GOCE DELCEV UNIVERSITY - STIP
FACULTY OF AGRICULTURE

JOURNAL OF AGRICULTURE AND PLANT SCIENCES

YEAR 2017 VOLUME 15, Number1/2

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Journal of Agriculture and Plant Sciences, JAPS, Vol 15
Successor of the Yearbook of Faculty of Agriculture of GDU, Vol 14

ISSN 2545-4447 print
ISSN 2545-4455 on line
Vol. 15, No. 1/2, Year 2017

GOCE DELCEV UNIVERSITY - STIP, REPUBLIC OF MACEDONIA
FACULTY OF AGRICULTURE

UDC 63(058)
ISSN 2545-4447 print
ISSN 2545-4455 on line

Journal of Agriculture and Plant Sciences, JAPS, Vol 15
Successor of the Yearbook of Faculty of Agriculture of GDU, Vol 14

YEAR 2017 VOLUME XV, Number 1/2

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CONTENT

INTRODUCTION

Journal of Agriculture and Plant Sciences (JAPS) is a successor of the national journal **Yearbook of Faculty of Agriculture of Goce Delcev University**. Starting from 2001, with the first edition of the Yearbook of the JNU Institute of Southern Crops (Vol. 1-5), since 2006 with the editions of the Yearbooks of Faculty of Agriculture (Vol. 6-14), we continue with our publishing activity through publication of the **Journal of Agriculture and Plant Sciences, Vol. 15 (JAPS)** as an international journal.

Science is the primary factor for the constructive development of each area of a modern society. The involvement of science in agriculture is one of our leading priorities. By doing this we promote the production of healthy food, we contribute to the development of the processing industry for agricultural products, we influence the management of our natural resources, and therefore, we influence directly in the development of the rural and urban environment. **Journal of Agriculture and Plant Sciences (JAPS)** is a semi-annual journal devoted to the advancement and dissemination of scientific knowledge concerning all basic and allied sciences to Agriculture.

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Editorial Board,
December, 2017

Editor in chief,
Prof. Liljana Koleva Gudeva, PhD

JOURNAL OF AGRICULTURE AND PLANT SCIENCES, JAPS, Vol 15, No. 1/2, 2017

Manuscript received: 09.10.2017
Accepted: 24.10.2017

In print: ISSN 2545-4447
On line: ISSN 2545-4455
UDC: 635.64-235:579.25
635.649-235:579.25
Original scientific paper

MULTIGENE CHARACTERIZATION OF '*CANDIDATUS* PHYTOPLASMA SOLANI' IN PEPPER AND TOMATO PLANTS IN THE REPUBLIC OF MACEDONIA

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Abstract

'*Candidatus* Phytoplasma solani' is a phytoplasma distributed worldwide, associated in the Euro-Mediterranean area with various diseases in most spreader and cultivated plants including grapevine, maize, potato, pepper, celery and tobacco.

Molecular multigene characterization was performed for the first time on phytoplasma strains collected from the Eastern part of Macedonia from symptomatic and asymptomatic pepper and tomato plants, during the summer period of the 2015/16 season. PCR amplification on specific *tuf*, *vmp*1 and *stamp* genes showed that the phytoplasma strains identified belong to subgroup 16SrXII-A, since their restriction patterns were indistinguishable from one another and from the patterns characteristic of the STOL (16SrXII-A) reference strain.

For samples positive for the *tuf* gene, it was possible to identify the *Hpa*II RFLP profiles associated with *tuf* type b genes.

This is the first field observation and molecular laboratory testing for the presence of stolbur phytoplasma in pepper and tomato in the Republic of Macedonia.

Key words: *pepper, tomato, stolbur phytoplasma, multilocus genetic analysis*

INTRODUCTION

Phytoplasmas are obligate, phloem-limited phytopathogens. They are pleomorphic prokaryotes without cell walls. Phytoplasmas are transmitted to plants in the process of feeding of their vectors, sap-sucking hemipteran insects, mainly leafhoppers, planthoppers, and psyllids (Bertaccini et al., 2014).

Since the identification of phytoplasmas by standard microbiological methods in routinely grown laboratory cultures was not possible until recently, they are classified in a system of groups and subgroups based on DNA fingerprints (RFLP patterns) of 16S rRNA genes (16S rDNA) (Lee et al., 1998). Based on 16S rRNA gene sequence identity and biological properties, group 16SrXII encompasses several species, including STOL – '*Candidatus* Phytoplasma solani' in subgroup 16SrXII-A, and '*Candidatus* Phytoplasma australiense', '*Candidatus* Phytoplasma japonicum' and '*Candidatus* Phytoplasma fragariae' within subgroup 16SrXII-B. 16SrXII-A phytoplasma strains are associated with stolbur disease in numerous cultivated and wild plants, hence they are commonly known as stolbur phytoplasmas (Bertaccini et al., 2014).

Bois noir phytoplasma was shown to be transmitted by the planthopper *Hyalesthes obsoletus*, which normally feeds on herbaceous weeds, and occasionally also on grapevines (Berger et al. 2009).

In Macedonia, agriculture uses well-established traditions in the production of vegetables, more specifically various pepper cultivars (Gudeva et al., 2007; Mitrev and Spasov, 1999). Till now, only grapevines, the most spreader cultivars in the Tikves region, are known as host plants for stolbur phytoplasma in Macedonia (Kostadinovska et al., 2014a, 2014b; Mitrev et al., 2008, 2012).

Strumica and Kocani are the best-developed regions in Eastern Macedonia for the cultivation of different varieties of pepper

(*Capsicum annuum* L.) and tomato (*Solanum lycopersicum* L.) in the open field and most often under greenhouse or glasshouse conditions. Great agrobiological diversity, of 129 domestic and 2205 imported varieties of *Capsicum* spp. (Gudeva and Trajkova, 2008), has been documented in the country; throughout the last 30 years, most of them have been successfully introduced for fresh consumption as well as for industrial processing.

The aim of this study was to check the distribution of this disease in Eastern Macedonia, in Strumica and in Kochani as the main pepper cultivation regions, and to identify and characterize the disease with modern and reliable molecular methods, including multilocus genetic analysis of the 16S rRNA gene.

MATERIAL AND METHODS

Collection of stolbur-symptomatic pepper and tomato plants

During our field surveys carried out from the beginning of August till the end of October 2015/16, pepper and tomato leaf samples were collected from 24 symptomatic and asymptomatic plants from 11 localities in Eastern Macedonia, in Strumica and Kocani regions (Figs. 1 and 2). Of all samples analysed, four sweet red pepper plants from Burievo (Strumica region, latitude 41° 25′ 24″ N, longitude 22° 45′ 54″ E) and one *C. annuum* L. var. *cerasiforme* (Mill.) Irish plant from Cesinovo (Kocani region, latitude 41° 52′ 18″ N, longitude 22° 17′ 24″ E) were identified and characterized for stolbur disease with the molecular methods described below.

Tomato plants were collected randomly without symptoms or with atypical symptoms, because we had difficulty finding tomato plants in the open field, as most tomato production in Macedonia is in controlled conditions (green- or glasshouses).

Figure 1. (a) Pepper field in the Strumica locality; (b) symptoms of stolbur phytoplasmas on a whole *Capsicum annuum* var. *cerasiforme* plant.

Table 1 shows the number of plants collected from the field with the presence of visual symptoms, and included characteristics of location, region and plant host.

Table 1. Field observation during the summer period 2015/16.

Number	Strain	Location	Region	Plant host	Symptoms*
1	1–5/15	Strumica	Burievo	Pepper plant	+++
2	8/16	Strumica	Prosenikovo	Healthy pepper plant	–
3	9/16	Strumica	Piperovo	Healthy tomato plant	–
4	10–13/16	Strumica	Dobrejci	Pepper plant	+?
5	14–18/16	Kocani	Cesinovo	Sweet pepper plant	++
6	25–30/16	Kocani	Ciflik	Pepper plant	++++
7	37–43/16	Kocani	Gorni Podlog	Pepper plant	++++

*Plants were chosen randomly, and symptoms were not clear phytoplasma symptoms: – negative; +? symptoms not clear; ++ good symptoms; +++ very good symptoms; ++++ obvious phytoplasma symptoms.

Total DNA extraction

Leaf veins, separated from laminas by a sterile razor, and all parts of the tomato and pepper plants were stored at –80 °C. Total nucleic acids were extracted from 0.5 g of frozen plant tissues by a cetyltrimethylammonium bromide (CTAB) extraction procedure (Angelini et al., 2001). The concentration of total DNA was measured with a NanoDrop spectrophotometer (Jenova Nano Spectrophotometer).

Molecular identification of stolbur phytoplasmas

'*Ca.* P. solani' strains from symptomatic and asymptomatic pepper and tomato plants were employed for further molecular characterization carried out on the *tuf* (encoding translation elongation factor Tu), *vmp*1 (encoding a putative membrane protein) and *stamp* genes (encoding an antigenic membrane protein).

The *tuf* gene was amplified in a nested PCR procedure using primer pairs tuf1f/r/ tufAYf/r (Langer and Maixner, 2004), and amplicons were subjected to RFLP analysis with *Hpa*II enzyme.

The nested PCR products of the *vmp*1 gene were obtained with StolH10F1/R1 (Cimerman et al., 2006) and TYPH10F/R (Fialova et al., 2009) primers.

Nested PCR for the *stamp* gene was done with StampF/R0 followed by StampF1/R1 primers (Fabre et al., 2011).

PCR and RFLP reaction conditions were as previously described (Lee et al., 1998; Quaglino et al., 2009). PCRs were performed by using *Taq* polymerase (Promega) in an automated thermal cycler (MasterCycler Gradient, Eppendorf). PCR and enzymatic digestion products were electrophoresed through 1% and 3% agarose gel, respectively, in TBE buffer, stained with ethidium bromide and visualized under a UV transilluminator.

Characterization of stolbur phytoplasmas through multilocus genetic analysis

Molecular characterization of phytoplasma strains was performed by nested PCR/RFLP-based assays of two phytoplasma genomic portions, including *tuf*, *vmp*1 and *stamp* genes.

RESULTS AND DISCUSSION

Symptomology of the pepper and tomato plants

During our field surveys carried out from the beginning of August to the end of October 2015/ 16, leaf samples were collected from 24 symptomatic/asymptomatic pepper and tomato plants from 11 localities in Eastern Macedonia. Among all plants analysed for stolbur disease, four sweet red pepper plants and one *C. annuum* var. *cerasiforme* were proven to be stolbur-positive with the molecular methods used in this study. In pepper plants, typical symptoms assessed in the leaves in the course of disease progress were yellowing, stunting and wilting. The fruits were smaller and without taste. The anthers and the filaments of the flowers were distorted and grown into one whole entity (Fig. 2). The roots of symptomatic plants were dry and/or not well developed (Fig 3.).

Figure 2. Pepper plants with distorted anthers and filaments of the flowers, and grown into one whole entity.

Figure 3. The roots of symptomatic plants were dry and/or not well developed.

Molecular identification of stolbur phytoplasmas

Due to variations in typical symptoms of stolbur phytoplasmas in the field, PCR-based amplification of 16S rRNA genes was performed to prove that some of the samples examined were affected by stolbur phytoplasmas. PCR amplification of specific *tuf*, *vmp*1 and *stamp* genes showed that the phytoplasma strains identified belong to subgroup 16SrXII-A, since their restriction patterns were indistinguishable from one another and from the patterns characteristic of the STOL (16SrXII-A) reference strain (*tuf* profiles are presented and *stamp* profiles are not presented in this study) (Fig. 4).

For the *tuf* gene, it was possible to identify *Hpa*II RFLP profiles associated with *tuf* type a (two strains) and *tuf* type b (16 strains), formerly named VK-I and VK-II (Langer and Maixner, 2004). Our samples were positive for *tuf* type b, VK-II (Fig. 4a and b).

Also, only PCR amplification was done to confirm *vmp*1 and *stamp* gene-positive profiles (Fig. 5).

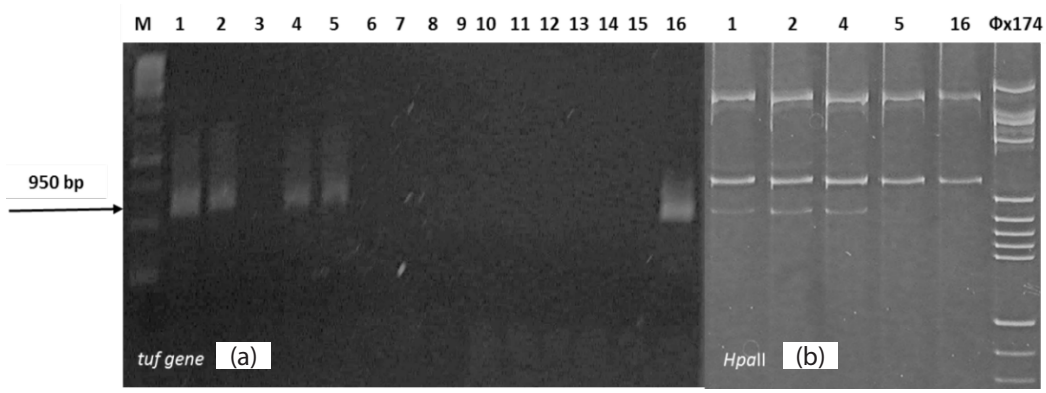

Figure 4. (a) PCR pattern of specific *tuf* gene for stolbur phytoplasma, including tomato and pepper plants: M – 1 kb DNA ladder marker; (1–7) pepper, Strumica, Burievo; (8) healthy pepper plant as negative control; (9) healthy tomato plant as negative control; (10–13) pepper, Strumica, Dobrejci; (14–16) pepper, Kocani, Cesinovo; (b) RFLP profiles from HpaII digestions of positive fTufAY/rTufAY PCR products (*tuf* gene) using 3% agarose gel: Φx174 – marker. b) RFLP profiles from *Hpa*II digestions of positive fTufAY/rTufAY PCR products (*tuf* gene) using 3% agarose gel: Φx174 – marker.

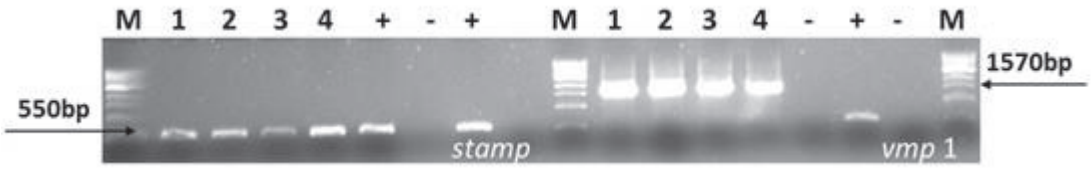

Figure 5. PCR pattern of specific *vmp*1 gene (1570 bp) and *stamp* gene (550 bp) for stolbur phytoplasma, including only positive pepper plants: M – 1 kb DNA ladder marker; (1–4) pepper, Strumica, Burievo; (–) healthy pepper plant as negative control.

CONCLUDING REMARKS

Stolbur disease is an old and well-known disease, mainly in Europe but in other continents as well. Symptoms in pepper vary depending on the pepper variety, geographical region and cultivation conditions. Although it has been noticed in Macedonia multiple times in the last 40 years, it has not been laboratory-tested and proven until this present study for pepper and tomato.

The present molecular multigene characterization is the first detection of stolbur phytoplasmas on garden crops (pepper and tomato) and corresponded to stolbur phytoplasma (16SrXII-A), the same one that we've already had in the vineyards (*tuf* type b).

Four of the 24 pepper and tomato plants analysed were molecularly identified and characterized with stolbur symptoms. Only samples collected from peppers showed positive PCR/RFLP profiles. In this study, we did not find tomato plants with positive profiles, so future field observations and laboratory analysis are required.

Therefore, it can be concluded that incidence of the disease is still sporadic in Eastern Macedonia, and crop damage is not yet substantial. Nevertheless, it is very important to emphasize the presence of stolbur disease in pepper plants in the country, and to keep the alarm turned on for eventual future local or countrywide outbreaks.

ACKNOWLEDGEMENTS

This article is professorially English correct by Proof-Reading-Service.com

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МУЛТИГЕНЕТСКА КАРАКТЕРИЗАЦИЈА НА *CANDIDATUS PHYTOPLASMA SOLANI* КАЈ ПИПЕРКА И ДОМАТ ВО РЕПУБЛИКА МАКЕДОНИЈА

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Резиме

Candidatus phytoplasma solani е фитоплазма дистрибуирана во светски рамки во евромедитеранската област поврзана со разни болести кај најраспространетите и култивирани растенија, вклучувајќи ги виновата лоза, пченката, компирот, пиперката, целерот и тутунот.

Во ова истражување молекуларната мултигена карактеризација за првпат беше изведена на соеви на фитоплазмите собрани во источниот дел на Република Македонија од симптоматски и асимптоматски растенија од пипер и домати во текот на летниот период во сезоната 2015/2016. PCR амплификацијата на специфичен ген за трите специфични гени *tuf*, *vmp*1 и *stamp* покажа дека идентификуваните соеви на фитоплазмата ѝ припаѓаат на подгрупата 16SrXII-A, бидејќи нивните ограничувачки шеми не се разликуваат една од друга и од шаблоните кои се карактеристични за референтниот вид на STOL C фитоплазмата (16SrXII-A).

За позитивни примероци на генот *tuf* беа направени типизација и идентификација на *Hpa*II RFLP профилите поврзани со гените од типот *tuf - type* b.

Ова е прво молекуларно лабораториско тестирање за присуство на столбур фитоплазма кај пипер и домат во Република Македонија.

Клучни зборови: *пипер, домат, столбур фитоплазма, мултилокусна генетска анализа*.

Journal of Agriculture and Plant Sciences, JAPS, Vol 15, No. 1/2, 2017

Manuscript received: 28.09.2017
Accepted: 09.11.2017

In print: ISSN 2545-4447
On line: ISSN 2545-4455
UDC: 502.175:[582.32:546.19
502.175:631.416.319
Original scientific paper

BIOINDICATION ABILITY OF *Hypnum cupressiforme* AND *Homolothecium lutescens* FOR DETERMINATION OF ARSENIC DISTRIBUTION IN ENVIRONMENT

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Abstract

Atmospheric dust emissions can be a threat for the environmental and human health. Long-term emission occurs in this area due to the Pb-Zn hydrothermal exploitation (*Sasa* and *Zletovo* mines) and copper ore exploitation and flotation (Bučim mine), in the area of Bregalnica river basin. The present study proposes a combined model based on: bioindication with moss species (*Hypnum cupressiforme* and *Homolothecium lutescens*), and universal kriging mapping for determination of arsenic distribution. For that purpose, 149 moss samples were collected from the area, and both moss species were used interchangeably. At the same sampling points, soil samples from the surface layer were also collected. Mass spectrometry with inductively coupled plasma (ICP-MS) was used for determination of total arsenic content in moss and soil samples. Prior to analysis, the samples were totally digested with the application of microwave system for samples digestion for moss samples and open wet digestion was used for total dissolution of soil samples. Spatial distribution maps were constructed for determination and localizing of narrower areas with higher contents of arsenic. The content of arsenic in moss tissue (regarding air-born dust) ranges from 0.05 mg/kg to 4.28 mg/kg, while distribution of arsenic in soil samples ranges from 3 to 261 mg/kg. Dominant lithogenic occurrence of arsenic was correlated with areas of Neogene pyroclastites (volcanism).

Key words: *moss, biomonitors, air pollution, ICP-MS*

INTRODUCTION

Atmospheric pollution represents solutions or suspensions of minute amounts of harmful compounds in the air (Valero, 2014). The degree and the extent of environmental changes over the last decades has given a new urgency and relevance for detection and understanding of environmental changes, due to human activities, which have altered global biogeochemical cycling of heavy metals and other pollutants (Greenwood and Earnshaw, 2005; Acton, 2013). Arsenic is one of the most prevalent toxic elements in the environment. The toxicity, mobility, and fate of arsenic in the environment are determined by a complex series of controls dependent on mineralogy, chemical speciation, and biological processes (Alloway, 1990). As a chemical element, arsenic is widely distributed in nature and can be concentrated in many different ways. In the Earth's crust, arsenic is concentrated by magmatic and hydrothermal processes and has been used as a "pathfinder" for metallic ore deposits, particularly gold, tin, copper, and tungsten (Alloway, 1990; Greenwood and Earnshaw, 2005; Keller et al., 2015). Monitoring toxic air pollutants is needed for understanding their spatial and temporal distribution and ultimately to minimize their harmful effects. In addition, to direct physical and chemical methods of air pollution monitoring, *bioindication* has also been used to evaluate air pollution risk (Aboal et al., 2010; Ares et al., 2012; Valero, 2014).

Mosses have been frequently used to monitor time-integrated bulk deposition of

metals/semimetals as a combination of wet, cloud, and dry deposition, thus eliminating some of the complications of precipitation analysis due to the heterogeneity of precipitation (Harmens et al., 2004, 2008, 2010, 2015). Moss data provides a better geographical coverage than measured deposition data and reveals more about actual atmospheric pollution at a local level (http://icpvegetation.ceh.ac.uk/). Latest data reported from Harmens et al. (2015) and Barandovski et al. (2015) indicates on the significant enrichments of some toxic elements.

The investigated area is characterized by several significant pollution sources of potentially toxic metals and other chemical elements in the environment: the copper mine and flotation "Bučim" near the town of Radoviš, the lead and zinc mines "Sasa" near the town of Makedonska Kamenica and "Zletovo" near the town of Probištip (Serafimovski et al., 2004; Alderton et al., 2005; Rogan et al., 2006; Dolenec et al., 2007; Rogan et al., 2008; Rogan-Šmuc et al., 2009; Serafimovski et al., 2011a, 2011b; Vrhovnik et al., 2013; Alderton et al., 2014; Serafimovski and Tasev, 2015; Vrhovnik et al., 2016; Stafilov and Šajn, 2016). The excavation of the copper minerals is carried out from an open ore pit, while in the lead-zinc mines the exploitation is underground, and the ore tailings are stored outdoors.

The focus of this research is on the uses of the two moss species *Hypnum cupressiforme* (Hedw.) and *Homalotecium lutescens* (Hedw.) Schimp. for monitoring atmospheric deposition of arsenic in mine environs. Sharing the same common name "fern moss" with other monitoring mosses, these species similarly have extensive branching allowing for a large exposed surface area for ion exchange. These features make *Hypnum cupressiforme* and *Homalotecium lutescens* likely candidates for use as a biomonitors. The primary objective of this study was to evaluate the suitability of two moss species as a bioindicator of arsenic on a regional landscape scale in potentially polluted area. Mosses as pollution bioindicators only give an overview of the areas where we found the presence of higher content of arsenic in atmospheric dust, but not a real measurement of the content in the ambient air.

MATERIAL AND METHODS

Moss/soil sampling protocol

Samples of the pleurocarpous moss species *Homalotecium lutescens* and *Hypnum cupressiforme* were collected in the investigated area. Researchers while setting up large-scale survey often face the problem that the location of the predicted sampling spot becomes subordinate to the presence/absence of the selected species (Fernández et al., 2015). This problem can be overcome by using more than one moss species within the same survey; however, it is clear that the concentrations of elements may vary considerably between species thus precluding comparison of the results obtained (Boquete et al. 2013). Interspecies comparison has been made by Balabanova et al. (2017b) improving the insignificant variation for arsenic accumulation between both moss species. Depending on the conditions and the accessibility of the locations the species which are available and typical for the region were collected. Random samples (in the very close vicinity of the pollution source) and samples according to sampling network (5 x 5 km) were collected from total of 149 sample locations, as presented in Figure 1. Detailed description of the collection of samples (according officially accepted techniques) is given by Fernández et al. (2015). At each location for moss sampling, topsoil (0-5 cm) samples were collected also according to the standard protocol given by Salminen et al. (2005).

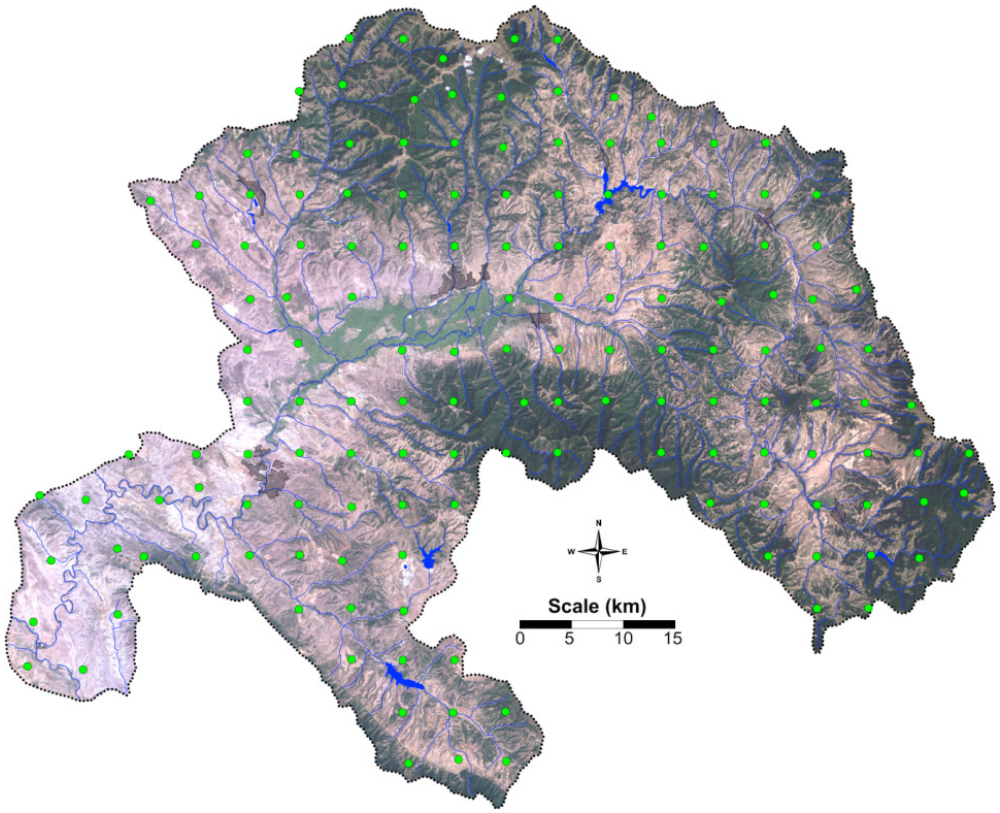

Figure 1. Moss/soil sampling network

Sample preparation protocol and spectroscopy analysis

Total digestion of moss samples was performed with application of microwave digestion system (CEM, model Mars). Precisely measured mass (0.5000 g) of moss samples was introduced into Teflon microwave vessels, than 5 mL concentrated HNO_3 (trace pure), and 2 mL H_2O_2 (30%, *m/V*) were added. The Teflon vessels were carefully closed and the microwave digestion method was applied. Digestion method was performed in to two steps for total digestion of moss tissue as previously given by Balabanova et al. (2010). After the digestion method was finished, digests were quantitatively transferred into 25 mL volumetric flaks.

