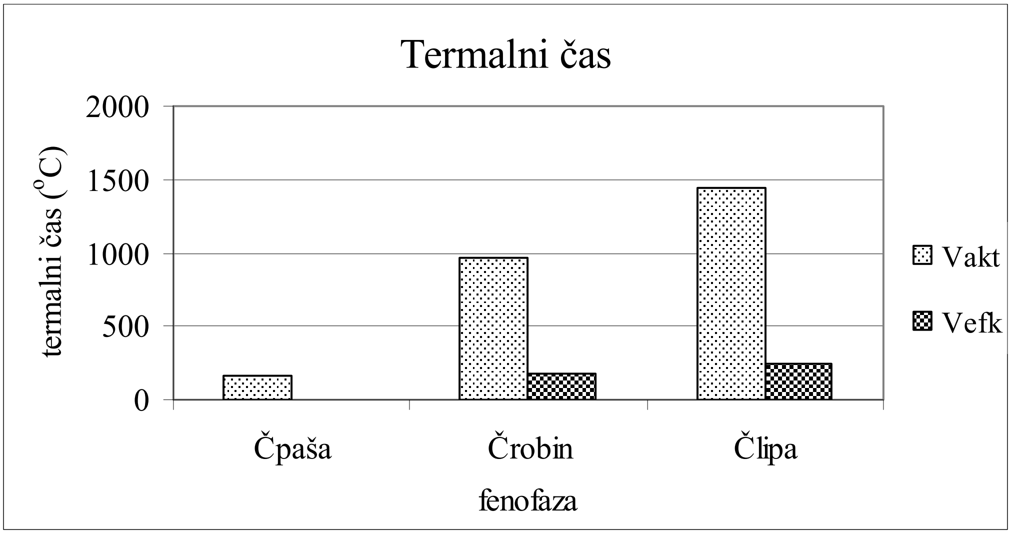
**Vsote aktivnih temperatur**: pri prvi paši čebel so potrebne $V_{akt}$ za nastop fenofaze 166 °C, (KV=43 %), pri paši čebel na robiniji 960 °C (KV=19 %) in pri paši čebel na lipi 1143 °C (KV=15 %). $V_{akt}$ so večje, če je nastop fenofaze kasnejši (seštevamo pozitivne povprečne temperature). Velikost $V_{akt}$ se veča glede na zaporedje nastopa fenofaz in glede na temperaturne razmere okolja.

**Vsote efektivnih temperatur:** Paša čebel na robiniji povprečno nastopi 23.5. (143. $J_d$), $V_{efk}$ potrebna za pojav te fenofaze, znaša 171 °C (KV=36 %). Paša čebel na lipi povprečno nastopi 4.6. (155. $J_d$), $V_{efk}$ znaša 248 °C (KV=29 %).

**Preglednica 2**: Termalni čas, potreben za nastop izbranih fenofaz pri čebelah
**Table 2**: Thermal time needed for the start of chosen phenophases for bees

| Fenofaza | Vsote akumuliranih toplotnih enot | ZD | TP (°C) | Termalni čas (v °C) | KV (v %) |
|---|---|---|---|---|---|
| $Č_{paša}$ | $V_{akt}$ | 1.1. | 0 | 166 | 43 |
| $Č_{robin}$ | $V_{akt}$ | 1.1. | 0 | 960 | 19 |
| $Č_{lipa}$ | $V_{akt}$ | 1.1. | 0 | 1143 | 15 |
| $Č_{robin}$ | $V_{efk}$ | 28.2. | 10 | 171 | 36 |
| $Č_{lipa}$ | $V_{efk}$ | 28.2. | 10 | 248 | 29 |

Na sliki 4 smo prikazali velikost $V_{akt}$ in $V_{efk}$ pri obravnavanih fenofazah.



**Slika 4**: Termalni čas ($V_{akt}$, $V_{efk}$), potreben za nastop fenofaz pri čebelah
**Figure 4**: Thermal time ($V_{akt}$, $V_{efk}$) needed for the start of phenophases for bees

3.3 FENOKLIMATSKO MODELIRANJE

S statističnim programom STATGRAPHICS Plus Version 4.0 smo z metodo backward selection napovedali fenološki razvoj izbranim fenofazam. V multiplo regresijsko analizo smo vključili povprečne mesečne, dvomesečne in trimesečne temperature zraka ter termalni čas.

**Preglednica 3**: Fenoklimatski modeli za napoved izbranih fenofaz pri čebelah; $r_P^2$ -prilagojen koeficient determinacije

**Table 3**: Phenoclimatic models for predicting the chosen phenophase for bees; $r_P^2$ - modified determination coeficient

| Fenofaza | Pojasnjevalne spremenljivke modela ($x_1$, $x_2$, ...$x_n$) | Regresijska konstanta (a) | Regresijski koeficienti za pojasnjevalne spremenljivke modela ($b_1$, $b_2$, ...$b_n$) | $r_P^2$ (v %) | Število dni vnaprej |
|---|---|---|---|---|---|
| $Č_{robin}$ | $T_{febmar,}T_{febapr}$ $V_{efk}$ | 133,0 | 739,1; -1110,6; 0,19 | 91,0 | 12 |
| $Č_{lipa}$ | $T_{febapr}$ $V_{efk}$ | 144,7 | -2,55; 0,10 | 53,2 | 14 |

Izdelali smo dva statistično značilna modela (paša čebel na robiniji in paša čebel na lipi), ki pojasnjujejo dovolj velik del variabilnosti (med 53 % in 91 %) časa fenofaz. Pojasnjevalne spremenljivke so temperature od februarja do aprila ter vsote efektivnih temperatur. Z modeloma lahko napovemo čas nastopa fenofaze od 12 do 14 dni vnaprej.

## 4 RAZPRAVA IN SKLEPI

Zimske in pomladne temperaturne razmere se iz leta v leto precej razlikujejo, zato so tudi med najzgodnejšim ter najkasnejšim dnevom nastopa določene fenofaze velike razlike. Fenološka opazovanja nam dajejo možnost proučevanja odzivnosti čebel na regionalne klimatske spremembe. Raziskave v zadnjih desetletjih so pokazale, da višje temperature v spomladanskem obdobju vplivajo na zgodnejši pojav fenofaz in daljše vegetacijske dobe tako v Evropi kot tudi drugod po svetu (Ahas in sod., 2000; Beaubien in Freeland, 2000; Menzel, 2000). Trend fenofaze prva paša tako znaša -19 dni/10 let, fenofaza paša na robiniji pa -10 dni/10 let.

Primerjava korelacij je pokazala, da so korelacije tesnejše, če uporabimo povprečja temperatur dveh ali treh zaporednih mesecev pred nastopom fenofaze; takšne korelacije uporabljajo tudi v drugih fenoloških raziskavah (Ahas in sod., 2000). Korelacije so negativne, r je v razponu od -0,45 do -0,70. Fenofaze so najboljše korelirane s $T_{marapr}$.

Akumuliran termalni čas računamo kot vsoto aktivnih ali efektivnih temperatur, ki jim je bil organizem izpostavljen. Za izračunan termalni čas je značilna velika variabilnost med leti, kar pomeni, da je poleg vpliva temperature potrebno upoštevati tudi druge dejavnike fenološkega razvoja. Čeprav je vsota akumuliranih toplotnih enot za posamezno fenofazo konstantna, pa lahko obstaja velika variabilnost med leti, kar je verjetno posledica dejstva, da se ZD iz leta v leto spreminja, v raziskavi pa smo za celotno obravnavano obdobje uporabili enoten ZD. Tudi koeficienti variabilnosti se med fenofazami zelo razlikujejo, pri najzgodnejših fenofazah so zelo veliki, kar je posledica dejstva, da čebele pri zgodnje pomladanskih fenofazah potrebujejo zelo majhne vsote akumulirane toplote za nastop posameznih fenofaz.

Procese rasti in razvoja, njihove interakcije ter interakcije z okoljem lahko opišemo z modelom oz. računalniškim programom. Z metodo regresije po korakih (backward selection) smo oblikovali fenoklimatske modele**,** kot pojasnjevalne spremenljivke smo vključili povprečne mesečne, dvomesečne in trimesečne temperature zraka ter termalni čas. Pri čebelah lahko z modeli pojasnimo od 53,2% pa do 91,0% variabilnosti časa pojava fenofaz, fenofaze lahko napovemo od 12 do 14 dni vnaprej.

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COBISS koda 1.01

**Agrovoc descriptors:** allium, buds, sampling, chromosome number, meiosis, mitosis, evolution, sexual reproduction, plants, genes, recombination

**Agris category code**: F30

# Problematika nabiranja cvetnih brstov za izdelavo preparatov mejoze

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## IZVLEČEK

Opisan je evolucijski pomen mejoze in vzroki za prevlado spolnega načina razmnoževanja pri višjih rastlinah ter pomen prerazporejanja genov (rekombinacija) v profazi prve zoritvene delitve. Predstavljen je razvoj mejoze iz mitoze in razlike med tema celičnima delitvama. Opisano je nabiranje cvetnih brstov čemaža za pripravo mejotskih preparatov. Cvetne brste čemaža smo nabirali v marcu in aprilu 2008. Največ mejotskih faz smo opazili pri cvetnih brstih nabranih v tretjem tednu v mesecu marcu. Optimalna dolžina cvetnih brstov je bila 1 do 1,5 cm. V aprilu so bili cvetni brsti preveč razviti, lahko smo opazovali le pelodna zrna.

**Ključne besede:** mejoza, *Allium ursinum*, cvetni brsti, nabiranje, redukcija kromosomske števila, razvoj mejoze iz mitoze

## ABSTRACT

## FLOWER BUD SAMPLING FOR MEIOTIC SLIDE PREPARATIONS

Evolutionary aspect of meiosis and reasons for dominate sexual reproduction in higher plants are described. Conjugation of homologue chromosomes and recombination of genes in prophase of the first meiotic division are presented. Evolution of meiosis from mitosis is discussed. The problems of flower bud sampling for meiotic preparations are presented. Flower buds of *Allium ursinum* were collected in March and April 2008. Best results were obtained by sampling flower buds in the third week of March. For meiotic preparations the optimal length of flower buds was 1 to 1.5 cm. In April the flower buds were over-developed and we could observe developing pollen grains only.

**Key words:** meiosis, *Allium ursinum*, flower buds, sampling, chromosome number reduction, the evolution of meiosis from mitosis

## 1 UVOD

### 1. 1 Odkritje mejoze

Mejozo je odkril in prvič opisal nemški biolog Oscar Hertwig (1849 – 1922) leta 1876, na nivoju kromosomov, pa jo je prvi opisal belgijski zoolog Edouard Van Beneden (1846 – 1910). Redukcijska delitev ali mejoza je tesno povezana s spolnim načinom razmnoževanja.

Najpomembnejše sestavine pri delitvah pod mikroskopom vidnih nitastih kromosomov so DNK in beljakovine. Mejoza ohranja število kromosomov iz generacije v generacijo.

### 1.2 Evolucijski pomen redukcijske delitve ali mejoze.

Redukcijska delitev ali mejoza zmanjša število kromosomov v gametah na polovico.

Pri redukcijski delitvi se izvrši tudi prerazporejanje (rekombinacija) genov (Louis in Borts, 2003; Ronceret in sod., 2009) ali prekrižanje nesestrskih kromatid

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(crossing over). To je proces, ki poveča genetsko pestrost potomcev in je vzrok, da spolni način razmnoževanja predstavlja evolucijsko prednost hitrejšega prilagajanja živih organizmov na spremenjene razmere okolja. Zato spolni način razmnoževanja prevladuje pri rastlinah in glivah (Shonn in sod., 2000).

Novejši pogled na evolucijski pomen mejoze je, da ta omogoča popravilo poškodovane DNK. Po tej hipotezi je primarna naloga genskega prerazporejanja pri mejozi popravilo poškodovane DNK (Smith, 2004). Sparjeni homologni kromosom je šablona, ki vodi popravilo poškodovane DNK, ki se na ta način pomladi.

Prve evkariotske vrste so bile enocelične haploidne oblike in so se razmoževale z mitozo. Enostavni evkarioti kot so protisti in glive se mitotsko razmnožujejo v haploidnem in diploidnem stanju. Diploidnost predstavlja skoraj gotovo sekundarno razvojno stanje. Enoceličarji iz katerih izvirajo prve mejotske delitve so obstajali pred več kot miljardo let, njihova sled je že dolgo zabrisana (Wilkins & Holliday, 2009). Začetna naloga homolognega kromosomskega parjenja (sinapsis) je bila presenetljivo zmanjšanje ne pa povečanje genske pestrosti.

## 2 MATERIAL IN METODE

Brste čemaža, ki je uspeval pri glavnem vhodu na laboratorijskem polju Biotehniške fakultete (BF) smo nabirali v mesecu marcu in aprilu 2008.

Mejozo lahko pri rastlinah opazujemo, če naberemo mlade neodprte cvetne brste. Brste fiksiramo v sveže pripravljeni mešanici etanola in ocetne kisline v razmerju 3:1 in jih spravimo v hladilnik.

Precej zamudno je iskanje prave razvitosti cvetnih brstov za opazovanje stadijev mejoze. Pri čemažu smo cvetne brste nabirali tedensko ves mesec marec in april 2008.

Za opazovanje preparatov iz cvetnih brstov pod mikroskopom z binokuarno lupo izberemo ponavadi mlade prašnice (moška linija mejoze). Zarodne celice mikrospor mečkamo na objektnih stekelcih, obarvamo z acetokarminom ter preparate ogrejemo nad gorilnikom (Göltenboth, 1978). Nato opazujemo zarodne celice pelodnih zrn pod mikroskopom pri 400 x povečavi. Slike stadijev mejoze smo posneli z raziskovalnim mikroskopom "Olympus" Provis z digitalno kamero na naši katedri. Slike smo digitalno obrezali in povečali s programsko opremo za digitalno analizo slike "Olympus" DP Controller.

Mejoza je bolj zapletena kot mitoza, ker nastopata dve zaporedni zoritveni delitvi in tudi stadijev je več. Da opazimo vse stadije je potrebno narediti več mikroskopskih preparatov iz različno razvitih (velikih) cvetnih brstov.

## 3 REZULTATI

Največ mejotskih stadijev smo opazovali pri cvetnih brstih nabranih v tretjem tednu v mesecu marcu. Optimalna je bila dolžina cvetnih brstov med 1 in 1,5 cm. V aprilu je bilo nabiranje neuspešno, ker je bila mejoza zaključena in smo lahko opazovali le pelodna zrna.

### Slike mejotskih stadijev čemaža

Diakineza je zadnji stadij profaze prve mejotske delitve (slika 1.) Nato sledi metafaza prve zoritvene delitve in anafaza, kjer pride do dejanskega zmanjšanja kromosomskega števila na polovico, saj se s pomočjo niti delitvenega vretena ločijo celi homologni kromosomi in ne sestrske kromatide, ki ostanejo spojene zaradi povečane kohezije (Petronczki in sod. 2003). Motnje pri parjenju homolognih kromosomov vodijo do nesparjenih kromosomov - univalentov in trivalentov. Motnje pri segregaciji kromosomov v mejozi (nondisjunction) vodijo do kromosomskih aberacij - anevvuploidij, pride lahko do monosomij ali trisomij (Petronczki in sod., 2003; Sanso in Wulff , 2007).