For digestion of soil samples, open wet digestion with mixture of acids was applied. Precisely measured mass of soil sample (0.5 g) was placed in Teflon vessels and 5 mL concentrated nitric acid, HNO_3 was added, until the brown vapours came out from the vessels. Nitric acid is very suitable oxidant for digestion of environmental samples. For total digestion of inorganic components, 5-10 mL hydrofluoric acid was added. When the digest became clear solution, 2 mL of $HClO_4$ was added. Perchloric acid was used for total digestion of organic matter. After 15 minutes cooling the vessels, 2 mL of HCl and 5 mL of H_2O were added for total dissolve of metal ions. Finally the vessels were cooled and digests quantitatively transferred to 50 mL volumetric flaks.

In this way the digested moss and soil samples were prepared for determining the contents of the different elements using mass spectrometry.

Mass spectroscopy analysis

SCIEX Perkin Elmer Elan DRC II (Canada) inductively coupled plasma mass spectrometer (with quadrupole as single detector) was used for measurement of the arsenic concentration in digested samples. Optimization was first performed using the normal mode, and then using the collision cell mode. Before the parameters of the collision cell were optimized, the cell was flushed with collision gases (5 mL/min) for at least 1 hour. Two certified reference materials M2 and M3 (Steinnes et al., 1997) and spiked intra-laboratory sample were analyzed at a combined frequency of 20% of the samples. The recoveries for arsenic content in all control samples were obtained as: 85.6%, 109%, respectively. The detection limits (DL) were calculated using the following equation: DL = ($3\times\sigma bl/S$), where σbl is the standard deviation of the background and S the sensitivity. The quantum mode for arsenic was found for ^{75}As isotope and the calculated DL was 0.0013 mg/kg.

Data processing

The obtained values for the arsenic contents in moss and soil samples were statistically processed using basic descriptive statistics. Data processing was performed using the statistical software Stat Soft (Version 11) (StatSoft, Inc., Tulsa, OK, USA). Using field observations, analytical and measurement data matrix was created. For each observation, the following variables were extracted: sample identification number, location, geographic coordinates, sample type. Since many statistical techniques are sensitive to non-normally distributed data, the Box-Cox transformation was performed. The Box-Cox transformation improves the feature better, especially for the skewness and normality of the data sets (Box and Cox, 1964). Line and bar/colon plots were constructed for better visibility of data distribution according to defined zones. The universal method kriging with linear variogram interpolation was applied for the construction of spatial distribution map for arsenic deposition/distribution in the investigated area. Seven classes of the following percentile values were selected: 0–10, 10–25, 25–40, 40–60, 60–75, 75–90 and 90–100.

RESULTS AND DISCUSSION

The basic statistics of analysed moss and soil samples (surface soil layer) for arsenic content is presented in Table 1. The distribution of arsenic in the analyzed samples ranges from 0.05 mg/kg to 4.28 mg/kg. Compared to data available from Barandovski et al. (2015) from the survey for the whole territory of the Republic of Macedonia, indicates significant enrichments (EF=2.25, regarding maximum values). The median value for the whole territory of the Republic of Macedonia (0.48 mg/kg) did not show significant variation from the same value from the present investigation (0.49 mg/kg). The minimum arsenic content was obtained for sample collected in the area with dominant occurrence of Paleogene flysh where the topsoil layer contains 17.3 mg/kg of arsenic. In order to monitor the lithogenic affect from the natural distribution of arsenic in soil, data for arsenic content in moss tissue were compared with the data for arsenic content in topsoil layer. The distribution of arsenic in surface soil samples ranges in 3.02-261 mg/kg (Tab. 1). Four sampling spots, where the soil samples were enriched with arsenic content (104, 105, 121 and 261 mg/kg) were not characterized with higher content of arsenic in moss samples (0.35, 1.15, 1.29 and 0.15 mg/kg, respectively). This encourages the fact that soil dusting does not significantly affect the air-introduced particle distribution in the investigated area. In order to reveal a significant enrichment of arsenic, maximum value was compared with maximum values from moss survey in other countries, such as Albania, Croatia, Bulgaria and Norway (Qarri et al., 2013; Špirić et al., 2013; Harmens et al., 2013; Steinnes et al., 2011). The calculated enrichments factors, regarding the maximum value for arsenic content in moss, are given as follow: 1.49, 4.28, 0.42 and 0.88, respectively.

Table 1. Descriptive statistics for elements content values in moss samples, N=149 (given in mg/kg)

Sample	Min	P_{10}	P_{25}	P_{40}	Md	P_{60}	P_{75}	P_{90}	Max
Moss	0.050	0.25	0.33	0.42	0.49	0.57	0.75	1.03	4.28
Topsoil	3.02	6.62	9.80	13.4	16.9	20.8	28.7	53.9	261
	X	**X(BC)**	**S**	**Sx**	**CV**	**A**	**E**	**A (BC)**	**E (BC)**
Moss	0.70	0.48	0.71	0.058	100	3.06	11.5	-0.02	0.46
Topsoil	26	17	26	2.1	100	3.91	23.6	0.001	0.27

Min – minimum; P_{10} – 10 percentile; P_{25} – 25 percentile; P_{40} – 40 percentile; Md – median; P_{75} – 75 percentile; P_{90} – 90 percentile; Max – maximum; X – mean; S –standard deviation; CV – coefficient of variation; A – skewness; E – kurtosis; BC-Box/Cox transformed data.

The data for arsenic content in moss samples additionally were processed according to different lithological units in the investigated area. Data were also processed and analyzed according to the generalized geological map given by Balabanova et al. (2016). Several lithological units were identified as dominant in the investigated area: Quaternary sediments, Neogene sediment and pyroclastite, Paleogene flysch, Pleozoic schist, Rifeous schist, Proterosoic schist, gneisse and granite. Mainly, arsenic do not participate significantly in the composition of the Earth's crust, although several minerals containing as its major constituents (Alderton et al., 2014). Dumurdžanov et al. (2004) explained that natural enrichment of arsenic may occur in areas where the Neogene vulcanite's are dominant geological units. The calculated median values of Box-Cox transformed data, according to the lithological units are given as follow: in area with dominance of Quaternary sediments - 0.73 mg/kg, for Neogene sediments the median value was 0.43 mg/kg and for Neogene pyroclastites was obtained the maximum value regarding the lithological units - 0.95 mg/kg. In the area with dominant occurrence of Paleogene flysch the median value was 0.49 mg/kg, which was very similar with arsenic distribution in areas with dominant occurrence of Pleozoic schist (0.44 mg/kg), Rifeous schist (0.55 mg/kg), Proterozoic schist (0.51 mg/kg) and Proterozoic granite (0.46 mg/kg). Lower median value was obtained for moss samples collected from area with dominant occurrence of Proterozoic gneisse (0.34 mg/kg). For better visualization of data distribution according to different lithological units bar plot was constructed (Fig. 2). Ohnuki et al., (2002) introduces data that suggest strongly correlation of As with Fe, Si and Ca in mine areas. Weathering of the rocks containing As probably generates the powder rock containing As and other elements (Alderton et al., 2014). They found that As accompanies Fe in the spatial distributions in moss; small particulates containing As and Fe are associated with the lower plants in a similar manner to the trapped silicate minerals (Ohnuki et al., 2002). However, in the area where the anthropogenic introducing of arsenic is not significantly enriched, this element can shows different distribution pathways correlated with the dust weathering. From the summary data available from Balabanova et al. (2017a) the distribution of arsenic in air-distributed dust, is strongly correlated with distribution of Co, Ge and V. The long-time deposition monitoring using attic dust, suggest very stable geochemical occurrence of arsenic in areas with polymethalic enrichments (Balabanova et al., 2010, 2011; Balabanova et al. 2016; Angelovska et al., 2016; Balabanova et al., 2017a; 2017b).

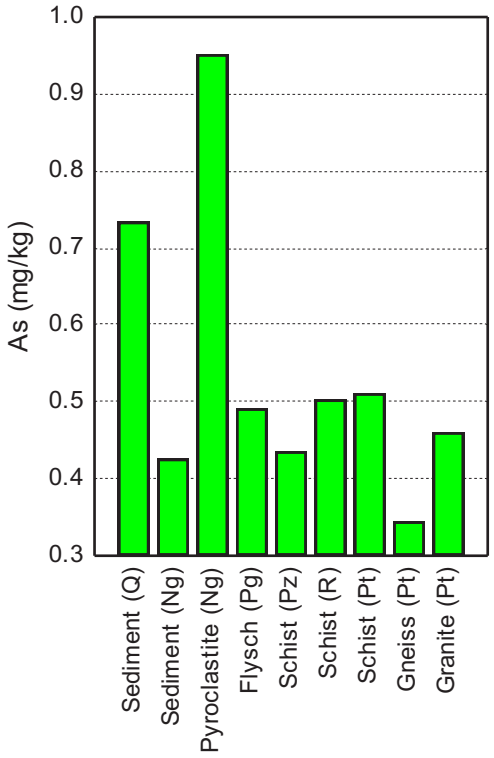

Figure 2. Arsenic distribution according to different lithological units in the investigated area

The constructed kriging map visualizes the areal distribution of arsenic in the Bregalnica River basin (Figs. 3 and 4). Arsenic deposition is with predominant occurrence on Neogene pyroclastites (Fig. 3). According to the generalized geology map (Balabanova et al 2017a), Kratovo-Zletovo region is the unique district in the region located along the continental margin and is closely related to the Tertiary volcanoes and hydrothermal activities in this area. Pb-Zn mine *Zletovo* is located in the area with dominantly presence of the Neogene volcanism appears sequentially and in several phases forming sub-volcanic areas. According to Dumurdžanov et al. (2004) the pyroclastites are most frequently found in the Kratovo-Zletovo volcanic area, where the dacites and andesites are the oldest formations. These polyphasal Neogene deformations through the insignificant movements associated with the volcanic activities had direct influence on the gradual formation of the reefs and the formation of deposits in the Zletovo area. Spatial patterns are extended in eastern direction, due to the most common winds from western direction with frequency of 199‰ and speed of 2.7 m/s (Lazarevski, 1993). This kind of geochemical *fingerprinting* occurs along the whole course of the Bregalnica river. Accordingly, the resulting areal distribution map used to support with high certainty the assessment for poly-metallic enrichments as ascribed to urbanization, including vehicular emissions and incinerators and industry. This area is characterised with poly-metallic enrichments (Ag-Bi-Cd-Cu-In-Mn-Pb-Sb-Te-W-Zn) for long-time air-dust deposition (Balabanova et al., 2017a). Furthermore, there is a strong interconnection between the anthropogenic and lithogenic fingerprinting. Arsenic distribution in topsoil layer of soil is strongly emphasised in the same area (Fig. 4). Basically, the element geochemistry intermediate between atmospheric emissions and lithogenic wind-blow dusting. Therefore, arsenic distribution can be used as proposed mechanism for possible tracking of anthropogenic poly-metallic enrichments in areas with dominant occurrence of old volcanism (Figs. 3 and 4). In the area where dominant lithological units relays on Paleogene volcanic sedimentary rocks (the area of Pb-Zn hydrothermal exploitation, Sasa mine) atmospheric emissions are significantly intensified (wind-blow dusting) compared to lithogenic enrichments in top-soil. Spatial attention also should be given for

the area so called Vladimirovo-Berovo, where arsenic contents in moss samples reaches more than 1 mg/kg (Fig. 3). Almost twenty years ago, Arsovski (1997) drew attention to poly-metallic enrichment in this area so called Vladimirovo-Berovo, during the tectonic investigation. The present investigation also interpolates this area as metallic's/metalloids enriched zone, with emphasis on the anthropogenic elements. This area is characterized by dominant occurrence of Neogene clastites, and this natural anomaly correlated with arsenic distribution continues along the whole course of the river Bregalnica. Enriched atmospheric depositions of arsenic also were found in the area of hydrothermal exploitation of cooper ore (Cu-mine "Bučim" near the town of Radoviš). This area was monitored in 2010, and authors reveal the occurrence of poly-metallic association Al-As-Cd-Cu-Fe-Pb-Zn as dominant anthropogenic marker for air-pollution (Balabanova et al., 2010).

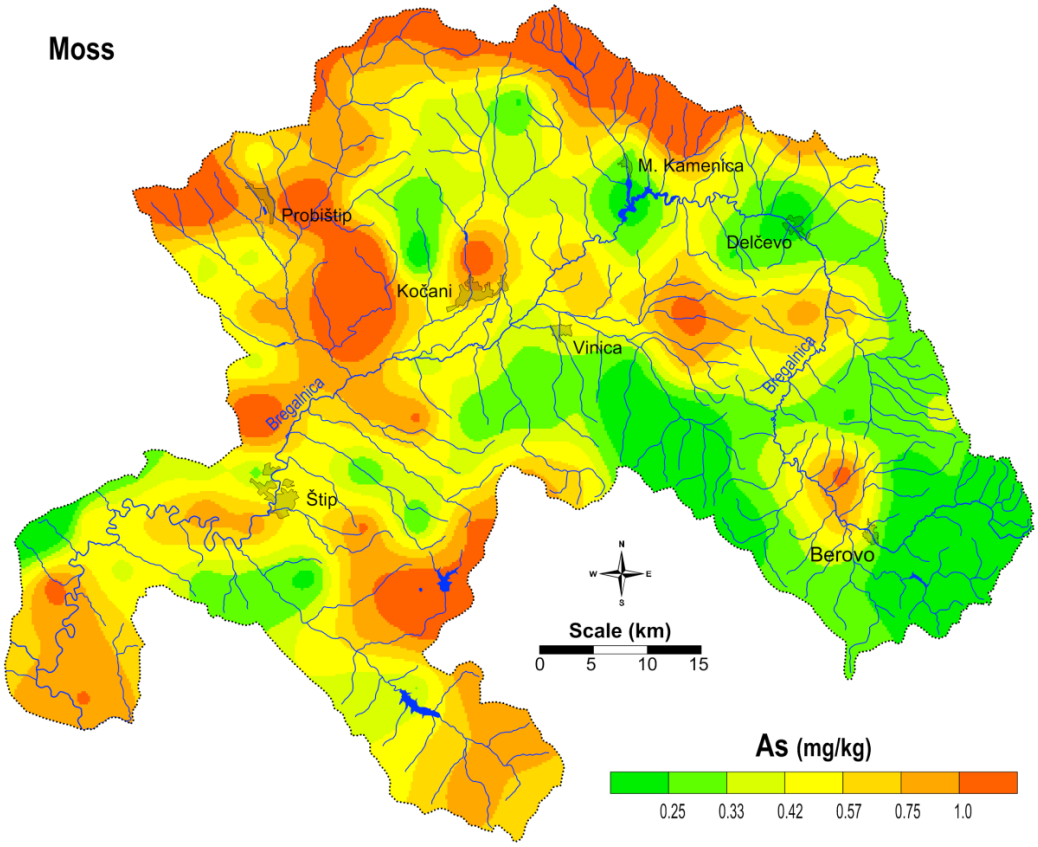

Figure 3. Areal distribution of arsenic in moss samples

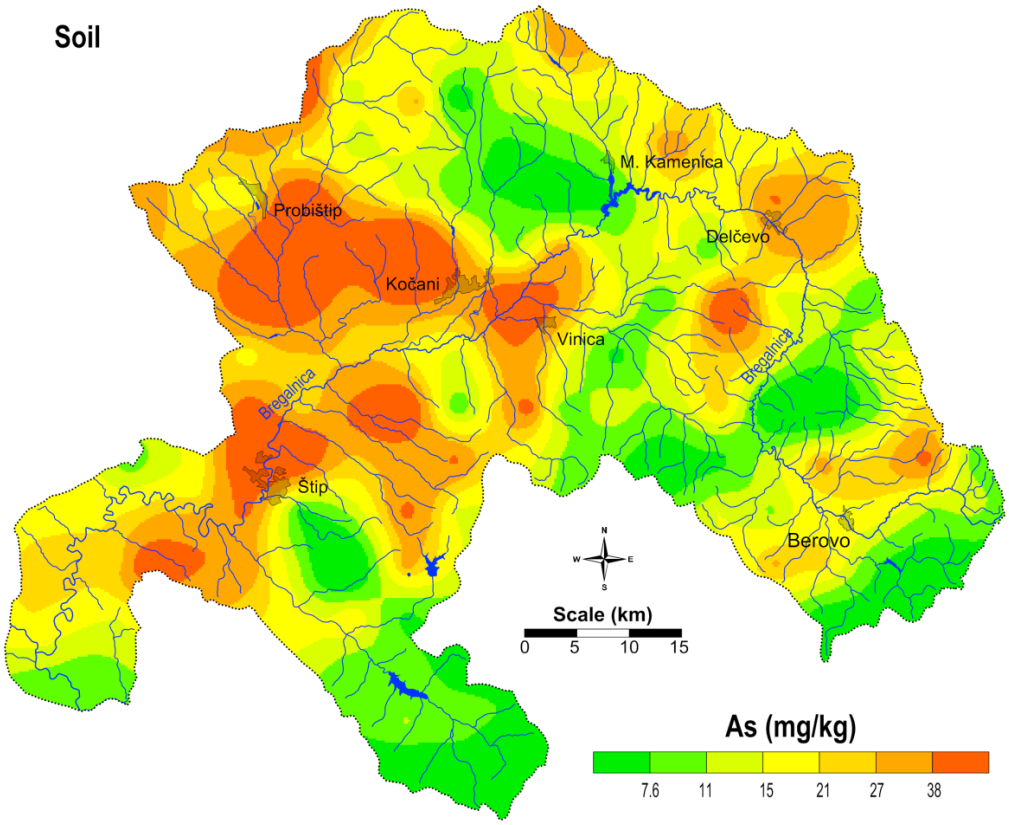

Figure 4. Areal distribution of arsenic in top-soil samples

CONCLUDING REMARKS

The present investigation points to strongly correlation of the lithogenic and anthropogenic atmospheric distribution of arsenic in the area of Bregalnica River basin. Both of the terrestrial moss species, *Hypnum cupressiforme* and *Homolothecium lutescens* were improved as a sensitive bio-indicative model for enriched arsenic deposition in air. This environmental media contain a mixture of material derived from in situ weathering of parent material and atmospheric input dominated by continental dust. The anthropogenic activities carried out in the areas of poly-metallic hydro-thermal exploitation (*Sasa*, *Zletovo* and *Bučim* mines) lead to increased content of arsenic. Atmospheric distribution of arsenic reaches to the maximum value of 4.28 mg/kg. Mainly, intensified atmospheric deposition of arsenic occurs in area with dominant occurrence of Neogene pyroclasites and clastites and Paleogene flysch. This indicates that arsenic distribution can be strongly correlated to the poly-metallic enrichments, which are due to hydro-thermal exploitation. The both moss species (*H. cupressiforme* and *H. lutescens)* were introduced as dominant bioindicator markers in the investigated area.

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БИОИНДИКАЦИСКА СПОСОБНОСТ НА *HYPNUM CUPRESSIFORME* И *HOMOLOTHECIUM LUTESCENS* ЗА СЛЕДЕЊЕ НА ДИСТРИБУЦИЈАТА НА АРСЕН ВО ЖИВОТНАТА СРЕДИНА

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Резиме

Атмосферските емисии на прашина во одредени услови претставуваат закана за животната средина и здравјето на луѓето. Во областа на сливот на реката Брегалница е утврдена долгорочна емисија на атмосферска прашина, којашто се должи на хидротермалната експлоатација на Pb-Zn руда (рудниците *Саса* и *Злетово*), како и експлоатација и флотација на бакарната руда (рудник *Бучим*). Ова истражување предложува примена на комбиниран модел базиран на биоиндикации со видови на мов (*Hypnum cupressiforme* и *Homolothecium lutescens*) и *кригинг* мапирање за одредување на дистрибуцијата на арсен. За таа цел беа собрани 149 примероци на мов од испитуваната област. И двата вида на мов беа собирани наизменично. На локациите каде што се собираа примероци на мов, исто така, беа собрани и примероци од почва од површинскиот слој. Масена спектрометрија со индуктивно спрегната плазма (ИСП-МС) беше користена за одредување на вкупната содржина на арсен во примероците на мов и почва. Пред да бидат анализирани, примероците беа целосно разложени со примена на микробранов систем за разложување на примероци (за примероците мов), додека за примероците на почва беше применет методот на отворена дигестија со смеша од киселини (мокро разложување). Карти на просторна дистрибуција беа конструирани заради одредување и локализирање на потесните области со повисока содржина на арсен. Содржината на арсен во мовното ткиво (во однос на прашината во воздухот) се движи од 0,05 mg/kg до 4,28 mg/kg, додека дистрибуцијата на арсен во примероците на почвата се движи од 3 до 261 mg/kg. Литогената дистрибуција на арсен значајно е поврзана за области со доминантно појавување на неогенски пирокластити (вулканизам).

Клучни зборови: *мов, биомонитори, загадување на воздухот, ИСП-МС*

Journal of Agriculture and Plant Sciences, JAPS, Vol 15, No. 1/2, 2017

Manuscript received: 12.10.2017
Accepted: 06.11.2017

In print: ISSN 2545-4447
On line: ISSN 2545-4455
UDC: 635.649.076:581.192
Short communication

COMPARATIVE ANALYSIS OF CAPSAICIN CONTENT IN PEPPERS (*CAPSICUM ANNUUM* L.) GROWN IN CONVENTIONAL AND ORGANIC AGRICULTURAL SYSTEMS

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Abstract

According to their chemical structure capsaicinoids are phenethylamine group of alkaloids and they are produced exclusively in the secondary metabolism of genus Capsicum, fam. Solanaceae. Pepper (*Capsicum annuum* L.) is economically one of the most important vegetable crop in the Republic of Macedonia. There is no methodical and long-term research in organic production of vegetables in the Republic of Macedonia until today. Still, largely speaking, there are current debates about the advantage of organic production as compared to conventional one in terms of morphology of products and especially their quality characteristics.

The aim of this paper is comparative analysis of capsaicin content in fruit extracts from (*Capsicum annuum* L.) produced in organic and conventional cultivation systems. Pepper genotypes Strumicka Kapija, Strumicka Vezena, Piran, Zupska Rana, Duga Bela and Kurtovska Kapija were subject of this study. Methanol (≥ 99.9%) was used as capsaicin solvent for extraction of capsaicin from dried pepper fruit material. The determination of capsaicin content was performed by chromatographic analyses with High Pressure Liquid Chromatography (HPLC).

Organically grown pepper fruits were characterized with higher capsaicin content than the conventional one. The genotype Strumicka Vezena was characterized with the highest capsaicin content in the both cultivation systems.

Key words: *Capsicum annuum L., pepper, capsaicin, organic cultivation system, conventional cultivation system*

INTRODUCTION

Peppers are grown because of their fruits which are used in human nutrition all year round as fresh, roasted, marinated for different dishes and salads, pickled, preserved, paste, juices and powder paprika spice. Since fruits contain capsaicin, it is used in pharmaceutical industry (Jankuloski, 1997; Bosland et al., 2012).

Organic agricultural production is an integral part of the sustainable agricultural development and it is well established in practice in the past years in Republic of Macedonia. Although the organic production is practiced on only 1% of total arable land in the country, there is a growing trend of practicing organic production which gives an expectation that organic agriculture production might be a leader in future development for the rest of the sustainable agriculture systems (National Plan for organic production, 2013-2030). As part of the sustainable agriculture, organic agriculture utilizes methods and practices which enhance soil fertility and in the same time minimize harmful impact on soil, water, air and health of farmers and consumers.

From the available literature, there is no methodical and long-term research in organic production of vegetables in Republic of Macedonia until today. Still, largely speaking, there are current debates about the advantage of organic production as compared to conventional one in terms of morphology of products and especially their quality characteristics.

This is a report about the first comparative

research in Republic of Macedonia on peppers (*Capsicum annuum* L.) grown in organic and conventional production systems.

The aim of this research is a comparative analysis of capsaicin content in extracts from fruits of six different pepper genotypes (Capsicum annuum L.) grown in organic and conventional system.