Končni stadij prve mejotske delitve je celična diada (slika 2.).

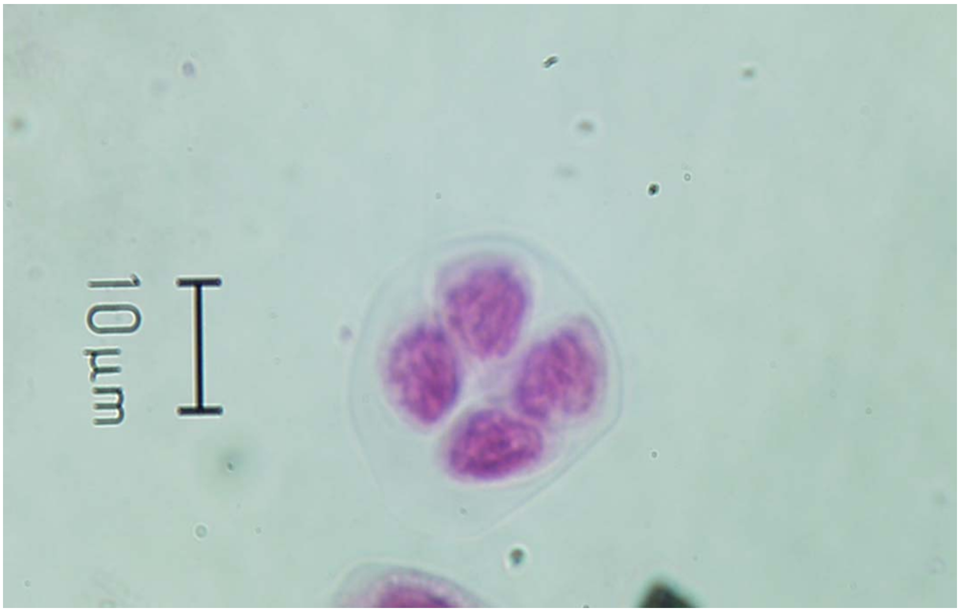
Sledi druga zoritvena delitev enaka mitozi in služi samo za povečanje števila gamet. Končni stadij (telofazo) predstavljajo 4 haploidna jedra ali celična tetrada (slika 3.). Zaradi pojava prerazporejanja genov, vsebuje vsako jedro nekoliko drugačne dedne zasnove.

**Slika 1**: Diakineza – zadnje obdobje profaze prve zoritvene delitve. Na mikroskopski sliki je sedem bivalentov (sparjenih homolognih kromosomov) čemaža (Allium ursinum L.), ki ima telesno kromosomsko število 2n = 14).

**Figure 1**:Diakinesis – the last period of prophase of the first meiotic division. The seven bivalents of ramsons (Allium ursinum L.) are shown, with the somatic chromosome number 2n = 14.

**Slika 2**: Celična diada je telofaza prve zoritvene delitve z dvema haploidnima jedroma.

**Figure 2**: Cell diade is the telophase of the first meiotic division with two haploid nuclei.



**Slika 3**: Celična tetrada je končni stadij druge zoritvene delitve s štirimi haploidnimi jedri.
**Figure 3**: Cell tetrade is the final phase of the second meiotic division with four haploid nuclei.

## 4 ZAKLJUČEK IN DISKUSIJA

Značilnost prve zoritvene delitve ali mejoze I je, da iz ene diploidne zarodne celice nastaneta dve haploidni jedri (celična diada). Pri drugi zoritveni delitvi ali mejozi II iz dveh haploidnih jeder nastanejo 4 haploidna jedra (celična tetrada).

Podobno kot pri mitozi ločimo štiri faze tudi pri mejozi. Osnovne značilnosti faz mejoze so podobne kot pri mitozi. Prva je profaza, sledi metafaza, nato anafaza in končno telofaza pri prvi in drugi zoritveni delitvi.

Mejotski kromosomi so drugače oblikovani kot mitotski kromosomi v telesnih celicah iste rastline. Mejoza se je zelo verjetno razvila iz mitoze (Wilkins in Holliday 2009) in ima štiri nove dogodke v primerjavi z mitozo:

- parjenje homolognih kromosomov,
- pojav genetskega prerazporejanja med nesestrskimi kromatidami,
- v prvi zoritveni delitvi se ne ločujejo kromatide ampak celi homologni kromosomomi,
- dedni material se v drugi zoritveni delitvi ne podvaja (brez S faze).

Mejoza se kot mitoza prične z podvajanjem DNK, tvorijo se celice z štirimi kromatidami za vsak homologni kromosomski par, dve kromatidi izvirajo iz materine rastline, dve iz očetove. Tudi pri mejozi obstajajo kontrolne točke (Jezernik in Komel, 1998), ki preverjajo predhodne dogodke v mejozi in nato sprožijo procese naprej ali jih ustavijo. V profazi I mejotske delitve obstaja pahitenska kontrolna točka (Roeder in Bailis 2000), nato še rekombinacijska kontrolna točka (Malone in sod. 2004) in druge kontrolne točke.

Največ mejotskih stadijev čemaža smo opazovali pri cvetnih brstih nabranih v mesecu marcu in približni dolžini cvetnih brstov 1 do 1,5 cm. Pri enaki dolžini cvetnih brstov v aprilu je bilo opazovanje neuspešno. Verjetno so mejotski stadiji pri čemažu vezani na koledarsko obdobje (dolžina dneva, temperature) in hkrati je tudi potrebna optimalna razvitost cvetnih brstov (med 1 in 1,5 cm).

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# Virulenčni faktorji glive *Verticillium albo-atrum*

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## IZVLEČEK

Gliva *Verticillium albo-atrum* je pomemben rastlinski patogen, ki lahko okuži več sto rastlinskih vrst in povzroča precejšnjo škodo v kmetijstvu. O mehanizmih patogeneze te glive je znanega zelo malo, pred kratkim pa je bil sekvenciran njen genom, kar ponuja dobre obete za raziskave na tem področju. V tej raziskavi sva pregledala literaturo o znanih genih gliv, ki sodelujejo pri napadu in pojavu simptomov bolezni, nakar sva v genomu *V. albo-atrum* z uporabo orodja BLAST poiskala homologe vseh najdenih genov in poskusila prepoznati razloge za virulenco te pomembne patogene glive.

**Ključne besede:** *Verticillium albo-atrum*, glivni efektorji, patogeneza, BLAST

## ABSTRACT

### VIRULENCE FACTORS OF THE FUNGUS *Verticillium albo-atrum*

The fungus *Verticillium albo-atrum* is an important plant pathogen, capable of infecting several hundred plant species and causing considerable agricultural losses. Pathogenesys mechanisms of this fungus are poorly understood, but the recent sequencing of its genome offers good foresight for conducting research on this topic. We have surveyed accessible knowledge on known pathogenesis related fungal genes and conducted a search of gene homologs of these genes in the genome of *V. albo-atrum* using BLAST. From retrieved data, we tried to form a possible explanation for the high virulence of this important pathogenic fungus.

**Key words:** *Verticillium albo-atrum*, fungal effectors, pathogenesis, BLAST

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## 1 UVOD

Talne glive rodu *Verticillium* povzročajo bolezni pri več kot 200 rastlinskih vrstah, ki rastejo *v* zmernem in subtropskem podnebju. Znaki bolezni so uvelost, kloroza, pobledelost listnih žil in nekroza. Napadajo pomembne poljščine in vsako leto povzročijo več milijard dolarjev škode v pridelku po vsem svetu. Vrste rodu *Verticillium* izločajo nizokomolekularne fitotoksine in encime, ki razgrajujejo rastlinsko celično steno. V svojem patogenem ciklu tvorijo posebne strukture imenovane mikrosklerociji in melanizirani miceliji, ki omogočajo dolgotrajni parazitizem rastline (Fradin in Thomma, 2006). Gliva *V. dahliae* ima sposobnost izredne prilagoditve na spreminjajoče se okolje in lahko parazitira rastline različnih ekoloških niš. Strategije nadzora teh bolezni so slabo razvite in neučinkovite zaradi slabega razumevanja biologije in ekologije glive *V. dahliae*. Glavni povzročitelj hmeljeve uvelosti je gliva *V. albo-atrum*, ki je tudi splošno razširjena, vendar se njen nabor gostiteljev močno razlikuje od gostiteljev glive *V. dahliae* (Bhat in sod., 1999, Qin in sod., 2006). D*a* bi ugotovili glavne lastnosti glive, ki omogočajo preživetje, patogenost in

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virulenco, so na inštitutu Broad posekvencirali celoten genom obeh zgoraj naštetih predstavnikov rodu *Verticillium*. Obe nukleotidni zaporedji genomov sta dostopni na spletnem strežniku Ameriškega nacionalnega centra za biotehnologijo (NCBI**)**.

### 1.1 Pregled glivnih efektorskih proteinov

Glivni efektorji so beljakovine ali majhne molekule, ki vplivajo na strukturo in funkcijo gostiteljskih celic (Ellis in sod., 2009). Vpliv glivnih efektorjev na rastlinsko tkivo se lahko izrazi kot bolezen (virulenčni dejavniki) ali pa kot sproženje obrambnih mehanizmov proti patogenu (avirulenčni dejavniki).

Patogene glive izločajo proteine, ki vplivajo na strukturo in funkcijo gostiteljskih celic, kot so npr. encimi in avirulenčni faktorji ter sintaze fitotoksinov. Encimi med patogenezo pospešujejo privzem kompleksnih ogljikovih hranil in privzem dušika iz nepreferenčnih virov rastlinskega organizma ter omogočajo glivi razstrupljanje in izogibanje obrambnim mehanizmom rastline. Encimi, ki jih izločajo številni patogeni rodovi gliv so npr. celulaze, kutinaze, celobiaze, ksilanaze, glukozidaze, celobiohidrolaze, lipaze in proteaze, pomembni patogeni dejavniki pa so tudi sami transkripcijski faktorji, ki regulirajo njihovo izražanje (npr. areA, xlnR in ctf1α; Bluhm in sod., 2008).

Nizkomolekularni fitotoksini so sekundarni metaboliti, ki jih proizvajajo glivne poliketidne sintetaze, in so pogosto ključni za infekcijo in virulenco ter z njima povezanimi simptomi bolezni. Fitotoksini lahko delujejo na različne celične tarče, lahko spremenijo gensko ekspresijo ali oslabijo integriteto celične membrane. Fotosenzibilizatorji so fitotoksini, ki tvorijo reaktivne kisikove oblike, te pa poškodujejo rastlinsko celico in lipidne membrane. Cerkosporin in elzinohrom vsebujeta perilenkvinonski kromofor, ki po absorpciji svetlobne energije preide v aktivirano tripletno stanje, to pa s kisikom tvori $O_2^-$ in $H_2O_2$. Cerkosporin izločajo glive rodu *Cercospora*, ki sintetizirajo poliketidno sintetazo CTB1. Same so pred toksinom zavarovane z ABC transporterjem (CFP1, kodiran v genu ctb4), ki aktivno izloča toksin iz celic. Gliva *Ramularia collocygni* izloča rubelin D, ki v rastlinski celici sproži peroksidacijo alfa-linoleične kisline. Epipolitiodioksopiperazini vsebujejo notranje disulfidne ali trisulfidne mostičke, ki omogočajo uničevanje gostiteljevih beljakovin. Primeri takih toksinov so sirodesmin PL, ki ga sintetizira *Leptosphaeria maculans*, in gliotoksini gliv rodov *Trichoderma* in *Aspergillus*. Številni fitotoksini inhibirajo encime, ki sodelujejo pri sintezi membranskih lipidov. Fumonizin gliv rodu *Fusarium* in AAL-toksin glive *Alternaria alternata* delujeta kot analoga sfingozina in tako inhibirata encima sfinganin–N-acetiltransferazo ter keramid sintazo. Posledično je moteno obnavljanje celične membrane, njena prepustnost pa se poveča. Fitotoksin ciperin je difenilni eter, ki ga izločajo številne patogene glive. Inhibira encim enoil reduktazo in posledično biosintezo lipidov. Gliva *Cercospora beticola* sintetizira betikoline ali rumene toksine, ki se v membranah sestavljajo v pore.

Črpanje esencialnih hranil kot je npr. železo je izredno pomembno za obstoj patogene glive. Pridobivanje železa iz rastlinskih celic poteka preko nizkomolekularnih kelatorjev imenovanih siderofori, kot je npr. ferikrocin iz *Alternaria brassicicola* in triacetilfuzarinin C (TAFC, iz *Aspergillus fumigatus*).

Blokiranje ATPaze vodi do popolnega zloma rastlinske celice. Ten-toskin sintetizirajo vrste rodu *Alternaria* in je ciklični tetrapeptid, ki se veže med α in β podenoto encima ATPaze v kloroplastu. Gliva *Cochliobolus heterostrophus* pa izloča T-toksin, ki vstopa v mitohondrij, kjer se veže na protein T-urf13, to pa sproži konformacijske spremembe in tvorbo pore. Posledično mitohondriji nabreknejo. Pri sintezi T-toksina sodelujejo trije biosintetski geni, in sicer dve poliketidni sintazi in ena dekarboskilaza (DEC1). Fitotoksin fuzikocin se veže na encim $H^+$ATPazo in jo stalno aktivira, to pa vodi do naraščanja izvencelične koncetracije reaktivne spojine $H_2O_2$. Fuzikocin sintetizira gliva *Phomopsis amygdale* z encimoma preniltransferazo (kondenzacija izoprenskih enot) in terpen ciklazo (ciklizacija C-20 prekurzorja).

Sprožitev apoptoze je ena glavnih strategij fitopatogenih gliv, saj omogoča hitro pridobivanje hranil iz rastline. Gliva *Fusarium graminearum* sintetizira fitotoksin deoksivalenol (DON); prvi encim v sintezni poti je trihodien sintaza, ki ciklizira farnezil pirofosfat do trihodiena. DON je gostiteljsko specifičen toksin, ki inhibira translacijo, ne da bi sprožil obrambni sistem rastline. Gliva je zaščitena proti toksinu z encimom trihotecen 3-O-acetiltransferazo (Tri101), ki acetilira DON v nenevaren 3-ADON.

Viktorin je ciklični pentapeptid iz glive *Cochliobolus victoriae*, ki povzroča rastlinsko rjo na ovsu. Toksin vstopa v mitohondrije rastlinskih celic in se veže na glicin dekarboksilazni kompleks. Posledično pride do cepitve DNA molekul, lipidne oksidacije, razgradnje encima RUBISCO in do inhibicije fotorespiracije.

Toksini iz družine citohalazanov (citohalazin A, citohalazin B in hetoglobozini) se specifično vežejo na aktinske filamente in tako blokirajo citokinezo. Gliva *Penicillium expansum* biosintetizira aminokislinsko hrbtenico haetoglobozina A in C s posebno hibridno neribosomalno poliketid sintetazo (CheA). Podobne hibridne poliketid sintetaze (ACE1) so odkrili tudi v genomu glive *Magnaporthe oryzae*.