The capsaicin (8-Methyl-N-vanillyl-trans-6-nonenamide), a major metabolite in *Capsicum* species, is the most common representative of broad family of capsaicinoids. It is a strong and stable crystal alkaloid which does not change the characteristics on temperature, thus it keeps the original strength in long-terms when heated or frozen. It is produced mainly in the placenta of *Capsicum* fruits. Different content of capsaicinoids in pepper fruits gives diversity of their flavour, from sweet to very hot (De Witt, 1999; Tod and Catt, 2002; De, 2004; Maksimova et al, 2014). The capsaicin owns high antioxidative activity which gives high recommendation for pepper consumption in everyday nutrition (Maksimova et al. 2013; Maksimova et al. 2014). The clinical trials have shown that the biological potential of capsaicin is due to its structure of alkaloid with different inhibitory effects: analgesic and redactor of pain (Bernstein, 1991; Tominaga, 1998; Cheng et al., 1999; Holt, 1999; Bunk, 2000; Ying-Yue et al., 2001; Košťálová, 2002); antimicrobial (Kurita, 2002); antibacterial (Kalia et al., 2012; Sinha et al., 2011; Ubulom et al., 2012); anticancer (Mori et al., 2006); cancerogenic (Dasgupta et al., 1998; Archer & Jones, 2002); anesthetic (Bernstein, 1991; De Witt, 1999; Cheng et al., 1999), cytostatic (Surh, 2002; Zhang et al., 2003). Even more, capsaicin can be used as biopesticide against the green peach aphid (*Myzus persicae* Sulz.) in organic production of different crops (Maksimova et al., 2013).

MATERIAL AND METHODS

Experiment site and design

The experiment was conducted on open field at Kamnik Bio Organic in Skopje during three successive years. The fields of Kamnik Bio Organic are certified for organic production since 2011.

Six different pepper genotypes were used as plant material in this study: Strumicka Kapija, Strumicka Vezena, Piran, Zupska Rana, Duga Bela and Kurtovska Kapija. All genotypes were sweet peppers, except Strumicka Vezena which is hot pepper.

The experiment was set up in completely randomized design in three replicates for both organic and conventional system. The experimental conventional and organic plots were in the near vacancy. Each genotype was represented with 45 plants in total for both production systems. During the vegetation period, conventional and organic production practices were accordingly applied. The described experimental design was applied during the three experimental years.

Plant material

Fruits from six genotypes of pepper (*Capsicum annuum* L.) grown under organic and conventional practices were used for determination of capsaicin content. For each extraction sample of five pepper fruits was used from each genotype, organically and conventionally grown respectively. Fruits from genotypes Strumicka Kapija, Kurtovska Kapija and Strumicka Vezena were collected in botanical maturity (deep red color of fruits), while fruits from genotypes Piran, Zupska Rana and Duga Bela were collected in horticultural maturity (greenish to yellow color of the fruits). The phenological phenophases of fruit collection was chosen according to pepper genotype characteristics and their utilization for human consumption.

Extraction method

The fruit pericarp with placenta was dried on 40°C until constant weight in laboratory oven with proper ventilation system.

A plant tissue extractor with semipermeable bags for extraction was used in the extraction procedure. Pure methanol with High Pressure Liquid Chromatography (HPLC) grade (≥ 99.9%) was used as solvent; 2 g of dry pepper tissue was extracted with 5 ml pure methanol with HPLC grade. The extraction has been performed at room temperature, for 24 hours minutes in dark conditions. The extract was filtrated through 0.45 um filter before injection in High Pressure Liquid Chromatography (HPLC) instrument.

Instruments for capsaicin content determination

Determination of capsaicin content was performed on Varian Pro Star HPLC system (pump model 230, autosampler model 410, PDA detector model 330 and column thermostat model 500). The separation of capsaicin from the other extracted components was performed with C18 column: Perkin Elmer C-18 150x4.6, 5 um. The mobile phase was methanol : water = 60:40 (v/v) with isocratic flow of 1.3 ml/min. Determination was performed on wavelength of 222 nm, injected volume 10 µl for total analysis time of 20 min.

Preparation of standard dilutions of capsaicin

Standard stock solution of capsaicin with concentration of 650 mg/l was prepared in methanol with HPLC grade (≥ 99.9%). Standards with concentrations 1.3 mg/l; 3.25 mg/l; 6.5 mg/l;13.0 mg/l; 16.25 mg/l; 50 mg/l; 100 mg/l; 200 mg/l and 300 mg/l capsaicin in methanol were prepared from the standard stock solution. For determination of the limit of detection (LOD) and limit of quantification (LOQ), we used calibration in low concentration area of calibration curve in range 1.3-13 mg/l. Maximum time for usage of the standards was one day.

Validation properties of the method

The calibration curve characteristics of applied methodology were y=21567x+130756 with correlation characteristics $p < 0.998$.

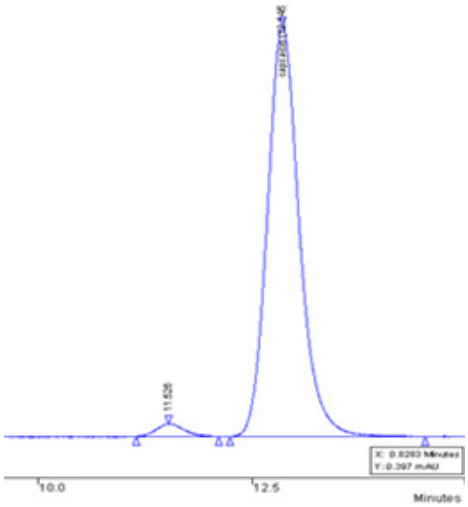

Figure 1. Chromatogram of standard capsaicin solution with concentration of γ = 10 mg/ml.

Detection of relative standard deviation (RSD) of the method is calculated from the calibration curve with extraction of 6 parallel repetition of pepper fruit samples from the same genotype and it is RSD 3%, while the LOD and LOQ are 0.5 mg/l and 1.5 mg/l, respectively (Fig. 1). Analytical yield of the method is calculated from the calibration curve, where the analytical yield on both levels with two parallel repetition with standard addition is determined. The analytical yield in both cases < 95%.

The results of the capsaicin content in six different in pungency pepper genotypes are expressed in mg capsaicin in dry matter.

Statistical analysis of data

The statistics of capsaicin content was done by the software programme IMB SPSS Statistics Software 19.0 (IBM SPSS Statistics 19 Brief Guide, 2010). The significance of differences between tested cultivation practices is established by the Duncan's Multiple Range Test. The evaluation of the effect of pepper genotypes and the effect of cultivation practices on capsaicin content in the three years experiment was made by the statistical analysis of univariance for each pepper genotypes and both growing practices. Additionally, pairwise comparison was applied for estimation of significance between capsaicin content of the two applied cultivation practices.

RESULTS AND DISCUSSION

Many researchers reported High Pressure Liquid Chromatography (HPLC) as an excellent method for determination of capsaicin content in pepper fruits (Perucka & Oleszek, 2000; Othman et al., 2011; Reyes-Escogido et al., 2011).

The capsaicin content in the organic system varied from 0.31 mg/g (Kurtovska Kapija) to significantly the highest 9.57 mg/g (Strumicka Vezena) (Tab. 1).

On the other hand, the capsaicin content in the conventional production system ranged from 0.41 mg/g (Strumicka Kapija) to 7.22 mg/g (Strumicka Vezena) (Tab. 2).

As expected the hot genotype Strumicka Vezena has shown the highest capsaicin content in both cultivation systems. The capsaicin content was influenced by the maturity of the fruits, since they were not harvest in the same maturity stage. Several studies have concluded that levels of capsaicinoids increase with maturation, remain constant or decrease slightly up to 60% after the maximum is reached. Yet, at all stages of growth, the total or individual capsaicinoids based on dry weight is far higher in the placenta than in the pericarp. This statement allows the conclusion that the main site of capsaicinoids synthesis is the placental tissue of the fruits (De, 2004).

Table 1. Capsaicin content in different pepper genotypes grown in organic production system.

Genotype	Capsaicin (mg/g dry matter)
Strumicka Kapija	3.96bc
Strumicka Vezena	9.57a
Piran	0.75c
Zupska Rana	1.24c
Duga Bela	0.79c
Kurtovska Kapija	0.31c

Means within the column having different letters are significantly different according to Duncan's test at $p<0.05$.

Table 2. Capsaicin content in different pepper genotypes grown in conventional production system.

Genotype	Capsaicin (mg/g dry matter)
Strumicka Kapija	0.41c
Strumicka Vezena	7.22b
Piran	0.79c
Zupska Rana	2.33c
Duga Bela	0.62c
Kurtovska Kapija	0.47c

Means within the column having different letters are significantly different according to Duncan's test at $p<0.05$.

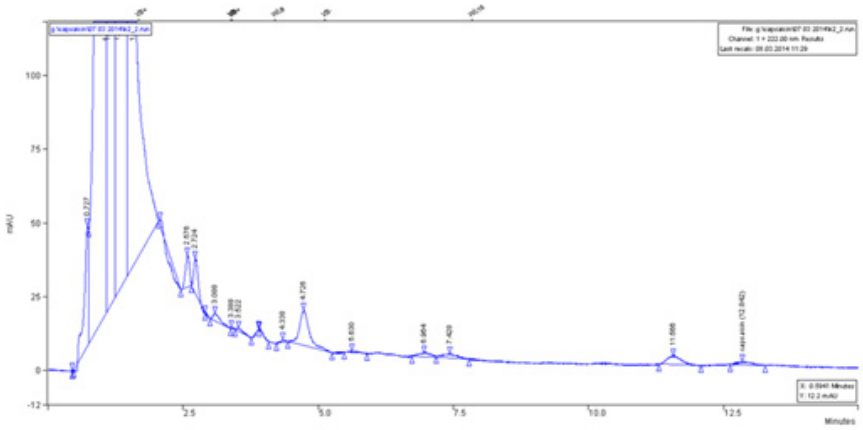

Figure 2. Chromatogram of analyzed sample of capsaicin content in Strumicka Kapija fruits.

The F test of the pepper genotypes effect on capsaicin content showed significant difference among tested genotypes during whole experiment, regardless cultivation practices applied (Tab. 3). When the same test was applied for the effect of cultivation practices (organic and conventional) on capsaicin content in pepper fruits, capsaicin content was higher in fruits obtained by organic then by conventional cultivation practices in all pepper genotypes but without significant difference (Tab. 4).

Table 3. Univariate analysis of the effect of six pepper genotypes on capsaicin content in their fruits.

Dependent Variable: Capsaicin						
	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Pepper genotypes	551.095	5	110.219	13.349	.000	.527
Cultivation practice	11.464	1	11.464	1.388	.243	.023

The F tests the effect of **Pepper genotypes** and **Cultivation practice**. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Table 4. Pairwise comparison between capsaicin content in pepper fruits and cultivation system applied.

Dependent Variable: Capsaicin				
(A) Cultivation system	(B) Cultivation system	(A-B) Mean difference	Std. error	Significance
Organic	Conventional	.798	.677	.243

Estimated marginal means of capsaicin of tested genotypes in organic and conventional cultivation systems showed higher capsaicin content in most of pepper genotypes under study (Figure 3).

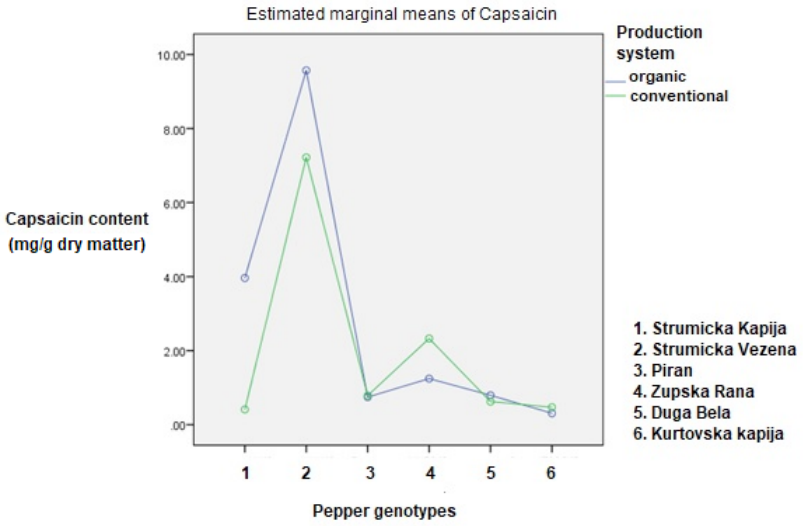

Figure 3. Estimated marginal means of capsaicin in six tested genotypes cultivated in organic and conventional production system.

Pepper fruits are rich with high number of bioactive compounds such as vitamin C and vitamin E, carotenoids and other polyphenolic compounds, but still the most of health beneficial and pharmaceutical properties and potential of peppers is due to capsaicin (Tominaga, 1998; Ying-Yue et al., 2001; Kurita 2002). There is a lack of research related to the content of nutritional and biologically active compounds as proteins, vitamins, ascorbic acid, minerals, fats and oils, phenolic compounds, aromatic substances in the fruits of varieties of *Capsicum* spp. in the Republic of Macedonia.

Maksimova et al. (2016) reported high capsaicin content in 13 hot pepper genotypes under study. Furthermore, they have not detected capsaicin in sweet pepper genotypes (Kurtovska Kapija and Zlaten Medal) when extraction was performed by maceration with 96% ethanol, which is opposite to our findings. Simonovska et al., 2014 reported detailed composition of red hot pepper fruits including capsaicinoids content in pericarp (5.38 mg/g), seeds (2.36 mg/g) and placenta (10.48 mg/g) of pepper genotype Horgoshka grown in Macedonian conditions which is in agreement with capsaicin content found in the pericarp and placenta of Strumicka Vezena. However, capsaicin content varies depending on pepper genotype, cultivation conditions and fruit phenological stage (Maksimova et al., 2016; De, 2004; Govindarajan & Sathyanarayana, 1991). Kraikruan et al. (2008) informed that capsaicin and dihydrocapsaicin contents were the highest in the first harvest in all cultivars and then they decreased in the subsequent harvests. The highest capsaicin content in fruits was found in cultivars grown at a high temperature and in nutrient-rich soils (Sung et al., 2005; Rahman & Inden, 2012).

CONCLUSION REMARKS

The capsaicin content found in six pepper genotypes grown in organic and conventional cultivation systems varies due to pepper genotypes diversity and applied cultivation practices. The highest capsaicin content was found in Strumicka Vezena grown in both cultivation systems. Overall, the capsaicin content found in organically cultivated genotypes was higher compared to conventionally grown which justifies the organic cultivation of pepper.

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КОМПАРАТИВНА АНАЛИЗА НА СОДРЖИНАТА НА КАПСАИЦИН ВО ПИПЕРКИ (*CAPSICUM ANNUUM* L.) ОДГЛЕДУВАНИ ВО КОНВЕНЦИОНАЛЕН И ОРГАНСКИ СИСТЕМ

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Резиме

Капсаициноидите кои според својата хемиска структура припаѓаат кон фенилетиламинската група на алкалоиди се создаваат од секундарниот метаболизам исклучиво на растенијата од родот Capsicum, фамилија Solanaceae. Пиперката (*Capsicum annuum* L.) по своето стопанско значење е една од водечките градинарски култури во Република Македонија. Досега во Република Македонија нема методолошко и долготрајно истажување за органско производство на зеленчук. Сè уште се водат дебати во поширока смисла на зборот за предностите на органското производство во споредба со конвенционалното во однос на морфологијата на продуктите и посебно нивниот квалитет.

Целта на овој труд беше да се направи компаративна анализа на застапеноста на капсаицин во екстракти од плодови одгледувани во органски и конвенционални системи. Генотиповите на пиперки кои се опфатени во истражувањето се: *струмичка капија*, *струмичка везена*, *пиран*, *жупска рана*, *дуга бела* и *куртовска капија*. Како екстрактант на капсаицинот беше користен метанол (≥ 99.9%) за екстракција на капсаицинот од сушени плодови на пиперка. Детерминацијата на содржината на капсаицин беше направена со хроматографска анализа со HPLC (високопритисочна течна хроматографија).

Плодовите од пиперка одгледувани во органско производство се одликуваа со повисока содржина од конвенционалните. Генотипот *струмичка везена* се одликува со највисока содржина на капсаицин и во двата системи на производство.

Клучни зборови: *Capsicum annuum L., pepper, капсаицин, органски систем на одгледување, конвенционален систем на одгледување.*

Journal of Agriculture and Plant Sciences, JAPS, Vol 15, No. 1/2, 2017

Manuscript received: 02.10.2017
Accepted: 20.10.2017

In print: ISSN 2545-4447
On line: ISSN 2545-4455
UDC: 632.982

Review paper

INSPECTION OF PESTICIDE APPLICATION EQUIPMENT

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Abstract

The Directive 2009/128 / EC of the European Parliament establishes a framework for the implementation of National Action Plan referring to the sustainable use of pesticides in every country in EU. One of the areas covered by the Directive relates directly to the introduction of mandatory monitoring and inspection of pesticide application machines using standard EN13790 and new one EN 16122. In the Republic of Macedonia at the end of 2014 began a process of harmonization of the old law for plants protection with new rules and regulations required by the European Commission. The new rules and regulations, inter alia, pay special attention to the pesticides application equipment and to their mandatory inspection at specific time intervals, protection of agricultural land from pollution and to the principles of environmental protection.

Key words: EN 13790, spray scanner, pressure gauge, agitator, flow nozzle

INTRODUCTION

The overuse and misuse of pesticides pose a threat to the environment and health hazards for the farmers in the agricultural production. Pesticide residues in food affect directly the health of consumers and the increasing number of infected people. In addition, exporters of food must comply with the standards on the minimum allowed presence of residues in processed foods, fresh fruits and vegetables. The non-compliance with these standards can have catastrophic effect on the increasing of export, which is one of the primary economic objectives of our country.

Precise application of pesticides is equally important for environmental reasons, the effectiveness of chemical protection as well as the need to reduce the input in production. By achieving greater precision of the application and thus the full effectiveness of the protection procedures, plant protection machines (sprayers, sprinklers) must have structural and technical correctness to achieve a suitable working potential (McFadden-Smith, 2003). Given the above mentioned facts, the laws on compulsory inspection of plant protection products under Directive 2009/128 / EC and 2006/42 / EC, which are based on EN 13790 (Rotteveel, 2012), are introduced in EU countries. This standard is currently under revision, as CEN (European Committee for Standardization) proposes standardization at the International Standardization Organization (ISO), Polveche (2014), so that a technical review will be carried out according to ISO 16122 in the future.

In the Republic of Macedonia at the end of 2014 began a process of harmonization of the old law for plants protection with new rules and regulations required by the European Commission. The new rules and regulations, inter alia, pay special attention to the pesticides application equipment and to their mandatory inspection at specific time intervals, protection of agricultural land from pollution and to the principles of environmental protection (Dimitrovski et al 2016). The law deals with the economic, health, environmental and social role of agriculture and establishes the principle of agricultural policy measures that are to be aimed at encouraging sustainable agricultural activities. The measures are aimed at maintaining the diversity of animal and plant species, conservation of soil and of its fertility and protection of natural conditions necessary for life in soil, water and air.

However, today the outdated technology in Macedonia, worn and poorly maintained machinery and pesticide application equipment cause directly the increased number of treatments, poor protection and uncontrolled spread of diseases and pests in the agricultural production.

PREVIOUS RESEARCH

At the end of the eighties a check on the proper operation of the pesticide application equipment and nozzles in the European Union began. The conducted tests showed which parts are most susceptible to defects.

Most researchers dealing with the issue of pesticide application equipment state that a technically correct machine can greatly contribute to reducing the risk of additional ecosystem pollution because all constituents are correct and work in the given criterion (Wegner, 2014, Tadic, 2013). Also, the authors (Almbauer et al., 2014) state that with a correct and properly calibrated machine the pesticide drift can be greatly reduced, i.e. loss of evaporation and wind.

Tests in Germany showed that the majority of defective pesticide application machines were due to defective nozzles. Out of 70000 tested pesticide application equipment, 19% of them, or 13,300 machines, showed that the nozzles were not proper (Reitz, S., Gamzlemeier, H., 1998). In Belgium between 1995 and 1998, 17466 machines were tested, of which 86% or 14895 machines were defective due to a defect in the manometer and nozzles (Langenakens, J., Pieters, M., 1999).

If the distribution of the fluid is incorrect, then there is a great chance for the appearance of pests, which causes additional economic costs and environmental problems. In Italy, a national law has not yet been adopted, which regulates the review, regulation and control of pesticide application equipment. Only in some Italian regions are guidelines issued by local governments. In most AAMS (Belgium) for review of pesticide application equipment and some solutions that comply with EN 13790 have been developed. The inspection of machines in use takes place in accordance with the European Norm 13790, which is in force since 2003, and is divided into EN 13790-1, which refers to boom sprayers and EN 13790-2, which refers to air assisted sprayers. European Norm 13790 contains rules and guidelines for determining the correctness and procedure of inspections of pesticide application equipment.

More serious testing of pesticide application machines in the Republic of Croatia was carried out at the end of the last decade, and bad results of the surface distribution of fluid in the operation of pesticide application machines were immediately noticed. (Banaj, Dj., Duvnjak, V., 2000). The failure of the nozzle is most likely due to the increase of the flow, i.e. the excess of the material due to the long-term use, which in the end causes unusual surface distribution of fluid (Tadic et al., 2010). The problem is more pronounced in nozzles with smaller ISO numbers, and Duvnjak et al. (1998) state that nozzles with a smaller aperture are more quickly consumed than nozzles with a larger aperture, and that the nozzles discharged have a much greater fluid flow in the nozzle center than the new nozzle. Even so, a big problem are installed nozzles of the non-recognizable producers. Because the flow in the new nozzle largely deviates from the ISO 10625 standard and the exploitation of technically obsolete machines that should already be replaced by new ones.

Regular inspection of the pesticide application equipment is necessary in modern agricultural production, which uses pesticides on large surfaces (Sedlar, A., 2016). Djukic, N., (2016) states that in order to ensure the production of eco-food, safe and healthy environment and reduce production costs, it is necessary to ensure controlled application of pesticides. Such application is possible only with machines for the application of pesticides that are in perfect working condition.

INSPECTION OF PESTICIDE APPLICATION EQUIPMENT BY APPLYING EN13790 STANDARD

Each technical inspection consists of a visual and operational test. Within the visual section, there are inspection of the PTO-s connection, the visibility of the mark on the fluid tank, the stability of the sprayer branch, the drainage of the fluid and the correctness of the valve. The operational test of the equipment includes test of the: Pressure gauge, pump flow and flow nozzle, liquid dispersion fluid, and fluid flow in the agitator.

The measurement of the correct operation of the pressure gauge is tested by a special

device, where the test gauge and pressure gauge are tested on the same device. According to the law all pressure gauges placed on pesticide application equipment must have a minimum diameter of 63 mm. Maximum breakthroughs that pressure gauge can produce by the standard is ± 0, 2 bar in the test range of 0 to 2bar, ± 10% in the test area more than 2 bar. Figure 1 shows the measuring device for the accuracy of the pressure gauge.

Figure 1. A measuring device for pressure gauge correctness

The pump capacity is measured using an electromagnetic fluid flow meter that fits into the valve construction and the pressure gauge. Each pump at the technical inspection has to have a capacity of 90% of the maximum capacity according to the factory data. Figure 2 shows the pump flow meter.

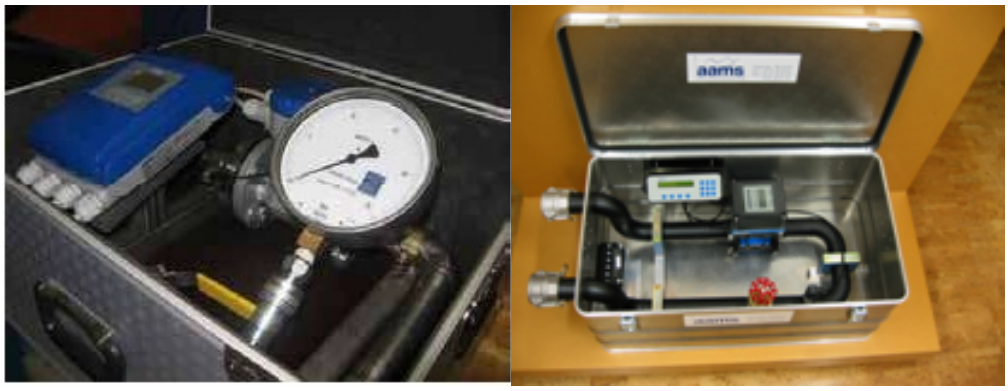

Figure 2. Electromagnetic device for pump flow measuring

Each nozzle according to ISO 10625 achieves its flow at a standard pressure of 2.76 bar. According to the technical inspection standard, each nozzle can achieve a deviation of ± 10% with respect to the nominal, in order to be correct. The nozzle flow measurement is distinguished by air assisted sprayers, which are made of stainless steel to which lenses are placed with a volume of 2000 ml. Liquid to the measuring bottle supplies plastic lines that are specially designed with buckles connected to the nozzles. The image of the device is shown in Figure 3.

Figure 3. A measuring device for mist blower nozzle flow

The nozzle flow measurement on the horizontal boom sprayers is performed by the individual digital meter shown in Figure 4.

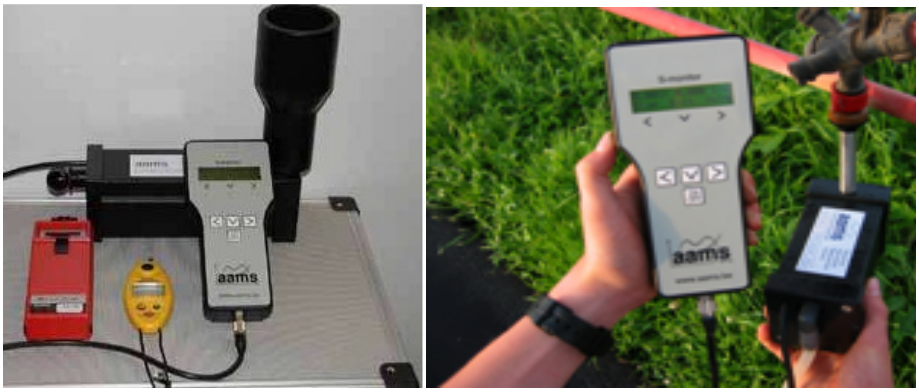

Figure 4. A measuring device for sprayer nozzle flow

Measuring of the liquid distribution is performed using a fully automated spray scanner. Newer versions of this device use a blue-tooth device to easily connect and manage real-time testing data. This device provides a very important liquid surface fluctuation coefficient, which should be below 20% of the total workflow of the sprayer by standard technical inspection. The image of the spray gun is shown in Figure 5.

For other equipment used for technical inspection, it is very important the test for surface fluid distribution is performed at wind speeds of less than 3 m / s. The PTO-s optical rotational speed gauges are also used, because the pump capacity testing is performed at 540 rpm. There are also mobile computers for enrolling machines in the database. The return of the fluid to the tank must be at least 5% from the volume of the tank and is measured by a flow meter (e.g. for a tank of 1000 l, the minimum return of fluid to the tank must be 50 l / min).

Figure 5. A measuring device for horizontal liquid distribution – spray scanner

CONDITIONS TO BE MET BY STANDARD EN 13790

For the practical application of the standard in the European Union it is necessary to establish central, regional and mobile laboratories, equipped with necessary instruments, which inspect the machines and appropriate software for collecting and analyzing data.

The task of the central laboratory is:

- Control of regional laboratories
- Issue of certificates for tested machines Writing annual reports on conducted inspections
- Training and issuing certificates to regional laboratories for each cycle of testing of three (five) years

In the composition of the central laboratory there are also adequate equipment consisting of:

Basic equipment consisting of: spray scanner, vertical partenator, instrument for measuring the flow of the spray fluid, Equipment for calibration of machines and software.

Regional laboratories perform the following tasks:

- Control of pesticide application equipment that are in use every three (five) years
- Prepare an inspection plan that is submitted to the Central Laboratory at the beginning of each year and requires certificates based on the report of the performed control
- Participate in the training organized by the Central Laboratory on the field.

These laboratories possess the following instruments: spray scanner, pump tester, instrument for measuring fluid flow of the sprayer and software.

Mobile laboratories are needed to test the pesticide application equipment in areas with inaccessible terrain, and they are also supplied with the same instruments.

Effective application of the standard allows:

- Easier access to the new markets
- Reducing the time to market exit and increasing its stake in it
- Bring new technologies to the market
- Financial risk management related to innovation
- Acceptance of innovations by clients and those who perform public procurements
- Technology transfer from developed countries in developing and transition countries
- Better assessment of new technologies

In addition, standards eliminate barriers to trade, support development, promote innovation, ensure product quality, increase safety and security, enhance visibility and enhance reputation, improve technical regulation, and foster national and international competition among suppliers the same economic branch.

The application of international, i.e. European standards has greatly contributed to the increase of competitiveness of the agricultural producers that apply them, in two ways. Firstly, the application of these standards in itself improves competitiveness because they have high international reputation and give confidence. Secondly, the application of these standards brings significant benefits to domestic and foreign suppliers as they make technical specifications in contracts more precise and clearer.

CONCLUSION

The production of healthy food is a basic task for modern agricultural production. According to the standards of the European Union, it is necessary to optimize the consumption of pesticides and to reduce their harmful impact on the environment, human health and animals. For this reason it is obligatory for every farmer in the European Union to make a technical inspection of the pesticide application equipment.

There is still no legislation in the Republic of Macedonia that prescribes the standards for inspection of pesticide application equipment. Therefore, at the end of 2014 began a process of harmonization of the old law for plants protection with new rules and regulations required by the European Commission. The law plans to draw up a national action plan based on the European directive 2009/128, which should include clearly defined objectives, measures and a timeframe for reducing the risk and impact of the use of pesticides on human health

in the shortest possible time. It is necessary to apply the European directive EN 13790, which prescribes the methods and equipment for plant protection machines to be inspected. In this way, the control over food production in the Republic of Macedonia will increase, and the country will be included in the countries where production of a healthy and safe food.

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ИНСПЕКЦИЈА НА МАШИНИТЕ ЗА АПЛИКАЦИЈА НА ПЕСТИЦИДИ

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Резиме

Директивата 2009/128/ЕС на Европскиот парламент воспостави рамка за имплементација на Национален акционен план кој се однесува на одржливо користење на пестицидите во секоја земја на ЕУ. Една од областите која ја покрива Директивата се однесува директно на воспоставување на задолжителна инспекција на машините за апликација на пестициди со примена на Стандардот EN13790 и новиот Стандард EN16122. Во Република Македонија кон крајот на 2014 година започна процесот на хармонизација на стариот Закон за заштита на растенијата со воведување на новите правила и регулативи според Европската комисија. Дел од новите правила и прописи директно се однесуваат на машините и опремата за апликација на пестициди, задолжителната инспекција во одредени временски интервали и заштита на земјиштето од загадување почитувајќи ги принципите за заштита на животната средина.

Клучни зборови: *директива EN 13790, спреј скенер, манометар, мешалка, проток на млазници*

Journal of Agriculture and Plant Sciences, JAPS, Vol 15, No. 1/2, 2017

Manuscript received: 01.10.2017
Accepted: 18.10.2017

In print: ISSN 2545-4447
On line: ISSN 2545-4455
UDC: 632.982(497.731)"2015"
Original scientific paper

CONDITION OF AIR ASSISTED SPRAYERS IN STIP REGION AND POSSIBILITY OF APPLYING EUROPEAN STANDARD EN 13790

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Abstract

The research was performed in Stip region in 2015. Stip region is located in the eastern part of the Republic of Macedonia where an intensive agricultural production takes place. Republic of Macedonia, as a candidate country for the European Union must harmonize its legislation with the European, including the European standard EN 13790. This standard includes, among other things, the introduction of mandatory inspection of machinery and equipment for pesticide application. Therefore, the main objective of this study was to collect data (number, model, age, safety ….) on the air assisted sprayers, as well as basic information about the owners of these machines (education, farm size, culture type, basic knowledge and trainings for safe and proper use of these machines etc.)

Key words: *Pesticides, inspection, visual flows, operational flows, nozzle*

INTRODUCTION

The overuse and misuse of pesticides pose a threat to the environment and health hazards for the farmers in the agricultural production. Pesticide residues in food affect directly the health of consumers and the increasing number of infected people. In addition, exporters of food must comply with the standards on the minimum allowed presence of residues in processed foods, fresh fruits and vegetables. The non-compliance with these standards can have catastrophic effect on the increasing of export, which is one of the primary economic objectives of our country.

With the new law on plant protection, the agricultural policy of our country pays special attention to the protection of agricultural land from pollution and to the principles of environmental protection. The law deals with the economic, health, environmental and social role of agriculture and establishes the principle of agricultural policy measures that are to be aimed at encouraging sustainable agricultural activities. The measures are aimed at maintaining the diversity of animal and plant species, conservation of soil and of its fertility and protection of natural conditions necessary for life in soil, water and air.

However, the outdated technology in Macedonia, worn and poorly maintained machinery and pesticide application equipment cause directly the increased number of treatments, poor protection and uncontrolled spread of diseases and pests in the agricultural production (Dimitrovski et al., 2016).

In the last few years, the European Commission adopted a whole set of rules (directives) and broadened the scope of all those regulations. According to the guidelines presented in the directives, all national regulations in the Member States must be harmonized when the countries introduce new laws. The Directive 2009/128 / EC of the European Parliament establishes a framework for the implementation of the National Action Plan in each country, which refers to the sustainable use of pesticides. One of the areas covered by the Directive relates directly to the introduction of mandatory monitoring and inspection of pesticide application equipment (Gil, 2006). Considering that in the Republic of

Macedonia the inspection is not mandatory, and as a country candidate it is bound to harmonize its regulations, the main goal of this research is to determine the current condition of the air assisted sprayers. The results are a good basis for further research and an opportunity to apply standardized procedures for mandatory inspection of machinery for pesticide application.

MATERIAL AND METHODS

The research was conducted in the Stip region in 2015 covering the municipality of Stip with larger villages: Tri Cesmi, Balvan and Argulica.

The instrument used during the field research was the questionnaire including data divided into three groups:

a) General information about the owner
b) General information on the pesticide application equipment
c) Visual and operational flaws of the machines

RESULTS AND DISCUSSION

In the Republic of Macedonia, despite the inventory of farmstead and agricultural machinery, there is no exact number of pesticide application equipment. In the questionnaire, according to the last list, there was no graph for these machines. That is why in Macedonia it is assumed that the number of these machines is about 14-15000. However, according to our initial research in the Stip region and wider, the number of pesticide application equipment is far smaller. The introduction of the EN 13790 standard, the identification of the required number of test stations, and the need for training for proper and safe operation with these machines were the motive for this first research in this field.

The aim of the research is to determine the current status of one part of air assisted sprayers and how much they meet the prescribed norms of the European standard EN 13790 and the new EN ISO 16122.

The Stip region is part of the Southeastern Mediterranean region, which is one of the major agricultural regions in the Republic of Macedonia. In this region, the most important are field crops (Fig. 1).

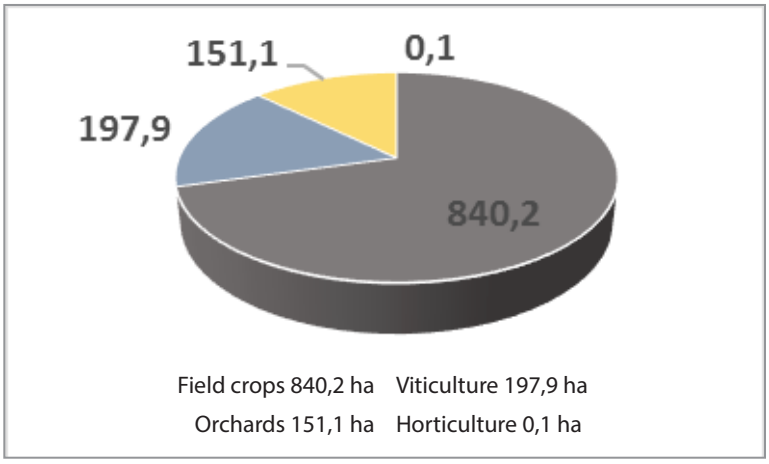

Figure1. Land structure in ha.

In the last 7-8 years as a result of the division of state land and subsidies given by the state, number of hectares with vineyard and orchards is growing. However, if irregular plant protection is carried out on these 1172 ha, on which various agricultural crops are grown, this directly affects the spread of pests and diseases, pollution of the environment and human and animal health.

Figure 2 and Figure 3 show general data about farmers who have pesticide application equipment. According to the data, it can be noted that, of the total number of farmers surveyed, 22 (88%) have secondary education, and most of them, in total 18 (72%), in this part of the Stip region are not registered farmers, because agriculture is not the main activity.

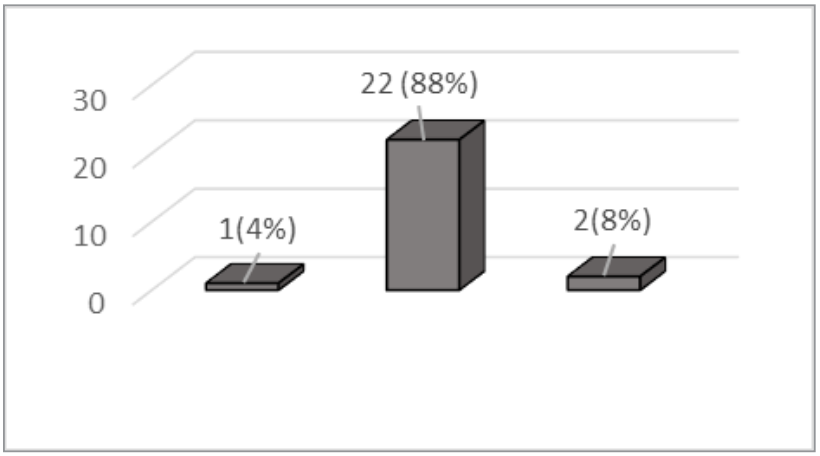

Figure 2. Education of interviued farmers.

The fact that all surveyed farmers have not attended any training on quality and on handling these machines is worrying. This means that these machines are used according to their personal experience or according to the experience of their relatives and friends which is often misleading and unsafe. However, all farmers have said they would like to participate in training to expand their knowledge, reduce plant protection costs, protect their health and reduce environmental pollution.

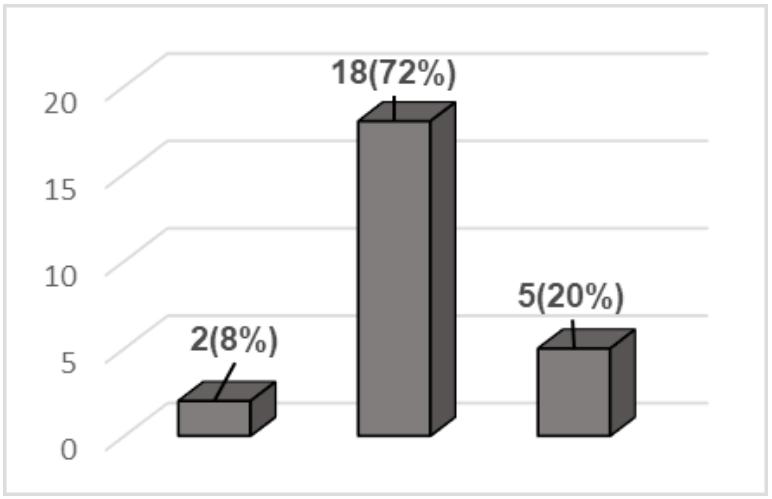

Figure 3. Regisrered farmers/firm.

The brands of pesticide application equipment, which are most prevalent in this part of the Stip region, are shown in Table 1. According to the data from the table, the most important brand is Agromehanika Kranj 15 (39.47%). Particularly satisfactory is the fact that 17 machines are aged up to 5 years, but also a large number of machines (14), are between 10-20 and over 20 years old. Improper maintenance and exploitation of these machines has a direct impact on poor quality plant protection and increased environmental pollution.

A large number of new pesticide application machines are the result of the state's help to farmers through subsidies to procure new machinery.

Table 1. Basic data for pesticide application equipment in part of Stip region.

Pesticide application equipment						
Number	Brand	Total %	Age			
			0-5	5-10	10-20	20<
1.	Agromehanika Kranj	15 (39.47)	8	4	1	2
2.	Evrotech	1 (2.63)	0	0	1	0
3.	Morava	9 (23.68)	2	1	3	2
4.	Agron Niš	7 (18.42)	7	0	0	0
5.	Metalbraneks Prokuplje	1 (2.63)	0	1	0	0
6.	Fisher	1 (2.63)	0	0	0	1
7.	Miterrer	2 (5.26)	0	0	0	2
8.	Vreček Kranj	1 (2.63)	0	1	0	0
9	Atomizatori	1 (2.63)	0	0	0	1
	Total	38 (100)	17	7	5	9

From the total number of tested machines in this region, the number of air assisted sprayers is 25. In Table 2, the number of air assisted sprayers is shown according to the way of hitching with the tractor and volume of the tank. According to the table, almost obsolete carried air assisted sprayers 13 and 12 trailered air assisted sprayers are used to protect the orchards and vineyards.

Table 2. Data for air assisted sprayers in part of Stip region.

Way of hitching	Carried	Trailered	Total
Tank	≤ 600 l	> 600 l	/
Total	13	12	25

Table 3. Visual flows on air assisted sprayers in part of Stip region.

Parts of the machine	Visual flaws of PAE		Total	%
	No parts	Modifications		
Chassis	/	4	4	13.33
Hitching device	/	/	/	0
Power take-off	/	/	/	0
Wheels / pneumatic tires	1	/	1	3.33
Tank	1	5	6	20.00
Agitator	/	/	/	0
Pump	/	2	2	6.67
Filters	2	/	2	6.67
Command valve	/	/	/	0
Manometer	1	/	1	3.33
Hoses	/	2	2	6.67
Sprayer boom	/	3	3	10.00
Nozzles	1	2	3	10.00
Ventilator	/	4	4	13.33
Total	6	24	30	100

Further research on air assisted sprayers relates to the operational and visual flows are shown in Table 3 and Table 4.

According to the results of Table 3, it can be concluded that after the visual inspection of the machine the biggest disadvantage and modification was found on the tank 6 (20%), chassis 4 (13.33%) and fan 4 (13.33%). Of the total of 30 flaws and modifications, visual defects are most often related to various adaptations of the armature with nozzles, broken or laminated cover on the tank, as well as the reinforcement of the chassis due to spinning and breaking on the machine itself. Of the total number of machines, 6 machines did not have any vital parts for proper operation and exploitation.

Analyzing the current state of air assisted sprayers it can be concluded that the most common malfunction in machines is determined in the manometer – 9 machines (36.00%), and one machine did not have any manometer at all (Tab. 4). It should be noted that this tool directly shows the operating pressure in the system and is one of the main control tools for proper and quality application of pesticides. Regarding the modifications of these machines they are usually observed in the hoses 8 (32.00%) of the machines. The great pressure and the quality of hoses affect directly the length of their use and the need for replacement (Declercq et al., 2012).

Table 4. Operational flows on air assisted sprayers in part of Stip region.

Parts of the machine	Operational flows			
	Functioning properly	%	Malfunctioning	%
Chassis	25	100	/	0
Hitching device	25	100	/	0
Power take-off	25	100	/	0
Wheels / pneumatic tires	21	84.00	4	16.00
Tank	25	100	/	0
Agitator	21	84.00	4	16.00
Pump	23	92.00	2	8.00
Filters	22	88.00	3	12.00
Command valve	19	76.00	6	24.00
Manometer	16	64.00	9	36.00
Hoses	17	68.00	8	32.00
Sprayer boom	21	84.00	4	16.00
Nozzles	21	84.00	4	16.00
Ventilator	20	80.00	5	20.00

During the conversation with the farmers about their experience the most common defects and problems encountered when using these machines were discussed. According to the results of the research it can be concluded that most defects occur in the hoses and pump of the pesticide application equipment (Tab. 5).

Table 5. Most common problems with air assisted sprayers in part of Stip region.

Chassis	Hitching device	Power take off	Wheels/ Pneumatic tires	Tank	Agitator	Pump	Filters	Command valve	Manometer	Hoses	Sprayer boom	Nozzles	Ventilator
1	0	0	0	1	0	7	3	0	0	9	1	5	1
4%	0	0	0	4%	0	28%	12%	0	0	36%	4%	20%	4%

Unfortunately, according to the first results, we can conclude that a large number of controlled air assisted sprayers can not meet the requirements of European Standard EN 13790 and the new EN ISO 16122. All surveyed machines have only one tank and many of them have modifications that are not in accordance with the above mentioned standards.

CONCLUDING REMARKS

According to the results of the research it can be stated that in the Stip region:

- The largest number of surveyed farmers is not registered farmers 18 (72%) and most of them have secondary education 22 (88%)
- None of the farmers interviewed visited training on the safe and proper exploitation of pesticide application machines (100%)
- The most common brand of pesticide application machines is Agromehanika Kranj 15 (39.47%)
- The largest number of machines is between 0-5 years old (17), but also a large number of machines (14), which are between 10-20 and over 20 years old
- The largest number of visual defects was noted due to various modification 6 (20%) due to broken or laminated cover on the tank
- Operating disadvantages were most pronounced 9 (36.00%) due to the defect of the manometer
- During the exploitation, farmers had the most problems with the intestines (36%) and the pump (28%) in pesticide application machines

In the Republic of Macedonia there is no compulsory inspection of pesticide application equipment. But as a candidate country, Macedonia is bound to apply and harmonize its laws and standards to the European Union laws and standards. The introduction of mandatory inspection, as well as other laws regarding the proper use and handling of waste pesticides, affects directly the protection of the environment and human health.

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СОСТОЈБА НА ОРОСУВАЧИТЕ ВО ШТИПСКИОТ РЕГИОН И МОЖНОСТ ЗА ВОВЕДУВАЊЕ НА ЕВРОПСКИОТ СТАНДАРД EN 13790

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Резиме

Истражувањата во овој труд се спроведени во Штипскиот регион во 2015 година. Регионот се наоѓа во источниот дел на Република Македонија, каде што се одвива интензивно зејоделско производство. Македонија како земја кандидат за членство во Европската Унија мора да ги хармонизира своите законски прописи со европските, вклучувајќи го и Европскиот стандард EN 13790. Покрај другото, овој стандард предвидува и задолжително воведување на инспекција на машините и опремата за апликација на пестициди. Поради овие причини, главна цел на ова истражување е прибирање податоци за број, модел, години на старост, безбедност и сл. на оросувачите, како и основните информации за сопствениците на овие машини (образование, големина на фармите, основни познавања и посетување на обуки за правилно и безбедно подесување и користање на овие машини итн.)

Клучни зборови: *пестициди, инспекција, визуелни недостатоци, оперативни недостатоци, млазници*

Journal of Agriculture and Plant Sciences, JAPS, Vol 15, No. 1/2, 2017

Manuscript received: 09.10.2017
Accepted: 20.10.2017

In print: ISSN 2545-4447
On line: ISSN 2545-4455
UDC: 634.64
Original scientific paper

PHYSICAL AND CHEMICAL CHARACTERISTICS OF POMEGRANATE (*Punica granatum* L.), CULTIVAR KARAMUSTAFA

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Abstract

Pomegranate is fruit species well-adapted to arid and semi-arid conditions and highly valued crop widely cultivated in Mediterranean and Near Asia countries. The most important pomegranate cultivars in Macedonia are "Bejnarija", "Lifanka" and "Karamustafa". The objective of this study was to evaluated physical and chemical parameters of pomegranate cultivar "Karamustafa", collected from two different location of the R. Macedonia. The physical parameters include fruit fresh weight, percentage of grain (%), weight of 100 berries (g), and percentage of juice (%). The cultivar "Karamustafa" (239.57- 304.79 g) belongs to a group of large fruits. The yield juice ranged from 47.83 % (Valandovo) to 50.63 % (Josifovo). Based on the content of the total acids (TA), the cultivar "Karamustafa" belongs to a group of sweet cultivar (TA<1).

Based on the results we found significant differences in the weight of the fruit, the content of the total acids and the content of the total polyphenols in the juice.

Key words: *Punica granatum L., fruits, juice, total acids, total polyphenols*

INTRODUCTION

The pomegranate (*Punica granatum* L.) belongs to the *Punicaceae* family, the genus *Punica* L. (Fond Quer, 1979). It is a diploid species whose somatic number is 2n=16, and haploid chromosomes = 8 (Westwood, 1982) or 2n=16 or 18 (Mars, 1998).

Pomegranate (*Punica granatum* L.) is an important commercial fruit crop that is extensively cultivated in port of Asia, Nort Africa and the more Mediterranean regions. All parts of the tree (leaves, flowers, and roots) are used for medical purposes for centuries (Godwa et al., 2009).

The total area of pomegranate cultivation in the world is well above 300.000 ha, which, more than 76% is found in these countries: India, Iran, China, Turkey and the USA (Melgarejo et al., 2012) with production higher than 3.000.000 t. The fruits are consumed fresh or processed into juice, jams, syrup and sauce (Fawole et al., 2011).

Consumption of pomegranate fruit and juice in the words is increasing very fast, because of its health benefits, high antioxidant capacity and the high content of polyphenols and anthocyanins (Zarei et al., 2010). Pomegranate juice is extremely nutritious, it has medicinal properties that include: sugars, citric and boric acid, pectin, vitamins, iodine, iron and other useful ingredients (Zarei et al., 2011).

Recent studies suggest that pomegranate juice contains anticancer, antimicrobial and antiviral components (Schwartz et al., 2009; Reddy et al., 2007; Kotwal, 2007). Pomegranate fruits' maturity is estimated by the skin colour, juice colour, and juice acidity (Al-Said et al., 2009), all of which vary by cultivar. Pomegranate fruits can be harvested when they reach a suitable size and the colour of skin achieves the desired pigment. The main parameters of maturity are TSS (total soluble solids), TA (total acids) and TSS/TA ratio (Pekmezci and Erkan, 2004). In the production of pomegranate pesticides and fertilizers are not used which means that fruits represent a very healthy food rich in natural nutritional and medicinal properties (Hassan et al., 2012).

In Macedonia, the pomegranate is grown on an area of 50 ha, and it has 700 tons annual production. The most of the area are orchards and some individual trees in the gardens. The pomegranate is traditionally grown in Valandovo region, which is under the influence of Mediterranean climate. In this region, climatic soil conditions (length of 240 vegetation days, annual average temperature > 14,8 C°, the annual temperature sum > 5.200 C°, soils fertile, etc.), enable the successful cultivation of pomegranate, which is present in this area for centuries.

To the best of our knowledge there are no published results about the determination of physical, morphological and chemical properties of pomegranate fruits, arils and juices in the R. Macedonia. Therefore, the main object of this study was the determination of physical, morphological and chemical parameters of pomegranate cultivar "Karamustafa", grown in two different location of R. Macedonia.

MATERIALS AND METHODS

Pomegranate fruits of "Karamustafa" species were collected from pomegranate orchards located in 10 year old orchard in Valandovo and 20 year old orchard in Josifovo area in R. Macedonia. Trees were planted at the spacing of 2.5 x 3.0 m.

Eight fruits of three different flower levels or a total of 24 per cultivar from Josifovo and Valandovo location were taken for analysis (Tab. 1, Tab. 3; I, II and III repetition).