Nekateri fitotoksini delujejo kot transkripcijski faktorji ali pa kot epigenetski modifikatorji. Yap1 sorodni protein je znan kot osrednji regulator kvasovke *S. cerevisiae*. V glivi *Ustilago maydis* pa je ta transkripcijski faktor vpleten v poces detoksifikacije s strani rastline proizvedenih reaktivnih kisikovih radikalov. HC-toksin glive *Cochliobolus carbonum* je ciklični tetrapeptid in deluje tako, da inhibira histon deacetilazo (HDAC), kar vodi do hiperacetilacije in posledično do sprememb v ekspresiji genov v rastlini. HC-toksin preko epigenetskih modifikacij stimulira privzem organskih in anorganskih molekul, predvsem nitrata, v koreninski sistem koruze. Gliva *Cochliobolus carbonum* vsebuje kompleksni lokus tox2, ki kodira HC-toksin sintetazo (HTS1) in protein, ki izloča HC-toksin (toxA), s čimer ščiti glivo pred lastnim toksinom (Möbius in Hertweck, 2009).

Rastline se branijo proti patogenim glivam, tako da s posebnimi receptorji prepoznavajo njihove ohranjene molekularne motive (ang. Pathogen Associated Molecular Patterns; PAMP; npr. hitin). Vendar so nekatere glive sposobne produkcije posebnih efektorskih proteinov kodiranih z avirulenčnimi geni (*Avr*), ki zaustavijo s strani PAMP stimulirani obrambni sistem. Skozi evolucijo so rastline razvile sekundarni sistem za prepoznavanje patogenov, in sicer preko prepoznavanja njihovih efektorskih proteinov z rastlinskimi proteini rezistence (R proteini). Hipoteza »gen za gen« trdi, da za vsak dominantni *Avr* gen patogene glive obstaja pripadajoči R gen v gostiteljski rastlini. Interakcija med proteinskima produktoma obeh pripadajočih genov vodi do aktivacije močnih rastlinskih odzivov kot je npr. hipersenzitivni odziv (HR; De Wit in sod., 2009). Avr geni so bili odkriti že pri številnih patogenih glivah kot npr. *Cladosporium fulvum* (Avr2, Avr4, Avr4E in Avr9), *Rhynchosporium secalis* (AvrRrs1/Nip1, Nip3 in Nip2), *Fusarium oxysporum* (Avr3/Six1, Avr2, Avr1), *Leptosphaeria maculans* (*AvrLm1–AvrLm9*), *Magnaporthe oryzae* (*Avr–Pita* geni, *Avr1–CO39, Ace1, Pwl* geni), *Magnaporthe grisea* (*Pwl* geni, *AVR-Pia, AVR-Pii* in *AVR-Pik/km/kp*), *Blumeria graminis* (*Avrk1* in *Avra10*) in *Melampsora lini* (*AvrL567 geni*, *AvrM geni*, *AvrP123 geni* in *AvrP4*). Za nekatere glivne efektorje še niso uspeli dokazati hipoteze »gen za gen«, npr. za *Ecp* gene iz *Cladosporium fulvum*, *Hum3*, *Pep1*, *Stp1* in *Rsp1* iz *U. maydis* ter nekatere *Tox* gene iz *Stagonospora nodorum* in Pyrenophora *tritici-repentis* (De Wit in sod., 2009).

## 2 MATERIAL IN METODE

Iz treh preglednih člankov iz posebne izdaje revije Current Opinion In Plant Biology, katere tema so bile interakcije rastlin z mikroorganizmi, sva izpisala imena vseh beljakovin oz. genov, za katere je znano, da so efektorji patogenih gliv (Ellis J.G. in sod., 2009; Hematy K. in sod., 2009; Möbius N. in Hertweck C., 2009). V programu Excel 2007 sva uredila seznam, v katerem sva posameznim efektorskim beljakovinam pripisala glivno vrsto, iz katere je bil efektor izoliran, ter referenco, ki je bila citirana v preglednem članku in se nanaša na določen efektor. Nato sva vse dosegljive po zgornjem postopku izpisane reference pregledala in izpisala morebitne novo najdene efektorske proteine. V bazi Protein na strežniku Entrez (www.ncbi.nlm.nih.gov/Entrez/) sva poiskala aminokislinska zaporedja v prejšnjem koraku najdenih efektorskih proteinov in jih prekopirala v skupno tekstovno datoteko.

S spletišča instituta BROAD (www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiDownloads.html) sva prenesla tekstovni datoteki v formatu FASTA s celotnim genomom seva glive *V. albo-atrum* VaMS 102 v obliki sosesk (contigs.fasta) ter z naborom vseh predvidenih genov (genes.fasta). V programskem paketu Blast 2.2.20 sva s programom formatdb.exe iz prenešenih datotek pripravila ločeni lokalni bazi za soseske in predvidene gene. Nato sva s programom blastall.exe (algoritem tblastn, največja dovoljena E-vrednost 0,01) izvedla iskanje možnih homologov v obeh bazah; kot vhodno datoteko sva uporabila tekstovno datoteko z v literaturi najdenimi efektorskimi beljakovinami (glej zgoraj). S celotnim naborom aminokislinskih zaporedij v literaturi najdenih efektorskih beljakovin sva izvedla poravnavo v programu ClustalX 2.0.12 (Larkin in sod., 2007), nato pa v istem programu na podlagi dobljene poravnave izračunala drevo po metodi združevanja sosedov (NJ; bootstrap=100). Za vse klade na drevesu z zadostno podporo (s parametrom bootstrap večjim od 50) sva izdelala nabore aminokislinskih zaporedij vseh članov določenega klada in vseh njihovih homologov v genomu *V. albo-atrum* in z njim izvedla poravnavo v programu Muscle v 3.6 (Edgar, 2007); na podlagi te poravnave sva izračunala drevo v programu ClustalX (metoda NJ, bootstrap=1000).

V bazi UniProt sva poiskala posamezne dostopne ontologije v literaturi najdenih efektorskih proteinov in jih zbrala v tabeli v programu Excel (geni iz *V. albo-atrum* pa so že vsebovali informacijo o predvideni funkciji v izhodiščni datoteki, dobljeni s spleta). Za vsako na podlagi evolucijske sorodnosti pridobljeno skupino glivnih efektorjev in njihovih homologov sva preverila, če se ujemajo tudi v ontologijah.

## 3 REZULTATI IN RAZPRAVA

### 3.1 Iskanje efektorskih proteinov v literaturi in iskanje njihovih homologov v genomu *V. albo-atrum*

Ob pregledu literature sva našla skupno 142 posameznih efektorskih beljakovin, in sicer 34 v izhodiščnih preglednih člankih in 108 v referencah, ki sva jih našla v teh preglednih člankih. Aminokislinsko zaporedje sva uspela najti le za 118 od teh beljakovin, od tega za 32 efektorjev iz preglednih člankov in 86 iz referenc. Ob izrisu filogenetskega drevesa vseh najdenih aminokislinskih zaporedij sva skupno število najdenih efektorjev zmanjšala, saj se je izkazalo, da je bila najina baza redundantna na račun različnih imen, pod katerimi so znani posamezni efektorji, in sicer za 11 efektorjev. Skupno število unikatnih najdenih aminokislinskih zaporedij znaša 107.