Physical characteristics

Fruit weight (g) and the weight of 100 arils (g) were measured using the analytical laboratory scale (Mettler Toledo XS). The percentage of peel (%) is calculated as the ratio between the peel weight and fruit weight multiplied by 100. The juice yield (%) is the squeezed juice (ml) of 100 g of fruit. Fruit color was determined by organoleptic method (Koppel et al., 2010). The juice analyzed the pH value, total soluble solids, the total acid content expressed in citric acid, the maturity index and the total phenolic compounds of the juice.

Statistical processing of the results of all three repetitions was performed. The correlation is represented by the statistical analysis of the results, using the software package for statistical data processing, SPSS 19.

Chemical analyses

Measurements were performed on fresh aril juice. Total soluble solids (°Brix) and salinity (%) were determined by using a digital refractometer (Krüss optronic DR 301-95) at 20°C, calibrated by using water distilled. Titratable acidity was estimated in titration with 0.1 N NaOH to the titration point of pH 8.3, monitored with a pH meter and expressed as citric acids content (g/L). Maturity index was calculated by dividing total soluble solids to titratable acidity. Juice's pH is measured with a pH meter ((100 ATC). Total polyphenolic content was measured by using the Folin-Ciocalteau method (Makkar et al., 1993).

The obtained results are processed by analyzing the variance using the programme package STATVIEW (SAS Institute Version 5.0). The differences between the mean values tested are LSD test for $p \leq 5$.

RESULTS AND DISCUSSION

The physical characteristics of the pomegranate fruit are presented in Table 1. From the results presented in the study, the fruits from the first repetition (I-first level of flowers) of "Karamustafa" cultivar from both locations (Valandovo, Josifovo) were the largest. On the other hand, the lowest fruits were determined from the third repetition (III level of flowers). In general, the average weight of fruits is 239.57 g in Valandovo and 304.79 g in Josifovo. According to the descriptor list for pomegranate (Bellini and Giordani, 1998), they belong to the group of large fruit cultivars (225-375 g). The difference in the weight of the fruit between the location is due to the age structure of the trees. The juice yield (%) varies from 47.83% in location Valandovo to 50.63% in Josifovo location, which is almost identical to some Croatian cultivars, 43.18-53.99% (Radunic and Gadze, 2011).

Table 1. Physical characteristics of pomegranate fruit of cultivar "Karamustafa" at the two locations.

Location	Repetition	Fruit weight (g)	Weight of 100 arils (g)	Juice yield (%)	Percentage of peel (%)	Color of fruit
Valandovo	I	285.43	27.86	48.26	42.14	Reddish yellow
	II	235.00	27.86	46.48	35.56	
	III	198.28	29.28	48.76	37.10	
	I-III	239.57	28.33	47.83	38.27	
Josifovo	I	421.25	38.75	50.36	33.68	Reddish yellow
	II	251.25	36.25	53.68	34.82	
	III	241.88	33.75	47.85	40.83	
	I-III	304.79	34.58	50.63	36.44	

The fruits of "Karamustafa" variety from the two sites were red in color, which is a characteristic of the variety.

The results of the correlation between the weight of the fruit, the weight of 100 arils, the percentage of peel and the juice yield were presented in Table 2.

Table 2. Pearson's coefficient of correlation between fruit weight ant weight of 100 arils, percentage of peel and yield of juice

Pearson's coefficient of correlation (ρ)					
Valandovo			Josifovo		
Fruit weight / Juice yield	Fruit weight / Percentage of peel	Fruit weight / Weight of 100 arils	Fruit weight / Juice yield	Fruit weight / Percentage of peel	Fruit weight / Weight of 100 arils
-,119	,791	-,817	-,034	,659	-,888

According to the Pearson correlation coefficient, we can conclude that there is a high correlation between the parameters. Negative values denote inverse proportional connection.

Among the physical parameters of the "Karamustafa" variety from the first location (Valandovo) shows a high correlation value. Both cultivars are highly dependent on the weight of the fruit and the percentage of the bark. The cultivar of the Valandovo location, ρ =, 791, while in the variety of the Josifovo location ρ =, 659. The dependence between the weight of the fruit and the juice yield is the lowest and the same is inversely proportional. The variety of the Valandovo is ρ = -, 119, while in the variety of the second location ρ = -, 034. Similarly, there is inverse relationship between the weight of the fruit and the weight of 100 arils. The variety of the Valandovo is ρ = -, 817, while for the variety of the Josifovo location ρ = -, 888.

Figure 1. Pomegranate "Karamustafa" (Valandovo).

Figure 2. Pomegranate "Karamustafa" (Josifovo).

Table 3. Chemical characteristics of pomegranate juice.

Location	Repetition	pH	TSS (^{0}Brix)	TA % (citric acid)	MI ^{0}Brix/TA	Salinity (%)	Total phenolic (mg/L)	Color of juice
Valandovo	I	3.31^{a}	13.8^{b,c}	0.56^{a}	2.49^{c}	12.7^{a,b}	978^{b}	Light pink
	II	3.30^{a}	13.5^{b,c}	0.55^{a}	2.44^{c}	12.5^{a,b}	981^{b}	
	III	3.32^{a}	12.1^{d}	051^{a,b}	2.20^{d}	12.8^{a,b}	980^{b}	
Josifovo	I	3.30^{a}	15.4^{a}	0.35^{c}	4.37^{a}	13.8^{a}	1014^{a}	Pink
	II	3.33^{a}	14.2^{b}	0.35^{c}	4.06^{a}	13.5^{a}	1015^{a}	
	III	3.32^{a}	14.0^{b}	0.35^{c}	3.94^{a}	13.5^{a}	1017^{a}	

Mean values followed by different lower-case letters in each column indicate significant difference among results at P≤ 0.05 by LSD test.
TSS - Total soluble solids, TA - titratable acids, MI - maturity index.

The results for the pH, total soluble solids (TSS), titratable acidity, maturity index, total polyphenolic of the pomegranate "Karamustafa" cultivar from the two different locations, are presented in Table 3. Significant differences (P≤ 0.05) were revealed among location for pH, total soluble solids, titratable acidity, maturity index and polyphenolic content.

As shown in Table 3, the highest total soluble solids content was in Josifovo, I (15.4 ^{0}Brix) and the lowest was in Valandovo, III (12.1 ^{0}Brix). The total acids content (equivalent to citric acid) is significantly higher in all fruits on the location Valandovo than Josifovo. Some of the Turkish cultivars range from 14.70^{0}Brix to 17.90^{0}Brix TSS and from 0.5% to 2.4% TA (Ozgen et al., 2008), Croatian autochthonous cultivars contain TSS from 13.0^{0}Brix to 15.55^{0}Brix TSS and from 0.44 to 1.64% total acids (Radunić et al., 2011), while Iranian cultivars from 12.85^{0}Brix to 15.05^{0}Brix TSS and 0.33% 2.44% total acids (Tehranifar et al., 2010). Based on the total acid content, the cultivars of pomegranate are divided into groups: sweet cultivars with <1 total acids, sweet-sour cultivars with 1-2% total acids and sour cultivars with> 2% total acids content (Bellini and Giordani, 1998).

The highest level of total phenolic compounds (1014-1017 mg/L gallic acid) was quantified in Josifovo. The level of the total phenolic content in Valandovo varied from 978 to 981 mg/L gallic acid. According to the results from our study, we can indicate that "Karamustafa" cultivar had higher value for total phenolic components than cultivar "Valencia" but significantly lower value than cultivars "Akko", "Hershkovitz" and "Wonderful" (Di Nunzio et al., 2013). However, the total phenolic content in pomegranate juice of 'Karamustafa" is higher in Turkish pomegranate juice 500-916 mg/L gallic acid equivalent (Tehranifar et al., 2010) but lower than in pomegranate juice found in Turkish markets (up to 2000 mg/L) (Tezkan et al., 2009).

CONCLUDING REMARKS

On the basis of the obtained results, we can conclude that the autochthonous cultivar "Karamustafa" belongs to the group of cultivars with large fruits, and according to the content of total acids in the group of sweet cultivars. A higher juice yield and higher content of the total phenols was obtained on the location Josifovo.

This is the first research for autochthonous cultivars pomegranate in the Republic of Macedonia. The research will continue in the direction of determining the potential of this and other pomegranate autochthonous cultivars for fresh consumption and for the production of juices.

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ФИЗИЧКИ И ХЕМИСКИ КАРАКТЕРИСТИКИ НА КАЛИНКАТА (*PUNICA GRANATUM* L.), СОРТА КАРАМУСТАФА

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Резиме

Калинките се добро адаптирани овошни видови во суви и полупустински услови и високоценети култури кои се одгледуваат во земјите на Медитеранот и во Азија. Најважните сорти калинка во Македонија се *бејнарија, лифанка* и *карамустафа*. Целта на оваа студија е да се евалуираат физичките и хемиските параметри на калинката сорта *карамустафа*, собрани од две различни локации во Република Македонија. Физичките параметри вклучуваат тежина на свежо овошје, процент на зрно (%), тежина од 100 бобинки (г), процент на сок (%). Сортата *карамустафа* (239.57- 304.79 g) припаѓа на група големи плодови. Сокот од родот се движи од 47,83% (Валандово) до 50,63% (Јосифово). Врз основа на содржината на вкупните киселини (ТС), сортата *карамустафа* ѝ припаѓа на групата со слатка сорта (ТА <1).

Врз основа на резултатите пронајдовме значителни разлики во тежината на овошјето, содржината на вкупните киселини и содржината на вкупните полифеноли во сокот.

Клучни зборови: *Punica granatum* L, *овошје, сок, вкупни киселини, вкупно полифеноли*

Journal of Agriculture and Plant Sciences, JAPS, Vol 15, No. 1/2, 2017

Manuscript received: 28.09.2017
Accepted: 16.10.2017

In print: ISSN 2545-4447
On line: ISSN 2545-4455
UDC: 637.5'65.054
Original scientific paper

CHEMICAL AND FATTY ACID COMPOSITION IN MEAT OF YOUNG CHICKENS DIFFERENT HYBRID LINES

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Abstract

This paper presents the results of the study on the chemical contents, the fatty acid composition and the quality loss thermic manufacturing of the calf meat from the hybrid lines Isa Brown and Dekalb. For this purpose, 7 samples of broiler chicken were taken from the hybrid lines Isa Brown and Dekalb. After the slaughter and primary manufacture, the inner fillet and drumsticks are separated. The inner fillet from the hybrid Dekalb contains less water and more proteins compared to the meat from the inner fillet from the hybrid Isa Brown. The meat from the drumsticks from the hybrid line Isa Brown contains more saturated and less unsaturated fatty acids compared to the meat from the hybrid Dekalb. At the meat taken from the inner fillet the contents of the saturated fatty acids is lower than the contents from the unsaturated which is higher at the hybrid line Isa Brown.

Key words: *Isa Brown, Dekalb, thermic manufacturing, pH*

INTRODUCTION

The chicken meat is considered to be a diet food for people from various target groups. Young chicken at the hard hybrid lines have higher muscle mass which corresponds to a higher body mass. The muscles are built of muscle fibers. The muscle fiber is the basic unit of the skeletal muscle and covers 75-90% of the overall muscle mass (Perich and Sar, 1986). The poultry muscle is built of two types of muscle fibers, bright and dark. Dark muscle fibers are richer with sarcoplasm and active oxidases while the light ones contain more glycogenase compared to dark ones. Therefore, in the dark muscles, the oxidase of some substances in the post-mortem corpus is enhanced (Djerić et al., 2009). The white meat contains more protein while the darker more fat (Rašeta and Dakić, 1984). The chicken meat compared to other types of meat in its composition contains a higher percentage of proteins and the lowest percentage of fat and can be used as a diet food (Vasilev and Kitanovski, 2005). According to Baltic et al. (2003), poultry meat on average contains 70.6-78.2% water 21% protein, 1.85-9.85% fat, about 1% mineral matter and its energy value is 700 kJ per 100 grams. According to Ristic et al. (2008) quoted by Todorovski (2015), the white meat from broilers contains 74.9% water, 0.6% fat, 23.6% protein and 1.2% ash, and white chicken meat contains 72.9% water, 1.35% fat, 25% protein 1.1% ash.

The fatty acids in poultry meat are important from a nutritional point of view. The content of fatty acids in poultry meat has been examined by many authors. According to (Cortinas et al., 2004) the drumstick meat contains the most commonly polyunsaturated fatty acids, then unsaturated and saturated. (Klarik et al., 2005) state that in the breast meat the most common are the polyunsaturated fatty acids and then the unsaturated and saturated. According to their tests in the meat of the inner fillet, the most present are the saturated fatty acids and then the unsaturated and polyunsaturated fatty acids.

The loss of mass during the thermic

treatment of the meat, as well as in the other types of meat and in poultry meat, depends on: the manner of the thermic treatment, the time of thermic treatment, the temperature, the surface of the piece of meat. At normally, thermally treated meat, losses range from 5 to 20%. According to Klarik et al. (2012), calving of breast meat in chickens is 22.65%.

The objective of our research is to examine the chemical compound, the fatty acid compound and the quality loss of thermic manufacture at the drumstick and inner fillet meat at young chicken at the age of 16 weeks from the hybrid lines Isa Brown and Dekalb.

MATERIAL AND METHODS

The sample materials for the research are 7 broiler chickens from the hybrid line Isa Brown and 7 broiler chicken from the hybrid line Dekalb. Both hybrids are supplied as one-day chicks and breed at the poultry farm on the floor. The chicks are grown on sawdust, fed controlled according to the recommendations of manufacturers of hybrid lines with hanging feeders whose height is regulated by the growth of the chickens. During the breeding, the chickens receive all the vaccines on the recommendation of the veterinary institute. Both hybrids are at the age of 16 weeks. Young chicks were taken from a farm in Kriva Palanka in cardboard boxes with ventilation openings, a special truck for poultry transport and a document that was examined by a veterinary doctor - inspector (health certificate). The slaughtering and primary processing of the chicken broilers was done according to all sanitary veterinary regulations. Before slaughter, the chicken broilers were subjected to an 8 hour fasting. After slaughtering and primary treatment, the hull of the basic parts was taken out (wings, drumsticks, hooves, breasts and back). Subsequently, each of the basic parts was specially processed so that the skin, bone and the meat were specially taken with a knife, and then measured on a digital scale with an accuracy of 0.01 g. Then the meat was separated from the bones of the inner fillet and the drumstick of all the chickens from the two hybrids.

Chemical composition

The chemical composition was examined (water content, protein, fat mineral matter). The water with the method of drying 105 °C; the fats according to the method of Gerber, proteins were examined by using the Kjeldahl method, and the mineral material by the method of incineration.

Fatty acid composition

The fat acid composition of the meat was determined by a gas chromatography. The method of work on the sample is AOAC 996.06 GC-FID-7890 A with appliance of the tool Gas chromatograph with Flame Ionizated Detector. The fat from the analyzed samples of meat are extracted by hydrolysis methods (acid hydrolysis). The pirogal acid is added so as to avoid liberation of the fatty acids. The methylation of fatty acids was performed according to AOAC GC - FID – 7890. The obtained methyl esters of fatty acid (FAMES) were analyzed by using the appliance of Gas chromatograph with Flame Ionizated Detector and a capillary column (SP 2560 100 mx0.25 mm to 0.25 μm). Operating conditions were: injector temperature 225 °C detector 285 °C. Initial temperature of 100 °C-keep 4 minutes increased by 3 °C every minute to a final temperature of 240, hold 15 minutes. The gas holder gas was helium with a flow rate of 0.75 ml / min. Certain FAMES from the analyzed meat were identified separately on the basis of comparison with the respective retention time (which is characteristic of their molecular mass as identification parameter) with retention time standards FAMES (which include cis and trans isomers of fatty acids) standard mix. The analyzed content of each fatty acid is expressed in percentage (%).

Difference in the mass

In determining the heat of the heat treatment of the meat from the digging in the two hybrids, separation of the meat from the bones of the drumsticks was carried out in the two hybrid hens and the measurement of the meat on an electronic scale. The determination of the quality loss of the thermic treatment of the meat from the drumsticks of the two hybrid lines was made after their thermic treatment at the temperature from 180°C for about 60 minutes (with its electrically heated body (electric cooker). The difference in the mass before and after the thermic treatment gives the quality loss of the thermic treatment of the meat of the drumsticks of the two hybrids.

Statistical analysis

The obtained results of the performed measurements were processed according to the usual statistical variations according to the method of ANOVA - EXEL 1997- 2003.

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of the drumstick and inner fillet meat of the hybrid lines Dekalb and Isa Brown is presented in Tables 1 and 2.

Table 1. Chemical composition of inner fillet at hybrids Dekalb and Isa Brown.

Chemical parameters	Dekalb Mean±SD	Isa Brown Mean±SD
Water %	74.332±1.059	75.592±0.773
Fat %	0.8±0.365	0.302±0.090
Ash %	1.182±0.023	1.136±0.046
Protein %	23.066±0.6	21.982±0.744
pH	5.832±0.031	5.814±0.059

Table 2. Chemical composition of the meat of drumsticks at the hybrids Dekalb and Isa Brown.

Chemical parameters	Dekalb Mean±SD	Isa Brown Mean±SD
Water %	73.47±0.577*	76.324±1.185*
Fat %	4.5±1.190*	1.6±0.532*
Ash %	1.132±0.060	1.154±0.47
Protein %	20.11±0;.573	20.384±0.730
pH	6.16±0.045	6.066±0.37

*Statistically significant differences

The examined chemical composition of inner fillet meat in the hybrids Dekalb and Isa Brown shows that the average water content of the Dekalb hybrid is 74.33, ±1.059 while in Isa Brown it is75.592±0.773, of which it can be concluded that the meat of inner fillet in the hybrid Isa Brown contains a larger percentage of water than that of the Dekalb. The average fat content of the Isa Brown hybrid is 0.8±0.365 and it is less than the fat content of meat in Isa Brown meat which is 0.302±0.090. The content of ash in the meat of the inner fillet in the Dekalb hybrid is larger than that of the Isa Brown hybrid and it is 1.182±0.023, while in Isa Brown is 1.136±0.046. The proteins are also more represented in the meat of the inner fillet in the Dekalb hybrid, 23.066±0.6, 21.982±0.744 in Isa Brown, pH in Dekalb is 5.832, while in Isa Brown 5.814.

The average water content of the meat of drumstick in the Dekalb hybrid is 73.47±0.577, while the Isa Brown hybrid is 76.324±1.185, which means that it contains a higher percentage of water from the meat in Dekalb. The salt of fat in

the drumstick meat is more prevalent in Dekalb with 4.5±1.190 opposed to that in Isa Brown 1.6±0.532. The larger ash content is found in the meat of drumstick in the Isa Brown hybrid with 1.154±0.47, and Dekalb1.132±0.060. And also the protein content is more present in the meat in this hybrid, 20.384±0.730 and Isa Brown that is 20.11±0.5873 in Dekalb. pH in the Hybrid Dekalb is 6.16, while at the hybrid Isa Brown is 6.066. From Table 1 in relation to the chemical composition of the inner fillet at hybrids Dekalb and Isa Brown there are no statistically significant differences. Table 2 shows that in meat from drumsticks in the hybrids of Dekalb and Isa Brown there are statistically significant differences in water and fat parameters ($p<0.05$). Dekalb water 73.47±0.577; Isa Brown water 76.324±1.185;Dekalb fat 4.5±1.190; Isa Brown fat 1.6±0.532.

Ristić et al. (2008) determined that the contents of water at the meat of breasts and drumsticks at the chicken is 75 % , fat at the infer fillet 0.6% at the drumsticks 3.9%, proteins at the meat of the inner fillet 23.6% at the meat of drumsticks 19.6% mineral material ant the meat of inner fillet 1.2 at the drumsticks 1.1%.

Glamoclija (2013) determined that the average content of water in the meat of the chicken is 74.6 %, fat 11.1%, proteins 12'1%, and mineral material in the meat 1 %. Todorovski (2015) researched the chemical compound of chicken meat from the hybrid lines Isa Brown and Dekalb which are excluded from production due to the ending of the production cycle of age of 84 weeks. He realized that the meat of the chicken from the hybrid line Isa Brown contains water 76.342%, proteins 20.384%, fat 2.968%, and mineral material 1.154%. The meat from the hybrid line Dekalb contains water 75.592%, proteins 21.928%, fat 2.302% and mineral material 1.163%.

Fatty acid composition

The fatty acid compound from the meat of drumsticks and inner fillet of the hybrid lines Isa Brown and Dekalb are presented in Table 3 and Table 4.

Table 3. Determining fatty acids at drumstick meat at hybrids Isa Brown and Deklab (%).

Fatty acids	Isa Brown %	Dekalb %
C14:0	0.461	0.332
C16:0	16.977	16.896
C16:1	4.002	4.034
C18:0	4.545	5.422
C18:1n9	42.126	39.965
C18:2n6c	31.196	32.823
C18:3n6	0.692	0.528
Saturated fatty acids	21.983	22.50
Unsaturated fatty acids	78.016	77.350
Polyunsaturated fatty acids	31.888	33.351

From Table 3 it can be concluded that the saturated fatty acids are more present at the hybrid Dekalb, while the unsaturated and polyunsaturated at Isa Brown.

Table 4. Determining the fatty acid compound of the meat of inner fillet at the hybrid Isa Brown and Dekalb (%).

Fatty acids	Isa Brown %	Dekalb %
C14:0	0.079	0.316
C16:0	18.956	21.038
C16:1	3.533	0.995
C18:0	6.093	7.466
C18:1n9	41.835	37.868
C18:2n6c	29.503	32.316
C18:3n6	ND*	ND*
Saturated fatty acids	25.128	28.80
Unsaturated fatty acids	74.871	71.179
Polyunsaturated fatty acids	20.503	32.316

ND* - Not determined

It can be seen from Table 4 that the Dekalb hybrid contains more saturated and polyunsaturated fatty acids, while the hybrid Isa Brown contains more unsaturated fatty acids. According to the studies of Cortinas et al. (2004), the saturated fatty acids in the meat of the drumstick are represented by 53.81%, unsaturated 89.34% and polyunsaturated 92.03%. while in the breast meat, saturated fatty acids are represented by 6.82%, unsaturated with 10.43% and polyunsaturated are represented by 13.29%. Klarik et al. (2005) found that fatty acids in inner fillet meat of the chicken are represented by 39.97%, saturated fatty acids 33.40%, unsaturated and 5.79%, polyunsaturated fatty acids. While in the drumstick meat, 39.27%, unsaturated 38.29% and polyunsaturated fatty acids are present with 20.79%. According to the research of (Kralić et al., 2002) the inner fillet meat contains saturated fatty acids 32.44%, monounsaturated fatty acids 37.59% and polyunsaturated fatty acids 21.26%. Barbir et al. (2014) indicate that in the bright and dark meat from the chickens the following fatty acids are present: SFA 40.4%, MUFA 35.3%, PUFA omega - 6 17.4%. The differences in the results of our examinations related to the chemical composition and fatty acid composition are probably the result of the fact that we examined young chicken at age of 14-16 weeks while the other authors examined broilers and laying hens.

Difference in the mass

The quality loss thermic manufacture of the meat of drumsticks at the hybrid lines Isa Brown and Dekalb are presented in Table 5.

Table 5. Determination of mean quality loss of meat from the legs hybrids Dekalb and Isa Brown

Drum sticks	Dekalb	Isa Brown	
Weight before heat treatment	0.0632	0.0708	NS
Weight after heat treatment	0.0408	0.0474	NS
% on loss of mass of the meat in heat treatment	35.724	33.364	NS

NS- No statistically significant differences

As it can be seen from the table 5 it can be concluded that the larger heat of heat treatment of meat from the digested has in the Dekalb hybrid 35.724% (0.0224 g), as opposed to the feces of the Isa Brown hybrid, which is 33.364% (0.0234 g). From Table 5 it can be seen that there are no statistically significant differences in the loss of the mass of heat treatment of the meat of drumsticks from Isa Brown and Dekalb.

CONCLUSION

From the above presented it can be concluded that the meat of inner fillet from the hybrid Dekalb contains less water and more proteins compared to the inner fillet meat from the hybrid Isa Brown. The drumstick meat from the hybrid Dekalb contains less water and more fat compared to the drumstick meat from the hybrid Isa Brown. The drumstick meat from the hybrid line Isa Brown contains more saturated and less unsaturated fatty acids compared to the meat of hoof from the hybrid Dekalb. At the meat of the inner fillet, the content of the saturated fatty acids is lower than the unsaturated fatty acids one which is higher at the hybrid line Isa Brown compared to the hybrid line Deklab.

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ХЕМИСКИ И МАСНОКИСЕЛИНСКИ СОСТАВ КАЈ МЕСО ОД МЛАДИ КОКОШКИ РАЗЛИЧНИ ХИБРИДНИ ЛИНИИ

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Резиме

Во трудот се дадени резултатите од испитувањето на хемискиот состав, маснокиселинскиот состав и кало на термичка обработка на месо од копани и кобилица добиено од хибридните линии *иса браун* и *декалб*. За целта се земени по 7 броја млади кокошки од хибридните линии *иса браун* и *декалб*. Од нив по колењето и примарната обработка се одвоени кобилиците и копаните. Месото од кобилица од хибридот декалб содржи помалку вода, а повеќе белковини во споредба со месото од кобилица од хибридот *иса браун*. Месото од копани од хибридот *декалб* содржи помалку вода, а повеќе масти во споредба со месото од копани од хибридот *иса браун*. Месото од копани од хибридната линија *иса браун* содржи повеќе заситени, а помалку незаситени масни киселини во споредба со месото од копани од хибридот декалб. Кај месото од кобилица содржината на заситените масни киселини е помала, а на незаситените масни киселини е поголема кај хибридната линија *иса браун,* во споредба со хибридната линија *декалб*.

Клучни зборови: *иса браун, декалб, термичка обработка, pH*

Journal of Agriculture and Plant Sciences, JAPS, Vol 15, No. 1/2, 2017

Manuscript received: 04.10.2017
Accepted: 31.10.2017

In print: ISSN 2545-4447
On line: ISSN 2545-4455
UDC: 635.64-278(497.74)
Original scientific paper

CORRELATION BETWEEN POPULATION DYNAMICS OF *TUTA ABSOLUTA* (LEPIDOPTERA: GELECHIDAE) AND CLIMATE, AT TOMATO IN PROTECTED AREA

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Abstract

This research confirmed the occurrence and presence of tomato leaf miner *Tuta absoluta* (Meyrick 1917) (Lepidoptera: Gelechidae) on tomato, in a protected area, in Southeast Macedonia, in the period of 2015 - 2016. Pheromones traps, as a secure method for monitoring and detecting the presence of *T. absoluta*, were used for collecting insects. The presence of *T. absoluta* was proved by morphological analysis of the male genital structure. The main objective of our research was to determine the correlation between the dynamics of populations of *T. absoluta* and climate factors in two production systems – integral and conventional, over two season spring /autumn, at tomatoes in a protected area. The correlation is displayed through statistical analysis of the results, by applying a software package for statistical processing of the results, SPSS 19.

Key words: *tomato leaf miner, pheromone traps, monitoring, integral production, conventional production*

INTRODUCTION

The South American tomato leafminer, *T. absoluta* (Meyrick 1917), is a micro lepidopteran moth belonging to the Gelechiidae family and is considered as one of the most devastating tomato pests (Arnó et al. 2010). It originates from South America and was detected for the first time in Europe in Spain in 2006 (Karadjova et al. 2013). This newly introduced pest has spread very quickly along the Mediterranean Basin and to other Central and Northern European countries, including the Balkan (Tosevski et al. 2011). Since the 1960s, this moth has become one of the key pests of tomato crops in South America (Garcia and Espul 1982). In Europe, the presence of *T. absoluta* was initially reported in Eastern Spain, at the end of 2006 (Urbaneja et al. 2007), then in Morocco (EPPO 2008) and in Tunisia (EPPO 2009d), as well as, in several European countries (Tosevski et al. 2011). It is now a devastating pest in tomato crops in South America, Europe, Africa and Asia (Tropea et al. 2012, Zappalà et al. 2013). In most countries where *T. absoluta* occurs, the main control strategy consists of frequent sprays with chemical insecticides (Öztemiz et al. 2013). Without control *T. absoluta* causes the yield loss to increase up to 100%, which leads to dramatic decrease of the fruit quality in both field and greenhouse tomato crops (Gilardón et al. 2002, Tropea et al. 2012). *T. absoluta* is a small moth, with a body length of around 7 mm and wing span of 10-14 mm. The ground colour of the adult moths is a greenish-brown or silvery gray, with darker patches on the fore wings. Wing apex is fringed with speckled brown scales. The eggs are oval-cylindrical, cream white coloured, 0.2x0.4 mm in size. The larvae of young insects are whitish-gray in early instars (L1- L3) with a black head. In later developmental stages (L4-L5), larvae become pinkish-green to green in colour with brown head (Goftishu et al. 2014). Temperature between 19 and 23 °C is most favourable for moth development (Pervin et al. 2014). Temperatures of 10 °C and below proved to be fatal for moth development. Temperatures above 23 °C appear to have a detrimental effect on the development of *T. absoluta* and their longevity (Silva et al. 2015).

MATERIAL AND METHODS

The experiment was set in July 2015, at the village Josifovo, Gevgelisko-Valandovski region of tomato in protected area. Gevgelisko-Valandovski region is located in the South-eastern part of Republic of Macedonia characterized by sub-Mediterranean climate which influence is mostly felt. The survey was conducted over two season, spring / fall. The first season started from July to October 2015, and the second season from April to July 2016. During these two seasons two climate factors were monitored and measured: temperature and precipitation. At first, the area in the greenhouse was split in two parts, using the dripping films-agril, therefore two production systems of tomato, were obtained, an integrated and conventional. Total 120 plants are placed in integral production, as well as in the conventional, in an area of 120 m^2. Because it was a commercial production, the plant protection was performed with pesticides with different active ingredients for conventional and integrated type of production, during the two seasons. The pesticides used in the conventional production were: methomyl, flubendiamide, chlorpyrifos and in integral production: abamectin, pymetrozine, as well as, zeofit forte (Ca 35% and Mg 7%, and includes iron, potassium, zeolite, etc.), 100% natural ecological product, in form of powder, used for plant nutrition and soil improving. After planting, water traps with pheromones were set up to attract males of *T. absoluta* (PHERODIS, *Tuta absoluta*, Biobest). The insects from the traps were collected in every 10 days and placed in 96% ethanol, for further morphological studies. Morphological characters of the trapped adults which, according to morphological characters, correspond to *T. absoluta* were examined under stereo microscope Leica DM 2500.

For all collected specimens the abdomen was carefully removed and macerated in 10% KOH for about 15 minutes. After maceration the male genitalia were extracted and analyzed. The shape of the valvae and vinculum, as most relevant characters for *T. absoluta* were studied according to the description provided by the Cooperative Agricultural Pests Surveys (CAPS) https://caps.ceris.purdue.edu/dmm/542.

The correlation between the dynamics of populations of *T. absoluta* and climate in both integrated and conventional production systems, over two seasons, spring/autumn, was performed by using the software package SPPS 19. Comparing the Pearson's coefficient of correlation we can see which parameters have higher correlation. If $\rho = 1$, it means that there is a perfect linear correlation, i.e. the growth of the one variable means growth of other variables. If $\rho = 0.5 - 0.9$, there is close correlation. If $\rho = 0.2 - 0.5$, then the correlation is weak, but there is still correlation between variables. Of course, negative values, $\rho = -1$ means inversely proportional connection.

RESULTS AND DISCUSSION

During the first season (July to October / 2015), total of 115 in integral production, and 179 males of *T. absoluta* in conventional production, were collected with pheromone traps. In the second season (April to July / 2016) total of 408 in integral production, and 272 males of *T. absoluta* in conventional production were collected. The analysis of the male genital structure proves the presence of *T. absoluta* in tomato crops in Gevgelisko-Valandovski region. The most informative character is morphology of valvae, which are digitate and setose apically, with inner margin prominent convex medially (Tosevski et al., 2011), which is also confirmed for the species that we have collected. The results obtained in this work were statistically processed, using the SPSS statistical software package 19. The analysis of the results was calculated with Pearson's correlation coefficient. Dependent critical variables in this research are the number of insects *T. absoluta* and climate factors: average monthly temperature (°C) and sum of monthly rainfalls (l/m^2).

Table 1, shows the main climate parameters measured in a period from July to October, 2015, in the locality Josifovo, the monthly average temperature (°C) and sum of monthly rainfalls (l/m^2), as well as the number of *T. absoluta* for each month separately, in both integral and conventional production. From the results we observed, there is a difference between the number of *T. absoluta* in the integrated

and conventional production in 2015. In the integrated production lower numbers of insects is noticed, apart the conventional type of production. In the integrated production the lowest number is in July (10), and highest in September (48), as well as in conventional, July (16) and in September (74). In view of climate parameters (Tab. 1), July is with the highest monthly average temperature (28.3°C) and sum of monthly rainfall (628 l/m^2), while in October the lowest monthly average temperature (15.5°C) is measured, and in August minimum sum of monthly precipitation is (23.5 l/m^2). In September, when there are optimal climatic conditions for insect development, the highest numbers of *T. absoluta*, in both integral and conventional production (48/74 respectfully) is noticed.

Table 1. The number of *T. absoluta* and climate parameters: monthly average temperature (°C) and sum of monthly rainfalls (l/m^2) in the period from July/October 2015, in conventional and integrated production of tomato.

Month	No. of *T. absoluta* in integrated production	No. of *T. absoluta* in conventional production	Average monthly temperature (°C)	Sum of monthly rainfalls (l/m^2)
July	10	16	28.3	628
August	24	47	27	23.5
September	48	74	22.5	183.8
October	33	42	15.5	167.7
July/October	28.75	44.75	23.32	250.75

From the results shown in Table 2 we can see the difference between the number of *T. absoluta* in integrated and conventional production in 2016. The integrated production shows a highest number of insects, compared to the previous season (April/July 2016). The lowest number of insects is noticed in April in both integral and conventional production (32/21), and the highest is in June (239/173). In view of climate parameters (Tab. 2) July is with the highest monthly average temperature (28.4°C) and the lowest sum of monthly precipitation (25 l/m^2), while in May the highest sum of monthly rainfall (342 l/m^2) is measured. In June, when there are optimal climate conditions for insect development, the highest number of *T. absoluta* in integrated and conventional production are shown respectfully (239/173).

Table 2. The number of *T. absoluta* and climate parameters: monthly average temperature (°C) and sum of monthly rainfalls (l/m^2) at the period from April/July 2016, in conventional and integrated production of tomato.

Month	No. of *T. absoluta* in integrated production	No. of *T. absoluta* in conventional production	Average monthly temperature (°C)	Sum of monthly rainfalls (l/m^2)
April	32	21	16.4	34.6
May	46	32	18.5	342
June	239	173	20.5	244
July	91	46	28.4	25
April/July	102	68	20.95	203.6

Using the software package SPPS 19, tabular display (Tab. 3) of correlation between the insect number and climate conditions, in two different production systems, over two season spring/autumn, in the period 2015/2016, was made. The influence of climate factors and system of production of tomato, to the dynamics of populations of *T. absoluta*, is represented by correlation in Table 3.

Table 3. Pearson's correlation coefficient (ρ) obtained from the correlation between insect *T. absoluta* and climate conditions, for two different production systems-integrated and conventional, in two season spring / autumn in the period of 2015 / 2016.

Pearson's coefficient of correlation (ρ)							
2015				2016			
Conventional production		Integrated production		Conventional production		Integrated production	
T. absoluta - Average monthly T	*T. absoluta* - Sum of monthly rainfalls	*T. absoluta* -Average monthly T	*T. absoluta*- Sum of monthly rainfalls	*T. absoluta* -Monthly average T	*T. absoluta* - Sum of monthly rainfalls	*T. absoluta* - Monthly average T	*T. absoluta* - Sum of monthly rainfalls
-0.333	-0.702	-0.578	-0.610	0.082	0.334	0.210	0.259

Much higher correlation between the number of *T.absoluta* and climate conditions, is noticed in 2015. Although this correlation has a negative value, however, there is a high correlation between variables and are inversely proportional. Unlike 2015, 2016 shows lower correlation value between variables, but it is positive. The analysis shows that there is a different correlation value between the number of insects and climate conditions, in two different production systems, integrated and conventional, over two seasons spring / autumn, in the period 2015/2016. The lowest correlation exists in conventional production in 2016, between the number of *T.absoluta* and average monthly temperature ρ = 0.082, while the highest negative value is in conventional production in 2015, between the number of insects and the amount of monthly precipitation, ρ = -0.702.

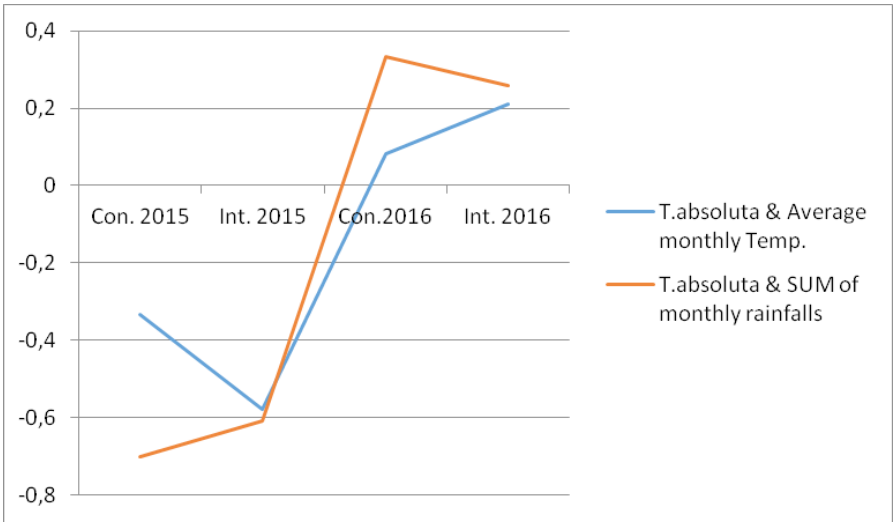

Figure 1. Correlation between *T. absoluta* and climate conditions, for two different production systems-integrated and conventional, in the period of 2015/2016

Figure 1 shows the highest correlation connectivity between *T.absoluta* and average monthly temperature, in integrated production, 2015 (-0.578), while this correlation in conventional production, 2015 was significantly lower (-0.333). It means that these two variables are inversely related. In 2016, higher correlation exists between *T. absoluta* and average monthly temperature in integrated production (0.210) while in the conventional production the correlation is significantly lower (0.082). It means that these two variables are directly proportional.

In both integrated and conventional production, in 2015 could be seen high negative correlation between *T. absoluta* and the sum of monthly precipitation (-0.610 / -0.702), while in 2016 this correlation is positive and lower (0.259 / 0.334).

CONCLUDING REMARKS

This research confirmed the occurrence and presence of tomato leaf miner *T. absoluta* (Meyrick 1917) (Lepidoptera: Gelechidae) on tomato, in a protected area, in Southeast Macedonia, in the period of 2015 - 2016. Pheromone traps as a secure method for monitoring and detecting the presence of *T. absoluta* were used for collecting insects. The presence of *T. absoluta* is proved by morphological analysis of the genital apparatus of males. The population dynamics, which depends on the climate conditions and the system of production, is displayed with statistical analysis of the received results by applying the software package for statistical analysis of the results SPSS 19. The results of the analysis showed that there is a different correlation value between the number of insects and climate conditions, in two different production systems, integrated and conventional, over two seasons spring / autumn, in the period 2015/2016. In the first season (2015), there is an inverse correlation between *T. absoluta* and average monthly temperature and the sum of monthly precipitation. In the second season, in 2016, there is a proportional relationship between the variables. The presence of *T. absoluta* in the South-eastern region of Republic of Macedonia confirms that there are optimal conditions for growth and development of the pest, because there reigns Mediterranean and sub-Mediterranean climate, which is completely suitable for the emergence of this type of insect.

ACKNOWLEDGEMENT

This study is part of the project "Entomofauna of tomato plants grown in greenhouses in conditions of conventional and integrated production", funded by the Research Fund at Goce Delcev University, Stip, Republic of Macedonia

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КОРЕЛАЦИЈА МЕЃУ ДИНАМИКАТА НА ПОПУЛАЦИЈА НА *TUTA ABSOLUTA* (LEPIDOPTERA: GELECHIDAE) И КЛИМАТА, КАЈ ДОМАТОТ ВВО ЗАШТИТЕН ПРОСТОР

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Ова истражување ги потврдува појавата и присуството на лисниот минер *Tuta absoluta* (Meyrick 1917) (Lepidoptera: Gelechidae) кај доматот во заштитен простор во југоисточна Македонија, во периодот од 2015 до 2016 година. За собирање на инсектите беа поставени феромонски мамки, кои се еден од најдобриот метод за мониторинг и детекција на *T. absoluta*. Присуството на *T. absoluta* е докажано со морфолошка анализа на машкиот генитален апарат. Главната цел на нашето истражување беше да се одреди корелацијата меѓу динамиката на популација на *T. absoluta* и климатските фактори во два производни системи – интегрален и конвенционален во текот на две сезони, пролет и есен, кај доматите во заштитен простор. Корелацијата е прикажана со статистичка анализа на резултатите, со примена на софтверски пакет за статистичка обработка на податоците, SPSS 19.

Клучни зборови: *лисен минер на доматот, феромонски мамки, мониторинг, интегрално производство, конвенционално производство.*

Journal of Agriculture and Plant Sciences, JAPS, Vol 15, No. 1/2, 2017

Manuscript received: 29.09.2017
Accepted: 17.10.2017

In print: ISSN 2545-4447
On line: ISSN 2545-4455
UDC: 633.18-152.4(497.7)"2014/2015"
Original scientific paper

GENETIC VARIABILITY FOR YIELD AND SOME YIELD-RELATED TRAITS IN RICE (*Oryza sativa* L.)

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Abstract

Fourteen rice varieties originating from Italy (arsenal, nembo, ronaldo, galileo, sprint, ulisse, krystalino, mirko, sfera, gloria, pato, creso, vasco and san andrea) were grown in agro-ecological conditions in the Republic of Macedonia in 2014 and 2015, in order to evaluate the genetic variability for yield and some important yield-related traits, like number of panicles per m^2, plant height, panicle length, number of grains per panicle, weight of grains per panicle and 1000 grains weight. Statistical analysis showed that phenotypic coefficient of variability was higher than the genotypic coefficient of variability for all evaluated traits. The highest values for genotypic and phenotypic coefficients were obtained for the number of grains per panicle (GCV = 48.51%; PCV = 55.02%) and plant height (GCV = 46.23%; PCV = 48.85%). High level of broad sense heritability for the plant height (89.57%), the number of grains per panicle (77.74%), panicle length (77.64%) and a number of panicles per m^2 (59.44%) was observed. High level of heritability coupled with genetic advance was noticed for the number of panicles per m^2, a number of grains per panicle and panicle length, which underscores the importance of these traits for the process of selection and improvement of rice genetics.

Key words: *variety, genotypic and phenotypic variability, broad sense heritability*

INTRODUCTION

In the Republic of Macedonia, there are favourable ecological conditions for high yield and quality rice production which can meet European and world quality standards. Among the factors of production, variety selection plays an important role in the productivity of rice in any location (Alam et al., 2009). A long period of time, the main rice varieties included in the process of production in the Republic of Macedonia were the Italian varieties san andrea and R-76/6, which account for over 90% of the entire production in the country. The rest are represented with selected domestic varieties (prima riska, montessa and biser-2). In the range of the selected domestic rice varieties, the main varieties are the Italian ones, also. The process of the production improvement depends on the continued providing of new germplasm possessing different genes with significant production properties. The introduction of new populations can be made easily from one region to another and may be used for further manipulation to develop new breeding lines (Ifftikhar et al., 2009). When new populations are introduced, especially from geographically distant regions, the evaluation is mostly focused on determination of their productivity and variability.

The understanding of genetic variability present in a given crop species for the traits under improvement is an imperative for the success of any plant breeding programme (Sankar et al., 2006). Parameters such as genotypic and phenotypic coefficients of variation (GCV and PCV) are a useful tool in detecting the amount of variability present in a given trait. Heritability (h^2) of a trait is important in determining its response to the selection. Genetic improvement of plants for quantitative traits requires reliable estimates of heritability in order to plan an efficient breeding program (Akinwale et al., 2011). Heritability coupled with high genetic advance (GA) would be more useful in predicting the resulted effect in the selection of the best genotypes for yield and its attributing traits (Singh et al., 2011).

The purpose of this research was to evaluate the genetic variability for yield and some important yield components in fourteen Italian rice varieties grown in Macedonia, identification of superior varieties that can be used as sources of genetic variability in future process of rice selection.

MATERIAL AND METHODS

Plant material and experimental design

During the period 2014 – 2015, fourteen rice varieties were evaluated in agro-ecological and production conditions of Macedonia. Thirteen of them were newly introduced varieties from Italy (arsenal, nembo, ronaldo, galileo, sprint, ulisse, krystalino, mirko, sfera, gloria, pato, creso and vasco), and the cultivar san andrea, also an Italian variety used for a long period as a main variety in commercial rice production in Macedonia. The experiment was performed on alluvial soil type in the region of Kocani. Each test area was 5 m^2 set in three repetitions in randomized block method. Standard agronomic practices were used.

Data collection

Ten randomly selected plants from each repetition have been analyzed for the plant height (cm), panicle length (cm), number of grains per panicle and weight of grains per panicle (g). The number of plants per m^2 was determined by counting the plants from m^2 of each repetition. 1000 grains weight has been determined to measure 1000 grains of each repetition. Grain yield obtained from the 5 m^2 was calculated in tha^{-1}. When calculating the 1000 grains weight and grain yield, the moisture content was reduced in 14%.

Statistical analysis

For the analysis of variance (ANOVA) the statistical package SPSS (2010) was used. The least significant difference (LSD) was calculated using Statistical analysis system software JMP (2002). The correlation between yield and yield components was calculated according to Singh and Chaudhary (1985) and the broad sense heritability was calculated according to Falconer (1989). Genotypic (σ^2g) and phenotypic (σ^2ph) variance were obtained from the analysis of variance table according to Comstock and Robinson (1952). The phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) were determined according to Singh and Chaudhury (1985). Genetic advance (GA) was calculated with the method suggested by Allard (1960)_and Singh and Chaudhury (1985).

RESULTS AND DISCUSSION

The results obtained from the analysis of variance for traits examined during the two years are given in Table 1. The effect of the variety shows significant values for all examined properties. Interaction of variety with the year also showed a significant effect for all examined traits except for 1000 grains weight, while the effect of the year was significant for all properties except for panicle length and weight of grains per panicle. The results showed that the selection of these traits can be effective for further improvement of rice genetics.

Table 1. Mean square for yield and some yield related traits examined in 14 rice varieties (2014-2015)

Traits	Source of variation			
	Variety (V)	Year (Y)	Interaction (V x Y)	Error
GY	3.13**	767.01**	2.75**	0.76
NP	16683.82**	939588.76**	6766.35**	5903.43
PH	5036.07**	673.22**	525.21**	31.42
PL	140.66**	0.48ns	31.45**	5.26
NGP	7050.77**	11677.89**	1569.44**	393.23
WG	4.03**	1.04ns	2.67**	0.44
1000GW	261.04**	219.93**	1.53ns	0.57

V: variety; Y: year; V x Y: Interaction between variety and year; GY: grain yield (tha^{-1}); NP: number of panicles per m^2; PH: plant height (cm); PL: panicle length (cm); NGP: number of grains per panicle; WG: weight of grains per panicle (g); 1000GW: 1000 grains weight (g); ns: no significant; **: significant at 1% level probability

Mean values for the examined traits are shown in Table 2. Among the examined varieties significant differences were identified for all evaluated traits. Superiority regarding grain yield of the variety was noticed at variety ronaldo (7.08 tha^{-1}), while the lowest grain yield was obtained from the variety gloria (4.77 tha^{-1}). The number of panicles per m^2 range from 360.17 for galileo variety to 537.50 for san andrea variety. The highest was variety san andrea (88.35 cm) and the shortest variety sfera (48.98 cm). A variety mirko was characterized with the longest panicle (16.63 cm), while krystalino has the shortest one (11.50 cm). Maximum number of grains per panicle showed variety krystalino (80.03), while the minimum number of grains per panicle was observed in variety gloria (44.88). The highest weight of grains per panicle was obtained from the variety galileo (2.58 g) and the lowest from sprint (1.59 g). The 1000 grains weight varies from 23.34 g to 42.79 g at variety mirko and gloria, respectively.

Significant differences obtained between varieties show the presence of genetic variability between them and provide a great opportunity for yield improvement.

Table 2. Means for yield and some yield related traits examined in 14 rice varieties (2014-2015)

Variety	GY	NP	PH	PL	NGP	WG	1000GW
Arsenal	5.23def	452.83a_e	54.45e	15.33abc	63.38bcd	1.79cd	28.12fg
Nembo	4. 92ef	377.00 de	61.82bc	12.68ef	73.28ab	2.30ab	31.35de
Ronaldo	7.08a	445.83a_e	54.37e	15.72ab	70.50abc	2.23abc	32.21d
Galileo	6.84abc	360.17e	54.57e	16.53a	62.27bcd	2.58a	42.34a
Sprint	5.73c_f	474.67a_d	61.28bcd	15.82ab	59.73b_e	1.59d	26.92g
Ulisse	6.21a_d	444.33a_e	61.27bcd	16.08ab	48.10ef	2.02bcd	41.90ab
Krystalino	5.98a_e	445.00a_e	56.72de	11.50f	80.03a	2.31ab	29.88def
Mirko	5.96b_e	514.20abc	57.31cde	16.63a	79.93a	1.84bcd	23.34h
Sfera	6.97ab	543.00a	48.98f	13.53de	61.33b_e	1.80cd	29.16efg
Gloria	4.77f	442.50b_e	57.38cde	15.17bcd	44.88f	1.96bcd	42.79a
Pato	5.23def	437.80cde	62.87b	13.87cde	51.02def	2.10bc	42.40a
Creso	5.99a_e	427.50cde	56.02e	15.05bcd	64.48bcd	2.09bc	31.66de
Vasko	6.17a_d	489.00abc	54.98e	15.13bcd	58.38c_f	2.04bcd	35.53c
San Andrea	5.82c_f	537.50ab	88.35a	16.38ab	55.00def	2.21abc	39.50b
Means	5.92	456.52	59.31	14.96	62.31	2.06	34.08
Minimum	1.97	281.29	46.79	9.14	26.93	0.81	30.94
Maximum	9.33	623.14	75.07	21.00	120.36	3.97	38.76
$LSD_{0.05}$	1. 12	99.40	4.85	1.41	14.15	0.48	2.68
CV (%)	11.27	13.01	4.89	5.65	13.58	13.84	4.70

Means followed by a common letter(s) within a column do not differ at 5% level; GY: grain yield (tha^{-1}); NP: number of panicles per m^2; PH: plant height (cm); PL: panicle length (cm); NGP: number of grains per panicle; WG: weight of grains per panicle (g); 1000GW: 1000 grains weight (g); CV: coefficient of variation

Genotypic variance, phenotypic variance, the genotypic coefficient of variability, phenotypic coefficient of variability, broad sense heritability, genetic advance and genetic advance, expressed as a percentage of the mean for evaluated traits, are presented in Table 3. High genotypic and phenotypic variance was obtained for the number of panicles per m^2 (1652.91 and 2780.64, respectively), number of grains per panicles (913.55 and 1175.13), plant height (751.81 and 839.34), 1000 grains weight (43.25 and 43.51) and panicle length (18.20 and 23.44, successively). Low values for a genotypic and phenotypic variance were observed only for weight of grains per panicle (0.23 and 0.67) and grain yield (0.06 and 0.52 respectively). Generally, the phenotypic variance is higher than genotypic for all examined traits.