Za 52 efektorskih proteinov so bili z uporabo orodja BLAST najdeni homologi v genomu *Verticillium albo-atrum*. V lokalni bazi sosesk celotnega genoma je bilo zaznanih 1605 posameznih zadetkov (lokalnih poravnav efektorskega gena in soseske). V lokalni bazi z naborom predvidenih genov pa je bilo najdenih 1555 poravnav, torej le 3,1% manj kot v bazi sosesk. Iz tega sklepava, da je bil genom bioinformacijsko zadosti kakovostno anotiran. Razlika v številu poravnav je morebiti v prisotnosti dveh ali večih delov posameznih genov na večih kontigih in s tem v prelomu poravnav. Za vseh 52 efektorskih proteinov so bili najdeni skupno 443 homologi. V genomu verticilija je predvidenih 10221 genov, torej izkazuje homologijo z glivnimi efektorji vsaj 4,3% verticilijevih genov.

Iskanje efektorskih proteinov v literaturi in njihovih homologov v genomu *V. albo-atrum*

Z iskanjem v bazi UniProt ter kombiniranjem z izpisanimi podatki o genih iz literature nama je uspelo okarakterizirati 48 efektorskih proteinov od 54, ki imajo homologe v verticilijevem genomu. Izvleček ontologije posameznih efektorjev je prikazan v preglednici 1.

### 3.2 Verticilijevi geni, udeleženi v sintezi in transportu toksinov

Slika 1 prikazuje filogenetsko drevo štirih skupin efektorskih proteinov, prisotnih v genomu *V. albo-atrum*. Močno so zastopane poliketidne sintaze (Slika 1A), ki se z izhodiščnimi efektorskimi proteini grupirajo v tri veje, od katerih ena vsebuje večino homologov. V celotni skupini je 24 možnih homologov iz genoma *V. albo-atrum*. Le 2 od teh nimata predvidene funkcije, predlagane ontologije preostalih pa ustrezajo ontologiji efektorskih proteinov, ki so grupirani v isto drevo (biosintezna pot glivnih toksinov; Preglednica 1).

Na drevesu manjkajo 4 izhodiščni efektorski proteini, ki naj bi bili udeleženi v biosintetski poti toksinov, in sicer PaFS, Pdx1, ToxC in ToxF. PaFS se skupaj s tremi avirulentnimi geni Avr-Pita1, Avr-Pita2 in Avr-Pita3 združi še z verticilijevim homologom geranilgeranil pirofosfatne sintetaze v klad, ki kaže nekaj sorodnosti z metaloproteinazami in nevtralnimi proteinazami (Slika 1B). PaFS je encim v izoprenoidnem biosinteznem procesu (Möbius in Hertweck, 2009), proteini Avr-Pita pa so metaloproteinaze (Ellis in sod., 2009; De Wit in sod., 2009); morda malo preseneča možnost, da so Avr-Pita sorodnejši verticilijevim homologom za sintezo izoprenoidov kot homologom metaloproteinaz. Pdx1 je udeležen v sintezi piridoksinskega obroča; v verticilijevem genomu ima le en homolog, katerega predvidena funkcija je ustrezna (PDX1, protein piridoksinske sinteze). ToxC je sintaza maščobnih kislin, ki sodeluje v sintezi HC-toksina, v verticilijevem genomu ima en homolog z ustrezno predvideno funkcijo. ToxF je aminotransferaza, tudi udeležena v sintezi HC-toksina; ima 4 homologe, vse z ustrezno predvideno funkcijo; le z enim ima zelo dobro poravnavo ($E < 10^{-60}$).

Tri12, ToxA in Cfp so membranski proteini, ki prenašajo proizvedene toksine iz glivne celice. Filogenetsko so si sorodni (Slika 1D). V verticilijevem genomu imajo 18 predvidenih homologov; Tri12 ima 9 homologov, ToxA in Cfp pa pripadata drugi veji, ki vsebuje še 3 njune statistično in ontološko dobro podprte homologe. Ctb4 in Fer2 pripadata enaki ontologiji, a sta filogenetsko bolj oddaljena; prvi ima 19 homologov, med najboljšimi zadetki so poliaminski transporterji. Fer2 je membranski transportni protein za železo, torej ima popolnoma drugo funkcijo kot preostali štirje zgoraj opisani; v verticilijevem genomu ima 4 homologe. Pri sintezi toksina cerkosporina v *C. nicotianae* sodelujeta tudi proteina Ctb2 in Ctb3, ki sta tudi zastopana pri verticiliju s 4 homologi.

**Preglednica 1.** Razporeditev v literaturi najdenih glivnih efektorskih proteinov glede na celični proces in molekularno funkcijo.

**Table 1:** Grouping of accessible fungal effector proteins by cellular process and molecular function

| Proces | Št. beljak. | Funkcija (število efektorjev;efektorji) |
|---|---|---|
| Biosinteza | 14 | Biosintetska pot glivnih toksinov (15; Ace1, CheA, Ctb1, Efpks1, Fuss, HTS1, HyNRPS, Nps6, PaFS, Pdx1, PKS5, SirP, ToxC, ToxF) |
| Metabolni proces | 11 | Oksidoreduktaza (1; Aox) |
| | | Prenos acilne skupine (2; Cps1, NRPS) |
| | | Razgradnja ogljikovih hidratov (7; α-glukozidaza, β-glukozidaza, Cbp1, celulaza, EIX, ksilanaza, Pls1) |
| | | Razgradnja maščob (1; ekstracelularna lipaza) |
| Oksidoreduktivni procesi | 8 | Katalazna aktivnost (1; katalaza) |
| | | Monooksigenazna aktivnost (1; Shh1) |
| | | O-metiltransferazna aktivnost (2;Ctb2, Ctb3) |
| | | Prenos acilne skupine (1; Pks1), |
| | | Vezava kovinskih ionov (3; Fer1, NoxA, ToxD) |
| Proteoliza | 3 | Metaloendopeptidaza (3; Avr-Pita1, Avr-Pita2, Avr-pita3) |
| Celična signalizacija | 1 | MAP kinaza (1; Vmk1) |
| Regulacija prepisovanja mRNA | 5 | Protein, ki veže cinkove ione (4; Cf1α, Ctb8, Nrf1, XlnR ) |
| | | Iz družine bZip (1; ToxE) |
| Prenos čez celično membrano | 5 | Prenos toksinov iz glivne celice (5; Cfp, Ctb4, Fer2, ToxA, Tri12) |
| Komunikacija med celicami | 1 | Hidrofobin (Vdh1) |
| Ni znano | 4 | AnPhiA, Gas1, Gas2, VdNep |

### 3.3 Glivni encimi s homologi v V. albo-atrum

V verticilijevem genomu so dobro zastopani tudi katalitični encimi, denimo hidrolaze z 18 homologi v vsaj dveh družinah proteinov (Slika 1C). Homologov α-glukozidaze je 7, homologov β-glukozidaze pa 23.

Cbp1 in Pls1 sodelujeta pri tvorbi posebne invazivne strukture pri *M. grisea* (Möbius in Hertweck, 2009). Pri verticiliju ima prvi vsaj 5 homologov, drugi pa enega statistično dobro podprtega.

Pri verticiliju najdemo še dva homologa alkohol oksidaze (ki je virulenčni dejavnik pri *Cladosporium fulvum*), 13 homologov aciltransferaz in 19 homologov ekstracelularnih lipaz. Proteini, ki vežejo kovinske ione, so tudi dobro zastopani; homologov že obravnavanega Fer2 ter NoxA, ki vežeta železo, je skupno 11, homologov ToxD, ki veže cink in je pomemben člen metabolne poti HC-toksina, pa je pri verticiliju 14.

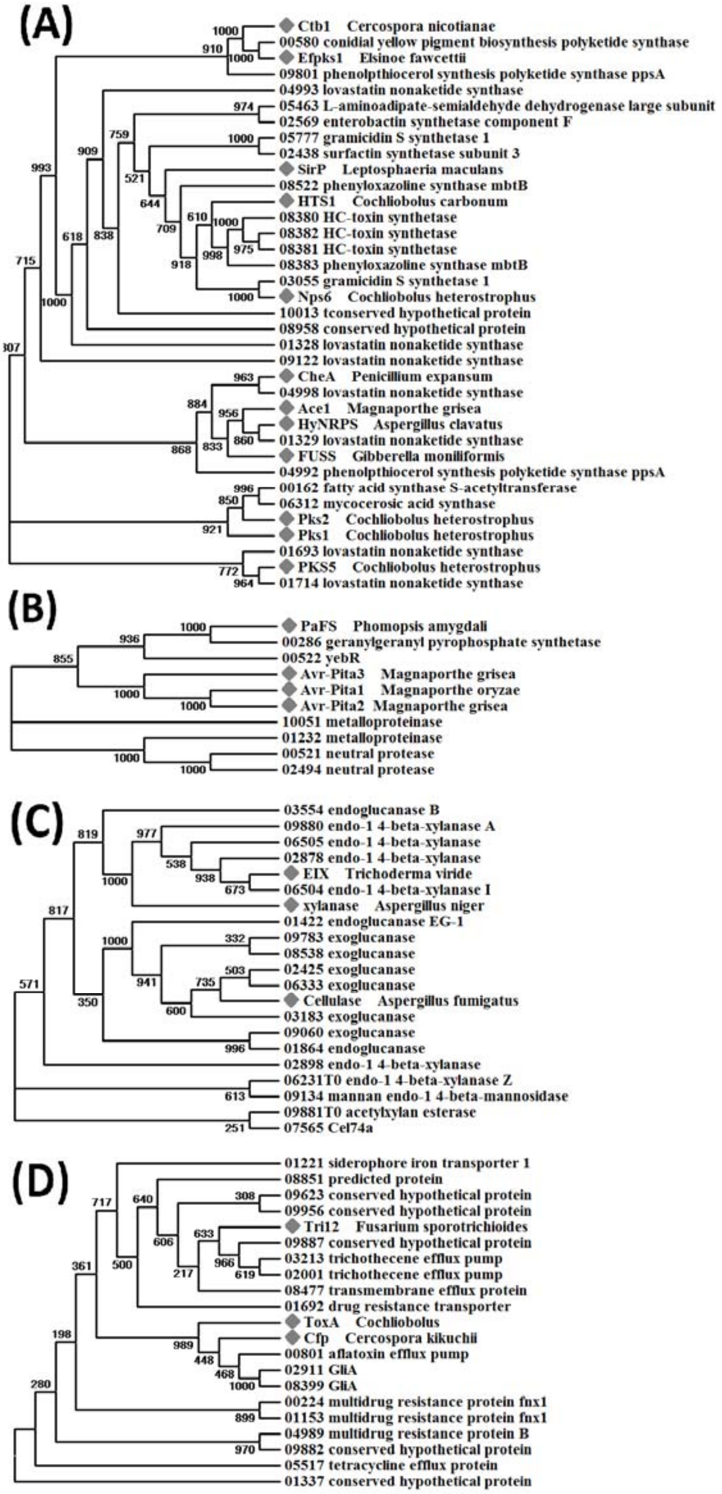
Katalaze so pomembne pri preprečevanju oksidativnega stresa glive in so v verticiliju prisotne vsaj 3; homologov monooksigenaze Shh1 iz glive *Fusarium oxisporum*, ki je tudi udeležena v preprečevanju oksidativnega stresa, pa je 8.

### 3.4 Verticilijevi homologi s patogenezo povezanih transkripcijskih faktorjev, proteinskih kinaz in proteinov za medcelično komunikacijo

Vmk1 je signalni protein (MAP kinaza), za katerega je znana vloga v celični signalizaciji pri tvorbi mikrosklerocijev (mirovnih struktur) pri glivi *V. dahliae* (Fradin in Thomma, 2006). V genomu *V. albo-atrum* je 74 homologov Vmk1, od katerih ima 5 zelo dobro poravnavo z Vmk1. Tudi Vdh1 je udeležen pri tvorbi mikrosklerocijev pri *V. dahliae*, le da gre za protein, udeležen v medcelični signalizaciji; v genomu *V. albo-atrum* ima 3 homologe, kar je razumljivo, saj obe sorodni vrsti gliv tvorita ta tip mirovne strukture (Fradin in Thomma, 2006).

Transkripcijski faktor Ctb8 omogoča prepisovanje genov za encime, ki sodelujejo v sintezi različnih glivnih toksinov; pri *V. albo-atrum* ima 6 homologov. Nrf1, ki sodeluje pri prepisovanju genov za sintezo sideroforov (Möbius in Hertweck, 2009), ima pri verticiliju 4 homologe.

XlnR in Cf1α sta transkripcijska faktorja, ki sodelujeta pri regulaciji sinteze katalitičnih encimov (ksilanaz ter kutinaz; Ellis in sod, 2009). Prvi ima skupno 15 homologov v verticilijevem genomu, od tega 2 z dobro poravnavo ($E < 10^{-100}$). Cf1α ima 31 homologov, od tega 2 z dobro poravnavo ($E < 10^{-120}$)



**Slika 1:** Filogenetska drevesa posameznih skupin v literaturi najdenih glivnih efektorjev (posamezni označeni z sivim karom) in njihovih homologov v genomu *V. albo-atrum*. (A) poliketidne sintaze, (B) proteinaze, (C) hidrolitski encimi, (D) transporterji toksinov.

**Figure 1:** Phylogenetic trees drawn for different groups of fungal effectors retrieved from literature (grey rectangles) and their homologs in the *V. albo-atrum* genome. (A) polyketide synthetases, (B) proteinases, (C) hydrolytic enzymes, (D) toxin transporters

### 3.5 Verticilijevi homologi proteinov z do sedaj še neznano molekulsko funkcijo

Za 4 v literaturi najdene glivne virulenčne dejavnike, ki imajo tudi zadetke v verticilijevem genomu, ni znano, v katerem biološkem procesu sodelujejo. Za protein AnPhiA je znano le to, da se nahaja v celični steni glive; zanj sva našla 1 homolog. Za med seboj sorodna Gas1 in Gas2 so bili najdeni 4 homologi, za VdNep pa 7 homologov. Pri slednjem gre za gene za proteine, ki se izločajo iz celice.

## 4 SKLEPI

Pričujoča raziskava genoma *V. albo atrum* je pokazala na možne osrednje vzroke virulence tega pomembnega glivnega patogena.Verticilij poseduje večje število homologov genov, za katere je znano, da sodelujejo v sintezi glivnih toksinov. Zraven tega ima homologe prenašalcev toksinov in genov, ki sodelujejo v lastni odpornosti glive nanje, in tudi možnih transkripcijskih faktorjev za izražanje teh genov. Verticilij ima homologe za večino znanih genov, povezanih s sintezo in izločanjem tako cerkosporina kot tudi HC-toksina.V genomu verticilija je obsežen nabor genov, ki sodelujejo v razgradnji rastlinske celične stene. Verticilij poseduje gene, ki mu omogočajo razgradnjo kisikovih prostih radikalov, ki so tudi način obrambe rastline pred patogenom. V verticilijevem genomu so tudi homologi za nekatere proteine, katerih delovanje še ni znano, a je bilo z genetskimi študijami pokazano, da imajo pomembno vlogo v poteku glivne virulence. Čeprav sva iz literature izpisala večje število znanih avirulenčnih genov, sva v genomu verticilija našla le eno družino takšnih genov (Avr-Pita1-3). Morebiti je to razlog za slabši obrambni odziv rastline na napad glivnega patogena

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# VSEBINSKA OBDELAVA PRISPEVKOV V ACTA AGRICULTURAE SLOVENICA let. 97 št. 3

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## SUBJECT INDEX BY AGROVOC DESCRIPTORS
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## POPRAVEK/ CORRIGENDUM

Za zvezek 97-2 pravilen opis slike na naslovni strani: Bučno polje v Prekmurju, Slovenija (ilustracija k članku na str. 145).