Table 3. Genetic parameters for yield and some yield related traits examined in 14 rice varieties (2014-2015)

Traits	σ^2g	σ^2ph	GCV,%	PCV,%	h^2,%	GA,%	GAM,%
GY	0.06	0.52	4.24	12.20	12.07	0.18	3.04
NP	1652.91	2780.64	8.91	11.55	59.44	64.57	14.14
PH	751.81	839.35	46.23	48.85	89.57	53.46	90.14
PL	18.20	23.44	28.52	32.37	77.64	7.74	51.74
NGP	913.55	1175.13	48.51	55.02	77.74	54.90	88.11
WG	0.23	0.67	23.13	39.79	33.78	0.57	27.67
1000GW	43.25	43.51	19.30	19.35	99.41	13.51	39.64

σ^2g: genotypic variance; σ^2ph: phenotypic variance; GCV: genotypic coefficient of variation; PCV: phenotypic coefficient of variation; h^2: broad sense heritability; GA: genetic advance; GAM: GA as % of mean; GY: grain yield (tha^{-1}); NP: number of panicles per m^2; PH: plant height (cm); PL: panicle length (cm); NGP: number of grains per panicle; WG: weight of grains per panicle (g); 1000GW: 1000 grains weight (g)

The phenotypic coefficient of variability was higher than the genotypic coefficient of variability for all examined traits. However, these differences are low, except when it comes to the grain yield, number of grains per panicle and weight of grains per panicle. Genotypic coefficient of variability and phenotypic coefficient of variability have high values for the number of grains per panicle (GCV = 48.51%; PCV = 55.02%), plant height (GCV = 46.23%; PCV = 48.85%), panicle length (GCV = 28.52%; PCV = 32.37%) and weight grains per panicle (GCV = 23.13%; PCV = 39.79%). Moderate variability was observed at 1000 grains weight with a genotypic coefficient of variability 19.30% and phenotypic coefficient of variability 19.35%. Grain yield and number of panicles per 1m^2 showed low variability (GCV = 4.24%; PCV = 12.20%; GCV = 8.91%; PCV = 11.55%, respectively). Variation present among the rice genotypes is very important for the improvement and developing of new cultivars (Rashid et al., 2014).

High values were observed for broad sense heritability in all examined traits (> 70%) except for the grain yield and weight of grains per panicle (12.07% and 33.78%, respectively). Heritability for the number of panicles per m^2 was 59.44%. High heritability indicates that the impact of environment had less influence on the traits and that such traits can easily be improved by the process of selection.

Genetic improvement (GA) ranged from 0.18% for grain yield to 64.57% for of the number of panicles per m^2. The highest values for GAM were obtained for plant height (90.14%), the number of grains per panicle (88.11%) and for panicle length (51.74%). Other examined traits showed moderate GAM, except the grain yield which has the lowest value. High heritability coupled with high GA shows additive gene effects, while high heritability coupled with low GA shows non-additive gene effects in the control of a trait.

The obtained results showed that plant height, panicle length, a number of grains per panicle and 1000 grains weight are important properties that need to be considered in the process of selection and improvement of rice.

High heritability with the high genetic advance for different traits in rice was also reported by Abdus et al., (2009), Akinwale et al., (2011), Pallabi et al., (2013), Pratap et al., (2012), Sadeghi (2011).

CONCLUDING REMARKS

The study has shown significant differences between tested varieties for all evaluated traits. The results indicate significant phenotypic and genotypic variance between varieties for the analyzed traits. The genotypic and phenotypic coefficient of variability was highest for the number of grains per panicle. Estimated broader sense heritability was in the range from 12.07% for the grain yield to 99.41% for 1000 grains weight. High level of heritability was obtained also for the plant height (89.57%), a number of grains per panicle (77.74%), panicle length (77.64%) and the number of panicles per m^2 (59.44%). High level of heritability for the panicle number per m^2, number of grains per panicle and panicle length is associated with high genetic improvement, which means that these traits can be successfully used in the process of

selection and rice genotype improvement.

Among the evaluated varieties, the highest potential to adapt to the production conditions of Macedonia and varieties with the highest yield are ronaldo, sfera and galileo. Based on the obtained results, these varieties can be used as parents in the selection programs to create new rice genotypes with better combinations of genes for the evaluated traits.

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ГЕНЕТСКА ВАРИЈАБИЛНОСТ НА ПРИНОС И НЕКОИ КОМПОНЕНТИ НА ПРИНОСОТ КАЈ ОРИЗ (*Oryza sativa* L.)

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Резиме

Четиринаесет сорти ориз со потекло од Италија (*arsenal, nembo, ronaldo, galileo, sprint, ulisse, krystalino, mirko, sfera, gloria, pato, creso, vasco* и *san andrea*) беа одгледувани во 2014 и 2015 година во агроеколошки услови на Македонија, со цел да се евалуира генетската варијабилност за приносот и некои позначајни компоненти на приносот (број на метлички на m^2, висина на стебло, должина на метличка, број на зрна во метличка, маса на зрна по метличка и маса на 1000 зрна). Фенотипскиот коефициент на варијабилност беше повисок од генотипскиот коефициент на варијабилност за сите анализирани својства. Највисоки вредности за истите се добиени за број на зрна по метличка (GCV=48.51%; PCV=55.02%) и висина на растение (GCV=46.23%; PCV=48.85%). Висока херитабилност во поширока смисла е пресметана за висина на растение (89.57%), број на зрна по метличка (77.74%), должина на метличка (77.64%) и број на метлички на 1 m^2 (59.44%). Висока херитабилност во комбинација со високо генетско подобрување е регистрирана за број на метлички на 1 m^2, број на зрна по метличка и должина на метличка, што го потенцира значењето на овие својства за селекцијата и подбрувањето на гентската основа на оризот.

Клучни зборови: *сорта, генотипска и фенотипска варијабилност, херитабилност*

Journal of Agriculture and Plant Sciences, JAPS, Vol 15, No. 1/2, 2017

Manuscript received: 79.10.2017
Accepted: 19.10.2017

In print: ISSN 2545-4447
On line: ISSN 2545-4455
UDC: 637.523.054/.055
Original scientific paper

THE INFLUENCE OF STARTER CULTURE OF LACTIC- ACID BACTERIA AND BIFIDO BACTERIA OVER THE SANITARY- HYGENIC, SENSOR AND PHYSICAL – CHEMICAL INDICATORS ON THE RE – BOILED – SMOKED DURABLE SAUSAGE

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Abstract

The objective of this research was to study the influence of the lactic-acid bacteria and bifid bacteria over the growth of the sanitary indicator bacteria *E.coli* during the process of draining of the boiled-smoked durable sausage. The influence of these starter cultures was also examined over the physical, chemical and sensor indicator of the final product. Two types of starter cultures were used for the experiment: monoculture *B. longum* (B_2) and combined culture in relation *B. longum* (B_2) : *L. plantarum* (L_6) - 2 : 1. For the study of their influence over the technological process and the qualitative features of the product, 10 kg sample of boiled-smoked durable sausage "Burgas" was produced. The obtained results are the basis for conclusion that the starter cultures repress the development of the *E.coli* from the beginning of the production of the boiled-smoked sausage and increase the sanitary-hygienic indicators of the final product. They also contribute to the formation of the sensor characteristics of the sausage and speed the reaching of the standard values of the physical chemical indicators.

Key words: *meat products, starter cultures, lactic acid bacteria, bifidobacteria*

INTRODUCTION

In the modern meat industry, the use of starter cultures is increasingly important. The artificially created starter cultures have additional qualities than those of the more classical species, as it helps to optimize the technological processes in the production of meat products and to produce smoother, safer and healthier products (Dellaglio et al., 1996; Demeyer et al., 2004; Toldra 2006).One of the most commonly used microorganisms as starter cultures are lactic acid bacteria. Their basic function is to achieve a rapid reduction in the pH of the bulk mass, which favours: 1) the safety of the product by deactivating the pathogens; 2) the stability and shelf life of the product by inhibiting adverse changes caused by micro-organisms that spoil the product; 3) creates biochemical conditions for achieving new sensory qualities of meat products by modifying the raw materials (Ammor & Mayo 2007.; Arihara 2006; Buckenhüskes 1993). At the moment, there are sufficient materials for the use of *Lactobacillus plantarum* in the production of sausages and their positive role on the leaks of biochemical processes (Erkkilä et al., 2001**;** Leroy et al., 2006; atarata et al., 2008; Pennacchia et al.,2004). Relatively little data exist about the use of bifidobacteria in the meat industry Indzhelieva (2015). Therefore, the examination of the effect of using bifidobacteria in the production of sausages is of a particular interest. The literature data and our previous research (Indzelieva et al.,2014; Indzelieva et al., 2015; Pidcock et al., 2002; Velkova – Jorgova et al.,2014; Vayst et al., 2008), had found that bifidobacteria develops successfully, both independently and in combination with lactic acid bacteria, in the bulk mass of sausages; and produce lactic and volatile fatty acids. Their metabolic products have high reducing properties that help to form and stabilize the colour of the sausage with a relatively low concentration of sodium nitrite.

These properties of bifidobacteria reveal a serious prospect of their use as starter cultures in the meat industry.

Beside the useful, the raw meat also contains various types of harmful organisms such as *E.coli, B. proteus, Salmonella, Cl. botulinum* and others. Literature data testify to the important role of starter cultures in regulating the breeding of pathogenic microorganisms in sausage (Danielsen et al., 2003; Rivera-Espinoza & Gallardo-Navarro 2010; Ruiz et all., 2008). The antimicrobial activity of some strains of lactic acid bacteria can be used as an alternative to chemical preservatives in the production of meat products, preventing microbial contamination and at the same time intriguing the consumer for a new attractive product (Schillinger et al.,1996).

In the bulk mass of sausages, the development of starter cultures and pathogenic microorganisms flows simultaneously with direct contact of bacterial cells.

The objective of this study is to examine the influence of the lactic acid bacteria and the bifid bacteria over the growth of the sanitary indicator bacteria of *E.coli* in the process of draining the boiled-smoked durable sausage. Also, the effect of these starter cultures over the physical, chemical and sensor characteristic of the final product is studied.

MATERIAL AND METHODS

In the experimental work, the clean cultures *Lactobacillus plantarum* (L_6) and *Bifidobacterium longum* (B_2) are used. The seeds are obtained from the private licensed laboratory for analyses and food control - Burgas. Based upon the previous studies it was determined that both seeds fulfil the necessary criteria for their usage both as independent cultures and combined.

For this research two types of starter cultures were used: monoculture *B. longum* (B_2) and combined culture in relation B. *longum* (B_2): *L. plantarum* (L_6) - 2 : 1. The activation of the dry bacteria concoction is made in oil-freed and sterilized milk on 121^{0}C for 13 minutes, which is then cooled to the temperature of 37^{0}C. The starter is obtained in quantity 0,1g on 1l. The soured milk remains in the thermostat on 37^{0}C in order to reach the level of acidity of 60-65^{0}C as well as thickening. The concoction on cooled to 5^{0}C.

In order to study their impact on the flow of the technological process and the quality of the product, a 10 kg sample was produced - a product of cooked-smoked permanent sausage "Burgas". The raw materials for 100 kg are: beef first category 20, veal meat first category 20, pork loose meat 10, pork half-fat meat 0.050, cooking salt 2.000, sodium nitrite 0.005, ascorbic acid 0.050, sodium tripolyphosphate 0.100. In contrast to the traditional technology, according to which the meat of large live animals is grinding on the wolf machine and together with the salting materials, it is left to mature for 2 - 3 days at 2 – 3^{0}C, in the sample product it is achieved for 6 hours in the process of drying under the influence of the starter culture. Beef and veal are prepared according to the technology for obtaining prawns for durable cooked-smoked sausages. The salting additives, pork loose and semi-fat meat are added until they are decanted with a particle size of 4 mm. At the end, the activated liquid starter culture is added in a quantity of 5% of the weight and with a concentration of 10 logs (cfu/ml). Also a control test without a starter culture is prepared. The finished bulk mass is filled on a hydraulic machine firmly, so as not to allow inside the charge table to have air voids. After that the sausages are crushed and dried at 22^{0}C for 6 hours. The number of lactobacilli and bifidobacteria in the filling was determined by plating respectively in MRS agar (Biocar diagnostics 089) and Bifidobacteria Selective Count agar base (BSC Propionate Agar Base the filled and dried sausages and are subjected to standard heat treatment, which includes heating, cooking and smoking. Drying is carried out at 15 – 25^{0}C and relative humidity of 75-80% up to reduce of water content according to the requirements of the standards.

A sensory analysis of 7 members, pre-selected and trained (Patarate et al., 2008) was performed to determine the sensory properties of the product. The assessors evaluated the features of the outer appearance, colour of the cross-section, aroma, taste, consistency and

general grade in each test by a hedonic scale from 1 (low intensity) to 9 (intense). The sessions were carried out at a sensory assessment laboratory equipped with individual assessment cabins for marks and equal lighting conditions at a temperature of 20 ° C. Each tester received 3 sausages (± 15 grams). Each sample was marked with a random three-digit number. Drinking water and unsalted bread were also provided for cleansing the mouth between tastings.

The determination of the lactic acid bacteria was made according to ISO 13721 [12]. The determination of *E. coli* was made according to ISO 6391:1997 [13]. The determination of bifido bacteria was made according to ISO 29981:2010(en) [14]. Data were transformed into log10 cfu/g before comparison of means.

RESULTS AND DISCUSSION

The results from the study of the influence of the starter cultures over the growth of *E.coli* in the process of draining the sample of boiled-smoked sausage are given in Table 1.

Table 1. The change in the quantity of *E.coli* in the bulk mass of the boiled-smoked sausage in the process of draining.

Time for draining, h	Content of bacteria in the bulk mass, log cfu/g						
	Контрола		*B. longum* (B_2)		*B. longum* (B_2): *L. plantarum* (L_6) 2:1		
	МКВ*	*E.coli*.	BFB*	*E.coli*	МКВ*	BFB*	*E.coli*
0	3.01	5.47	4.90	5.22	5.22	4.49	5.20
2	3.52	5.03	6.72	5.01	6.25	6.26	4.46
4	3.91	4.86	7.50	3.72	7.10	7.32	3.34
6	4.68	4.02	8.46	2.31	7.62	8.80	1.05

МКВ* - number of lactic acid bacteria in the bulk mass; **BFB*** - number of bifid bacteria in the bulk mass

From the obtained results, it can be seen that during the whole process of extraction during the experiment tests there is an intensive development of *L. plantarum* (L_6) and *V. longum* (B_2). The development of the beneficial microflora is also accompanied by the destruction of *E. coli*. At the end of the extraction, the amount of *E. coli* in tests with a combined starter culture is barely 1.05 log cfu/g, with *C. longum* (B_2) - 2.31 log cfu/g, and in the control sample - 4.02 log cfu/g. The reason of the antagonistic effect of the naturally occurring cultures is probably due to the accumulation of compounds with antibiotic action, the change in the physical chemistry conditions in the bulk mass of the sausage (pH, oxide-reductive potential), and also the competition in relation to the energy substrate. In this way, the development of the microflora from starter cultures prevents the growth of *E. coli* in the very early stages of the production of boiled-smoked sausage and increases the sanitary-hygienic indicators of the finished product. *Salmonella spp*. and *L. Monocytogenes* were not found in the boiled-smoked sausages from the experiment tests in 25 g of the final products.

As a result of our previous research, it is shown that the weight of the sausage is a favourable environment for the development of the combined starter culture, with an optimum starter culture of 5%. It has been established that starter cultures with bifidobacteria have a significant influence on the speed and dynamics of the pH change, the accumulation of volatile fatty acids and free amino acids in the bulk mass of the sausage during the period of extraction (Indzelieva et al., 2015; Indzelieva 2015).The results from the table. 1 show that the development of microorganisms from starter cultures is accompanied by the destruction of E. coli bacteria, and this effect is more emphasized with the use of the combined starter culture. All these beneficial effects from the use of starter cultures with bifido bacteria also affect the organoleptic characteristics of the final product. The results of the organoleptic evaluation are shown in Table 2.

Table 2. Sensor mark of the boiled-smoked sausage produced with starter cultures, n=6 *(x ± SD)*.

Indicator	*B. longum* (B_2): *L. plantarum* (L_6) 2:1	*L. plantarum* (L_6)	Control (without starter culture)
Outside appearance	8.32 ±0.04	8.29±0.05	7.21±0.04
Color of cross-section	8.54±0.05	8.31±0.05	7.20±0.03
Aroma	8.46±0.05	8.43±0.04	6.42±0.05
Taste	8.11±0.04	8.15±0.05	6.55±0.02
Consistency	8.19±0.08	7.82±0.07	6.40±0.04
General evaluation	8.25±0.04	8.10±0.03	6.75±0.03

x – mean, *Sd* – standard deviation; n- number of pieces

The obtained results for changes in the sensory properties of the control and experimental tests of the tested product show that the used starter cultures have a major influence on the formation of those qualitative indicators that increase them. The displayed data show that, in terms of indicators, the colour of cross-section, aroma, taste, consistency and general evaluation, there is a statistically significant difference between the samples from the control and experimental tests.

The surface of all the studied sausages is dry, clean and smoked. The type of cut area is determined immediately after the cut-off. The data in the Table.2, show that in regard to the sensory feature of the intersection colour "in the experimental sausages, statistically different higher grades were determined (dully 8.54±0.05 и 8.31±0.05) compared to the control test (7.20±0.03). In this connection, it has to be pointed out that in our conducted studies the pH values in the samples with incorporated yeast showed decrease (Indjelieva et al., 2015). Furthermore, pH values also depend on the composition of the starter culture. In the test sample with a combined starter culture lowering of pH to the optimum value of 5.4 was achieved within 6 hours. About at the same time pH value in the sample *B. longum* (B_2) was 5.52 while in control was 5.72 (Indjelieva et al., 2015). According to a number of authors, this circumstance plays an extremely important role in the specific formation and stabilization of the colour of meat products (Leroy et al., 2006: Pidcock et al., 2008, Ruiz Moyanos et al., 2008).

The observed changes in the flavour values of the studied sausages show significantly higher grades in experimental researches produced with starter cultures. The results of the sensory analysis do not contradict the ones which are obtained by determining the aromatic compounds with auxiliary gas chromatography, (Indjelieva et al., 2015). These results confirm the fact that the addition of starter cultures creates the necessary conditions for the proper activity of the flavouring processes as a result of which the specific taste of the finished products is formed.

One of the requirements for the starter cultures for the sausage is to produce substances which improve the organoleptic properties of the product. The accumulation in the medium and the product of non-volatile organic acids, in particular lactic acid, volatile fatty acids, amino acids, is associated with the formation of a specific odour and taste of sausages (Indjelieva et al., 2015).

It was found that the examined sausages produced by starter cultures obtained a higher mark for taste (Table 2). In addition, the taste of the experimental tests is evaluated with significantly higher values compared to those of the control test. The more commonly used bacterial strains have the ability to manage proteolytic processes during digestion and ripening and contribute to the pleasant and characteristic taste of sausage.

A similar trend is also observed in the sensory trait of consistency, where significant differences have been identified, as there are significant differences in the grades between the control and experimental trials. The higher levels of the organoleptic evaluation of the consistency of the experimental samples are associated with the occurrence of destructive processes in the pulp mass and mainly partial hydrolysis of the meat whites under the influence of the introduced starter cultures.

The complex sensor mark shows similar tendency that the tests produced with a starter culture obtained significantly higher marks compared to the control test.

The analyses of the obtained results is a basis to conclude that the applied starter cultures provide the necessary conditions of the proper activity of the bio-chemical processes, and as a result a sensory characteristic of the sausage is formed which promotes the final product with a high sensor mark.

The experiment results from the research of the changes of some chemical indicators included in the normative documents as a dry material of Sodium Chloride and nitrites content are shown in Table 3.

Table 3. Chemical indicators of the finished product cooked-smoked and dried sausage "Burgas", n = 6 *(x ± Sd)*.

Indicator	*B. longum* (B_2): *L. plantarum* (L_6) 2:1	*L. plantarum* (L_6)	Control (without starter culture)
pH	5.73±0.02	5.79±0.03	5.90±0.01
Dry matter, % of general mass	65.98±0.05	65.12±0.04	62.75±0.03
Proteins, % of dry matter	26.94±0.05	26.28±0.05	25.26±0.03
Fats, % of dry matter	61.83±0.04	62.78±0.06	64.82±0.05
Sodium chloride, % of general mass	2.80±0.01	2.86±0.03	2.95±0.04
Nitrite, mg/100g product	3.12±0.01	3.21±0.01	4.08±0.01

x – mean, *Sd* – standard deviation; n- number of pieces

From the results it can be seen that the used starter cultures do not have an impact on the indicators of the proteins and fat in the final product from both the control and the experiment tests.

The results show that the contents of nitrites in the sausages produced with starter cultures is significantly lower compared to the control test 3.12±0.01 and 3.21±0.01 and 4.08±0.01 (Tab. 3). Considering the results from the previous studies (Indzelieva et al., 2015) it can be concluded that the starter cultures which contain bifid bacteria speed the achievement of the standard values of the physical and chemical indicators.

CONCLUDING REMARKS

1. The development of the micro-flora of the starter cultures prevents the growth of *E.coli* at the very early stage of the production of the boiled sausage and increases the sanitary and hygienic characteristics of the final product.
2. The obtained results of the sensor analyses and the control and examination tests of the researched product show that the applied starter cultures have a significant influence over the formation of the qualitative indicators and they even increase them.
3. The starter cultures which contain bifid bacteria speed the achievement of the standard values of the physical and chemical indicators.

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ВЛИЈАНИЕ НА СТАРТЕР КУЛТУРА ОД МЛЕЧНО-КИСЕЛИ БАКТЕРИИ И БИФИДОБАКТЕРИИ ВРЗ САНИТАРНО-ХИГИЕНСКИТЕ, СЕНЗОРНИТЕ И ФИЗИЧКО- ХЕМИСКИТЕ ПОКАЗАТЕЛИ НА ВАРЕНОДИМЕНИ ТРАЈНИ КОЛБАСИ

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Резиме

Целта на ова испитување беше да се проучи влијанието на млечнокиселите бактерии и бифидобактериите врз растот на санитарно-индикаторните бактерии од *E. coli* во процесот на цедење на варенодимен траен колбас. Беше испитувано и влијанието на овие стартер култури врз физичко-хемиските и сензорни показатели на готовиот производ. Во експериментот беа користени два типа стартер култури: монокултура *B. longum* (B_2) и комбинирана култура во сооднос *B. longum* (B_2) : *L. plantarum* (L_6) - 2 : 1. За испитување на нивното влијание врз протекувањето на технолошкиот процес и квалитативните карактеристики на производот беа произведени 10 kg модел на варенодимен траен колбас бургас. Добиените резултати ни даваат основа да констатираме дека стартер културите го потиснуваат развитокот на *E. coli* уште во почетокот од производството на варенодимен колбас и ги зголемуваат санитарно-хигиенските показатели на готовиот производ. Исто така, придонесуваат за формирањето и подобрувањето на сензорните карактеристики на колбасот и го забрзуваат достигнувањето на стандардните вредности на физичко-хемиските показатели.

Клучни зборови: *месни производи, стартер култури, млечно-кисели бактерии, бифидобактерии*

Journal of Agriculture and Plant Sciences, JAPS, Vol 15, No. 1/2, 2017

Manuscript received: 04.10.2017
Accepted: 31.10.2017

In print: ISSN 2545-4447
On line: ISSN 2545-4455
UDC: 635.649.076:581.192]:543.544.068.7
Original scientific paper

CAPSAICIN AND DIHYDROCAPSAICIN VARIABILITY IN *CAPSICUM* SP. CULTIVARS FROM REPUBLIC OF MACEDONIA REVEALED BY VALIDATED HPLC METHOD

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Abstract

Capsaicinoids are large group of analogues synthesized in hot peppers, *Capsicum annuum* L. as secondary metabolites. Hot peppers are widely used in nutrition but their exploitation could be increased because of capsaicin's pharmacological properties, as analgesic, antidiabetic, hypolipidemic and antitumor agent. Therefore, the aim of this study was to determine capsaicinoids (capsaicin and dihydrocapsaicin) in ethanolic extracts obtained from the fruits of 15 different genotypes and evaluate their variability. Quantification of capsaicinoids extracted from peppers cultivated in Republic of Macedonia has been performed by a validated simple and sensitive HPLC method. Although capsaicin has been known as the highest represent in the group of capsaicinoids, it was found in this study that genotypes that contain higher amount of total capsaicinoids has even higher content of dihydrocapsaicin than capsaicin. The ratio of capsaicinoids in the extracts obtained by Soxlet method was similar to that of extracts obtained by maceration. In the extract obtained from *vezena dolga,* capsaicin has been represented with 42.80% and 45.99% dihydrocapsaicin. Their content in the extract from *dzinki* was 31.44% and 45.41% for capsaicin and dihydrocapsaicin, respectively, and in the extract from *vezena kusa* capsaicin has been represented by 28.85% and dihydrocapsaicin by 48.82%. Since, the biological activity of dihydrocapsaicin has not been clearly reported; these data can be very useful for breeders of hot peppers aimed in further extraction of capsaicin for medicinal purposes.