In issue 97-2, correct description of Cover photo: Pumpkins field in Prekmurje, Slovenija (illustration to paper on page 145).

## ZAPIS/ NOTE

Na proslavi 50 letnice Oddelka za živilstvo Biotehniške fakultete, Univerze v Ljubljani je uredništvo Acta agriculturae Slovenica – Zbornik BF prejelo pisno zahvalo Oddelka za živilstvo.

At the celebration of the 50th Anniversary of the Food Science and Technology Department, Biotechnical Faculty, University of Ljubljana, the editorial and technical stuff of Acta agriculturae Slovenica received Acknowledgment Document from the Food Science and Technology Department.

# NAVODILA AVTORJEM

## Prispevki

Sprejemamo izvirne znanstvene članke, predhodne objave in raziskovalne notice s področja agronomije, hortikulture, rastlinske biotehnologije, raziskave živil rastlinskega izvora, agrarne ekonomike in informatike ter s sorodnih področij v slovenskem, angleškem in nemškem jeziku, znanstveno pregledne članke samo po poprejšnjem dogovoru. Objavljamo prispevke, podane na simpozijih, ki niso bili v celoti objavljeni v zborniku simpozija. Če je prispevek del diplomske naloge, magistrskega ali doktorskega dela, navedemo to in tudi mentorja na dnu prve strani. Navedbe morajo biti v slovenskem in angleškem jeziku.

Pri prispevkih v slovenskem jeziku morajo biti preglednice, grafikoni, slike in priloge dvojezični, povsod je slovenščina na prvem mestu. Naslovi grafikonov in slik so pod njimi. Slike in grafikoni so v besedilu. Priloženi morajo biti tudi jasno označeni izvirniki slik. Na avtorjevo željo jih vračamo, s tem da je želja pisno sporočena ob oddaji gradiva in ponovno v teku 30 dni po izidu. Latinske izraze pišemo ležeče. V slovenščini uporabljamo decimalno vejico, v angleščini decimalno piko. Prispevki v angleščini morajo imeti povzetek v slovenščini in obratno. Prispevki v nemščini morajo imeti tudi povzetka v slovenščini in angleščini.

Prispevki naj bodo strnjeni, kratki, praviloma največ 12 strani. Uporabljamo Microsoft Word 97 (Windows); pisava Times New Roman, velikost strani 16,2 x 23,5 cm, velikost črk besedila 10, v obsežnih preglednicah je lahko 8; izvlečki in metode dela Arial velikost 8, levi in desni rob 2,1 cm, zgornji rob 1,3 cm, spodnji rob 1,6 cm,

## Prva stran

Na prvi strani prispevka na desni strani označimo vrsto prispevka v slovenščini in angleščini, sledi naslov prispevka, pod njim avtorji. Ime avtorjev navedemo v polni obliki (ime in priimek). Vsak avtor naj bo označen z indeksom, ki ga navedemo takoj pod avtorji, in vsebuje polni naslov ustanove ter znanstveni in akademski naslov; vse v jeziku prispevka. Navedemo sedež ustanove, kjer avtor dela. Če je raziskava opravljena drugje, avtor navede tudi sedež te inštitucije. Na željo avtorjev bomo navedli naslov elektronske pošte.

Pod naslovi avtorjev je datum prispetja in datum sprejetja prispevka, ki ostaneta odprta. Sledi razumljiv in poveden izvleček z do 250 besedami. Vsebuje namen in metode dela, rezultate, razpravo in sklepe. Sledijo ključne besede.

Izvlečku v jeziku objave sledi naslov in izvleček s ključnimi besedami v drugem jeziku.

## Viri

V besedilu navajamo v oklepaju avtorja in leto objave: (priimek, leto). Če sta avtorja dva, pišemo: (priimek in priimek, leto), če je avtorjev več, pišemo: (priimek in sod., leto). Sekundarni vir označimo z “navedeno v” ali “cv.”. Seznam virov je na koncu prispevka, neoštevilčen in v abecednem redu. Vire istega avtorja, objavljene v istem letu, razvrstimo kronološko z a, b, c. Primer: 1997a. Navajanje literature naj bo popolno: pri revijah letnik, leto, številka, strani; pri knjigah kraj, založba, leto, strani. Za naslove revij je dovoljena uradna okrajšava, za okrajšanimi besedami naj bodo vedno pike. Navedbo zaključimo s piko. Za primere upoštevajte objave v Zborniku BFUL.

## Oddaja

Avtorji prispevke oddajo v dveh izvodih, enega z dvojnim razmakom med vrsticami in največ 35 vrst na strani, in na disketi. Priložijo tudi izjavo s podpisi vseh avtorjev, da avtorske pravice v celoti odstopajo reviji.

Prispevke recenziramo in lektoriramo. Praviloma pošljemo mnenje prvemu avtorju, po želji lahko tudi drugače. Če uredniki ali recenzenti predlagajo spremembe oz. izboljšave, vrne avtor popravljeno besedilo v 10 dneh v dveh izvodih, enega z dvojnim razmakom. Ko prvi avtor vnese še uredniške pripombe, odda popravljeno besedilo v enem izvodu in na disketi ter vrne izvod z uredniškimi popravki.

Prispevke sprejemamo vse leto.

## NOTES FOR AUTHORS

### Papers

We publish original scientific papers, preliminary communications and research statements on the subject of agronomy, horticulture, plant biotechnology, food technology of foods of plant origin, agricultural economics and informatics; in Slovenian, English and German languages while scientific reviews are published only upon agreement. Reports presented on conferences that were not published entirely in the conference reports can be published. If the paper is a part of diploma thesis, master of science thesis or dissertation, it should be indicated at the bottom of the front page as well as the name of the supervisor. All notes should be written in Slovenian and English language.

Papers in Slovenian language should have tables, graphs, figures and appendices in both languages, Slovenian language being the first. Titles of graphs and figures are below them. Figures and graphs are part of the text. Clearly marked origins of figures should be added; they can be returned if author desires. Latin expressions are written in italics. Decimal coma is used in Slovenian and decimal point in English. Papers in English should contain abstract in Slovenian and *vice versa*. Papers in German should contain abstracts in German, Slovenian and English.

The papers should be condensed, short and usually should not exceed 12 pages. Microsoft Word 97 (Windows) should be used, fonts Times New Roman, paper size 16.2 x 23.5 cm, font size in main text 10; in large tables size 8 could be used, abstracts and material and methods Arial size 8, right and left margin 2.1 cm, upper margin 1.3 cm and lower margin 1.6 cm.

### First page

The type of the paper should be indicated on the first page on the right side in Slovenian and English language following by title of the paper and authors. Full names of authors are used (first name and surname). Each name of the author should have been added an index, which is put immediately after the author(s), and contains address of the institution and academic degree of the author, in the language of the paper. The address of the institution in which the author works is indicated. If the research was realised elsewhere, the author should name the headquarters of the institution. E-mail is optional.

Under the address of the authors some space for dates of arrival and acceptance for publishing should be left. A comprehensive and explicit abstract up to 250 words follows indicating the objective and methods of work, results, discussion and conclusions. Key words follow the abstract.

The abstract in the language of the paper is followed by the title, abstract and key words in another language.

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Papers are reviewed and edited. First author receives a review. If reviewers suggest some corrections, the author should forward them in 10 days and in two copies, one of them with double space. After the first author considers the editor’s notes, the corrected paper should be sent in one copy and on a diskette.

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