Key words: *capsaicinoids, hot peppers, liquid chromatography, validation parameters*

INTRODUCTION

Hot peppers belong to the genus Capsicum, which is comprised of more than 200 varieties grouped into more than 30 species, out of which five are domesticated: *C. annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L. and *C. pubescens* (Hernandez et al., 1999). There are many different genotypes of hot pepper, *Capsicum annuum* L., (Solanace), cultivated in Republic of Macedonia, which present an important vegetable in food and agriculture for a long time. They have been grown mainly because of their fruits, which are widely used in nutrition and appreciated for their pungency, color and flavor aroma (Aranoff et al., 2008). Even more, hot peppers have shown many significant biological activities such as anti-inflammatory (Demirbilek et al., 2004), antioxidant (Maksimova et al., 2014), and hypocholesterolemic (Alvarez-Parrilla et al., 2012) or cytotoxic properties (Maksimova et al., 2016). Hot peppers have been characterized by the presence of vanillyl amide conjugates, known as capsaicinoids, which are absent or replaced by their non-pungent esters, isosteres (capsinoids) in the mild types of peppers (Kobata et al., 1998).

Capsaicinoids occur in the placental tissue of pepper fruits (Iwai et al., 1979) and their

biosynthesis depends on a complex and still not fully characterized enzymatic pathway. The two major capsaicinoids, responsible for up to 90% of pungency, are capsaicin and dihydrocapsaicin (Govindarajan et al., 1987), with at least nine more minor capsaicinoids occurring in pepper fruits (Suzuki et al., 1980; Kozukue et al., 2005). The type and amount of each capsaicinoid affect both the degree and the characteristics of pungency (Todd et al., 1977; Krajewska et al., 1988). Capsaicinoid levels depend on the genotype and also change during fruit development (Perucka and Materska, 2007). Moreover, environmental and nutritional conditions which occurred during the cultivation of peppers can affect the capsaicinoid content. For instance, significant differences in pungency were found in double-haploid chili plants grown in five different plots of the same field (Estrada et al., 2000), and the total capsaicinoid content in those pepper fruits developed in summer was found to be larger than in those fruits developed in autumn (Estrada et al., 2002). Also, the production of five capsaicinoids in four pepper genotypes was found to depend both on the field location and on the year (Harvell et al., 1997).

Hot peppers are usually consumed in our country in nutrition because of their pungency. Pungency or "hottness" that capsaicin causes when it is consumed *per os* can be measured as Scoville heat units (Scoville Heat Unit, SHU), according to a graduated scale that was set up for the first time by the American pharmacist Wilbur Scoville. SHU is a value that gives information about how many times the pepper extract should be diluted in water to lose the pungency or not to be sensed organoleptic. Pure capsaicin has pungency, which has been measured as 16 million SHU.

Reviewing the first scientific papers on the discovery of capsaicin from Micko et al. (1898) and characterization of its structure and nomenclature of Nelson, (1910), to the more recent data in which some of the methods for its extraction and quantification of capsaicin were proposed (Perucka and Materska, 2007), it was perceived that the content of capsaicin can be different depending on the genotypes used for its extraction and several factors used through the cultivation of the plants.

Thus, the present study aimed to evaluate the content of capsaicin and dihydrocapsaicin in 15 genotypes of cultivated peppers, applying HPLC method, because of their common use in nutritional aims and the possibility of capsaicin use in medical purposes. Through this analysis the variability of these two capsaicinoids could be assumed. The data obtained in this study could give further direction to the breeders of this culture about the genotype that is most appropriate to be used in nutrition or in medicinal purposes.

MATERIALS AND METHODS

Plant material

Fifteen different genotypes of *Capsicum annuum* L. with their local names: *vezena dolga, feferona, bombona, zlaten medal, fortense, dzinki, sivrija, kurtovska kapija, piran, vezena kusa, gambi, aiseff f1, hybrid 13514, hybrid 13515; hybrid 14530*, were cultivated on two different locations of Stip (41,746° N, 22,199° E) and Strumica (41,437° N, 22,643° E) in 2012 and 2013. Their fruits have been collected at the end of August in phenological phase of botanical maturity. They have been dried and grounded and then used as a plant material. Two of these genotypes (*zlaten medal* and *kurtovska kapija*) were not pungent and they have been taken as negative control.

Chemicals

All eluents, buffers, and standard solutions were prepared with analytical grade type I water (Milli-Q Synthesis, Millipore). Capsaicin (8-methyl-N-vanillyl-trans-6-nonenamide, ≥ 97%), dihydrocapsaicin (8-methyl-N-vanillylnonamide, ≥ 90%), ethanol (≥ 96.0%), acetonitrile (LC-MS grade), were purchased from Sigma-Aldrich.

Extraction methods

The extraction method chosen must be fast, inexpensive, versatile and efficient and should have an easy performance and no toxicity. The most widely used solvent for extracting capsaicin is hexane, which is very toxic and produces residual solvent (Gao et al., 1996, Martins et al., 2014, 2015.)

Therefore, in this study we have chosen ethanol, as a non-toxic polar solvent, which is efficiently extractive agent with the lowest side toxic effects on the human organism. Many studies have examined the effectiveness of various methods of extraction and confirmed that the efficiency of extraction by conventional methods (maceration, percolation, Soxlet-extraction) and some novel methods (ultrasound, microwave extraction and extraction with supercritical fluids) is similar (Collins et al., 1995; Goci et al., 2013). Considering this data, two conventional extraction procedures have been taken in the experimental work: maceration followed by vacuum filtration and Soxlet extraction. For the maceration process 0.200 g of plant material were measured and mixed with 25 mL of ethanol (96% *v/v*). The extraction procedure has been performed on a water bath at a temperature of 50°C for 5 hours. The obtained extracts were filtrated then by vacuum pump and Gouch filter N#4. The extraction with Soxlet apparatus have been performed by use of 0.800 g plant material in 100 mL ethanol, for 5 hours at 80 ± 2°C. The obtained extracts were used for further analysis.

Method for quantitative determination of capsaicinoids by using a High Pressure Liquid Chromatography (HPLC)

Reverse-phase liquid chromatography has been used as a method for quantitative determination of capsaicinoids in ethanolic pepper extracts. According to the literature, many authors confirmed that C-18 column can be used for effectively partition and quantification of capsaicinoids, and so this column was also used in this research (Othman et al., 2011, Perucka and Oleszek, 2000).

Fruits, dried and grounded, were used as a plant material for Soxlet extraction by using a 96 % (*v/v*) ethanol as a solvent (70°C, for 5 hours) and capsaicinoids have been quantified by use of RP-HPLC (reverse-phase high performance liquid chromatography) system, on Zorbax SB-C18 column (5µm, 250 x 4.6 mm), mobile phase: H_2O/CH_3CN, 50:50 (*v/v*), flow rate: 1.5 mL/min. A suitable DAD (diode array detector) detector followed progress of chromatographic separation at 220 nm.

Equipment: chromatographic analyses were conducted on Agilent 1200 HPLC system, (Agilent Technologies Palo Alto, CA, USA), which contained: binary pump (Model Agilent 1100 series Pump), autosampler (Model Agilent 1100 series G-1329 ALS), DAD detector (Model Agilent series G-13158 Diode Array Detector), connected to Agilent ChemStation software.

The analytical method was validated by using the protocols set out in the International Conference on Harmonization (ICH) guidelines. The required validation parameters, specificity, linearity, accuracy, precision, limit of detection, and limit of quantification, were studied for capsaicin and dihydrocapsaicin.

RESULTS AND DISCUSSION

Quantitative determination of capsaicinoids has been performed by using an isocratic, reverse-phase high performance liquid chromatography, according to a method described by Othman et al., (2011).

UV spectra of capsaicin and dihydrocapsaicin in the wavelength range from 200 to 400 nm, were recorded by a Diode Array Detector (DAD), whereby they have shown two peaks characteristic for capsaicin and dihydrocapsaicin, on 228 and 280 nm. The wavelength of 228 nm, where the absorption maximum was measured for capsaicin and dihydrocapsaicin, was chosen to designate these two compounds in their standard solutions as well as in the extracts obtained from various genotypes of hot peppers.

A typical chromatogram (Fig.1) of the standard solution of capsaicin and dihydrocapsaicin (in equimolar concentration of 10 µg/mL) showed that the time required for elution of capsaicin was 7.65 minutes, while for dihydrocapsaicin was 10.82 minutes. Identification of capsaicin and dihydrocapsaicin in the extracts was based on comparison of their retention times with those obtained for the standard solutions.

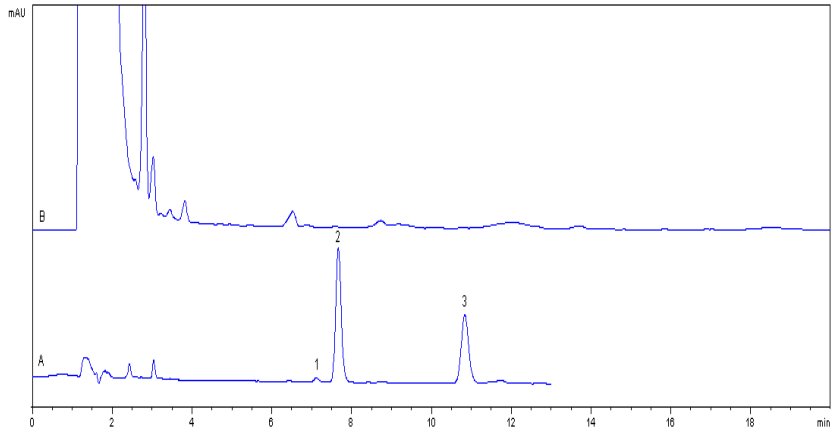

Figure 1. Chromatogram of A) standard solution, a mixture of capsaicin and dihydrocapsaicin (at equimolar concentrations 10 µg/mL), B) ethanolic extract of negative control, pepper genotype gold medal obtained by Soxlet extraction. Assignation: 1. nordihydrocapsaicin, 2. capsaicin, 3. dihydrocapsaicin.

The obtained results referring to the validation of the method for quantitative determination of capsaicinoids indicate that this method has been characterized with sufficient linearity, accuracy and precision. Correlation coefficient (R^2 = 0.999) indicated that there is a good linearity for tested concentration range for capsaicin (1.52 – 380.00 µg/mL) and for dihydrocapsaicin (1.12 – 279.00 µg/mL). Limits of detection (LOD) were 0.075 and 0.109 µg/mL, and limits of quantification (LOQ) were 0.230 and 0.331 µg/mL for capsaicin and dihydrocapsaicin, respectively. The high levels of analytical yield of 98.88 ± 2.87% for capsaicin and 98.62 ± 2.46% for dihydrocapsaicin indicated that the method is accurate. Values obtained from the examination of the repeatability of the method (RSD ≤ 2.0%) indicate that the method is precise (RSD ≤ 2.0%) and suitable for determination of the concentration of capsaicin and dihydrocapsaicin in extracts of chili peppers.

The results for the concentration of capsaicinoids (Fig.2) in the examined extracts have shown that the content of total capsaicinoids ranged from 23.27 to 6516.20 µg/g dry weight of pepper, which corresponds to the prescribed content in the literature (Othman et al., 2011, Gnayfeed et al., 2001). Genotype *feferona* presented the highest content of capsaicin, 2708.091 ± 48.75 µg/g dry weight, followed by genotype *dzinki* and *bombona* with 1725.625 ± 31.06 and 1040.431 ± 18.73 µg / g dry weight, respectively. Lowest capsaicin content has been measured in genotype *gambi*, 8.700 ± 0.16 µg/g dry weight, while in two genotypes that were not pungent and were used as controls (*gold medal* and *kurotvska kapija*), capsaicin have not been detected.

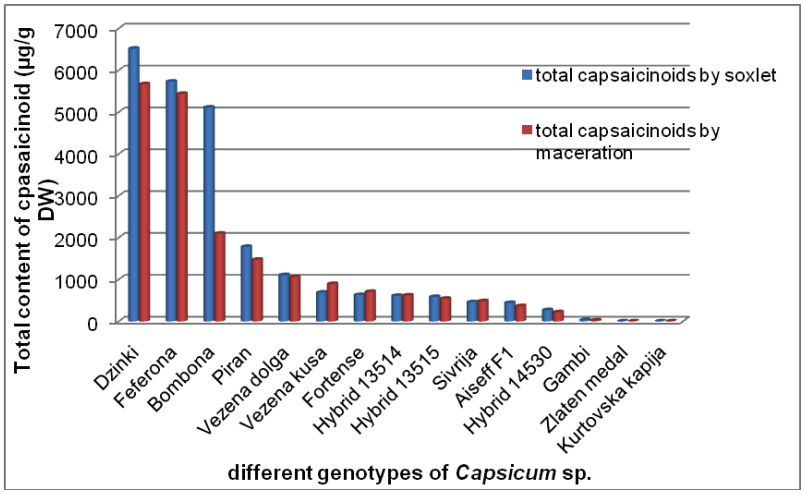

Figure 2. Total capsaicinoid content in two different extraction methods.

The results obtained from the analysis of ethanolic extracts by Soxlet extraction have shown that capsaicin is present again with the highest concentration in the extract obtained from the genotype *fefrona* (2835.190 ± 51.03 µg/g dry weight), and followed by *bombona* and *dzinki* with concentration of capsaicin 2437.991 ± 43.88 µg/g dry weight and 2048.533 ± 36.87 µg/g dry weight, respectively. The ratio of capsaicinoids in the extracts obtained by maceration (Fig.3) was similar to that of the extracts obtained by Soxlet method (Fig.4). In the extract obtained from *vezena dolga,* capsaicin has been represented with 42.80% and 45.99% dihydrocapsaicin. Their content in the extract from *dzinki* was 31.44 and 45.41% for capsaicin and dihydrocapsaicin, respectively, and in the extract from *vezena kusa* capsaicin has been represented by 28.85% and dihydrocapsaicin by 48.82%.

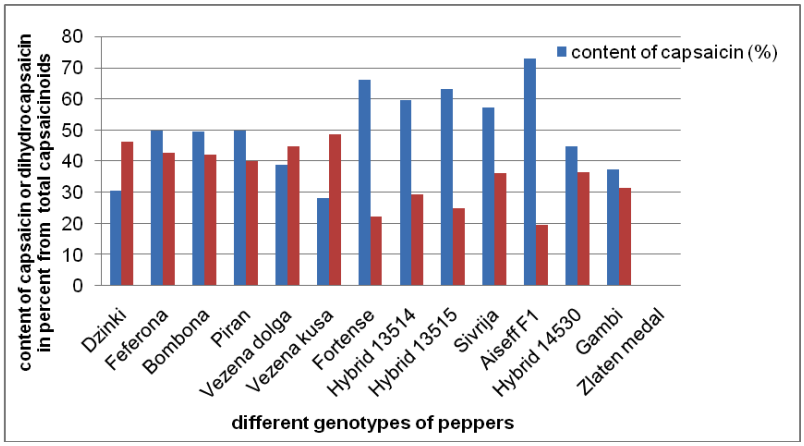

Figure 3. Variability of capsaicin and dihydrocapsaicin (% from total capsiaicinoids) in maceration extracts.

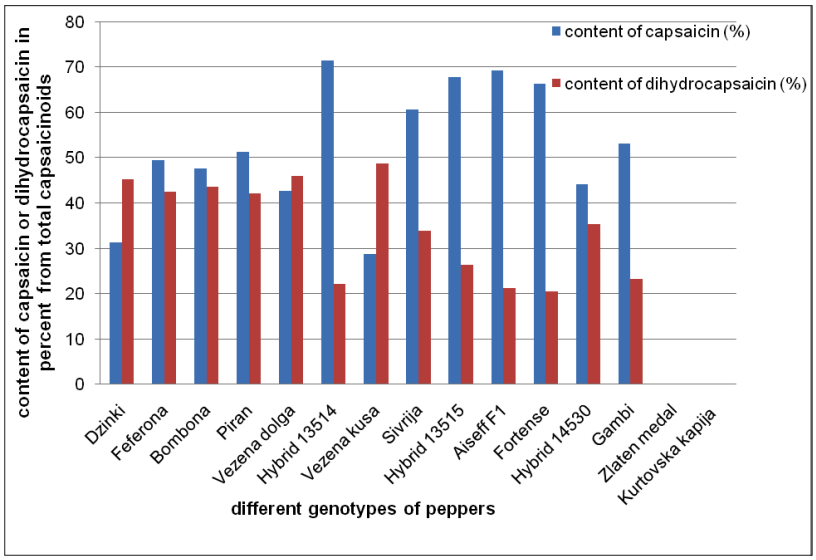

Figure 4. Variability of capsaicin and dihydrocapsaicin (% from total capsiaicinoids) in Soxlet extracts.

According to Suzuki and Iwai, (1984) capsaicin is usually represented by 69%, and dihydrocapsaicin with 30% of total capsaicinoids. Other analogues are represented in such a small amounts.

The results given for the quantitative content of capsaicinoids in hot pepper ethanolic extracts give a good basis for further investigation of the biological/pharmacological properties of capsaicin and pepper extracts.

CONCLUDING REMARKS

The analyzed Macedonian genotypes of hot peppers have characteristic high level of pungency capsaicin, which makes it promising for use, not only in food, but also in medicines. However, the development and efficient capsaicin extraction method for pharmaceutical and alimentary industry depends on the method, herbal material and optimization technique.

All the genotypes, except *gambi, zlaten* medal and *kurtovska kapija* can be used in extraction of capsaicin and dihidrocapsaicin, but the most appropriate are: *feferona, vezena dolga* and *dzinki*. From the genotypes that were taken for analysis, it was concluded that the genotype *feferona* contains the highest percentage of capsaicin.

This study has shown that genotypes which are characterized by higher content of total capsaicinoids may contain more dihydrocapsaicin than capsaicin. So, in the case of *vezena dolga,* capsaicin has been represented with 42.80% and 45.99% dihydrocapsaicin. Their content in the extract from *dzinki* was 31.44% and 45.41% for capsaicin and dihydrocapsaicin, respectively, and in the extract from *vezena kusa* capsaicin has been represented by 28.85% and dihydrocapsaicin by 48.82%. Since, the biological activity of dihydrocapsaicin has not been clearly reported; impermanence of capsaicinoids content can be very useful data. The variability of capsaicin and dihydrocapsaicin presented in these pepper genotypes can be exploited from breeders of these cultivars in order to improve content of capsaicin, which can be further used in medicinal purposes.

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ВАРИЈАБИЛНОСТ НА КАПСАИЦИН И ДИХИДРОКАПСАИЦИН ВО *CAPSICUM* SP. ОД РЕПУБЛИКА МАКЕДОНИЈА ОДРЕДУВАНИ СО ВАЛИДИРАН HPLC МЕТОД

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Резиме

Капсаициноидите се голема група на аналози кои се синтетизираат во лутите пиперки Capsicum annuum L. како секундарни метаболити. Лутите пиперки се користат во исхраната, но нивната експлоатација може да се зголеми поради фармаколошките својства на капсаицинот, како аналгетик, антидијабетичен, хиполипидемичен и антитуморен агенс. Затоа, целта на оваа студија беше да се одредат капсаициноидите, и тоа капсаицин и дихидрокапсаицин, во етанолни екстракти добиени од плодовите на 15 различни генотипови на лути пиперки и да се процени нивната варијабилност. Квантификацијата на капсаициноидите екстрахирани од пиперки одгледувани во Република Македонија се извршени со потврдениот HPLC метод. Иако капсаицинот е познат како највисок во групата на капсаициноиди, во оваа студија беше утврдено дека генотипите кои содржат повисоки количини на вкупни капсаициноиди имаат уште повисока содржина на дихидрокапсаицин отколку капсаицин. Односот на капсаициноидите во екстрактите добиени со Soxlet методот е сличен на оној на екстракти добиени со мацерација. Во екстрактот добиен од генотипот везена долга капсаицинот е застапен со 42,80% и 45,99% дихидрокапсаицин. Нивната содржина во екстрактот од џинки изнесувала 31,44 и 45,41% за капсаицин и дихидрокапсаицин соодветно, а во екстрактот од везена куса капсаицинот е претставен со 28,85%, а дихидрокапсаицин со 48,82%. Бидејќи биолошката активност на дихидрокапсаицинот не е сосема јасна, податоците добиени од овие истражувања можат да бидат многу корисни за одгледувачите на лути пиперки со цел за понатамошно искористување на капсаицинот во медицински цели.

Клучни зборови: *капсаициноиди, лути пиперки, течна хроматографија, валидациски параметри*

Journal of Agriculture and Plant Sciences, JAPS, Vol 15, No. 1/2, 2017

Manuscript received: 02.10.2017
Accepted: 16.10.2017

In print: ISSN 2545-4447
On line: ISSN 2545-4455
UDC: 632.95:581.143.5
Original scientific paper

MICROPROPAGATION OF ORNAMENTAL SPECIES *BRASSICA OLERACEA* CV. KYOTO RED GIVEN AND *AGERATUM* SP.

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Abstract

Ornamental red cabbage (*Brassica oleracea* cv. Kyoto red given) and ageratum (*Ageratum* sp.) are important ornamental plants which are typically grown in balconies, yards, parks and other open area spaces during the summer and winter period. Approximately, about 156 ornamental species/from different genera/ are produced via *in vitro* culture in different commercial laboratories worldwide. *In vitro* culture of plants is a key tool in plant biotechnology which utilizes the plant cell totipotency.

In this research the effects of different concentrations and combinations of BA, GA_3, IAA, and NAA on meristem buds and cotyledons as starting explants of ornamental red cabbage and ageratum were studied. The highest percentage of frequency of shoot formation from meristem buds was obtained on MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA_3 and MS + 2 mg/l BA for the ornamental red cabbage. On the contrary, the meristem buds of ageratum showed the best percentage of frequency of shoot formation on MS + 2 mg/l BA + 0.1 mg/l NAA. Cotyledons from ornamental red cabbage gave the highest frequency (56%) of shoot formation when initially cultivated on MS + 2 mg/l BA + 0.1 mg/l NAA, while the ageratum cotyledons responded with necrosis on all media utilized in the research. This research is a basis for further study about the enhancement of regeneration of different plant explants from different, economically important, ornamental species.

Key words: *ornamental red cabbage (Brassica oleracea cv. Kyoto red given), ageratum (Ageratum sp.), in vitro, growth regulators, meristem bud, cotyledon, shoot*

Abbreviations: BA (6-Benzylaminopurine), GA_3 (Gibberellic acid), IAA (Indole-3-acetic acid), NAA (alpha-Naphthaleneacetic acid)

INTRODUCTION

The commercial production of ornamental plants is growing worldwide. Its monetary value has significantly increased over the last two decades and there is a great potential for continued further growth in both domestic and international markets (Jain, 2002). Major pot plants such as Begonia, Ficus, Anthurium, Chrysanthemum, Rosa, Saintpaulia, and Spathiphyllum are being produced in the developed countries. About 212.5 million plants including 157 million ornamental plants amounting to 78% of the total production were reported (Rout et al., 2006). Ornamental industry has applied immensely *in vitro* propagation approach for large-scale plant multiplication of elite superior varieties. As a result, hundreds of plant tissue culture laboratories have come up worldwide, especially in the developing countries due to cheap labour costs. However, micropropagation technology is more costly than conventional propagation methods, and unit cost per plant becomes unaffordable compelling to adopt strategies to cut down the production cost for lowering the cost per plant (IAEA-TECDOC-1384, 2004).

Ornamental red cabbage (*Brassica oleracea* cv. Kyoto red given), also known as 'flowering' cabbage, is round shaped cabbage characterized by blooming in red and white colors during autumn period which makes it a desirable plant for different floristic decorations. It is plant of cold weather and it needs low temperatures to give the best leaf colors. As it is grown in late summer and early autumn, this species has less diseases and pests compared to spring species (Bajaj and Nietsch, 1975).

The name of the genus Ageratim (*Ageratum* sp.) is of antique Greek origin (ageratos = forever young) which is related to the blooming duration. The flowers of this species are mainly in shades of blue, but pink, lavender and white variations can be also found. The ageratum flowers are fluffy with nice fragrance. Each flower group is composed of 5 to 15 flowers. It blooms from late spring to early frost. The ageratum needs well drainage soil, but dry conditions are adverse for its growth. It does not have serious disease or pest problems, although mites can cause problems, particularly during dry and hot weather conditions (Stephens, 2007).

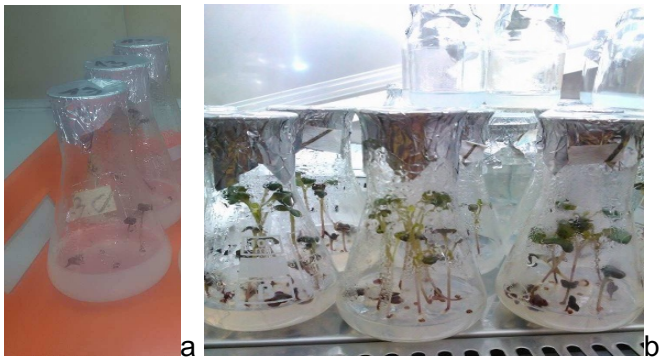
Shoot regeneration of different Brassica species was achieved from various tissues and organs including hypocotyls, cotyledons, roots, leaves, peduncle segments, callus and cell cultures, thin cell layers and protoplasts (Cardoza and Stewart, 2004). However, there are almost no literature sources on micropropagation of ageratum, which, beside ornamental, owns great medicinal values (Stephens, 2007).

MATERIALS AND METHODS

The research described in this paper was conducted at the Department of Plant Biotechnology, Faculty of Agriculture, Goce Delcev University – Stip. As starting explants were used meristem buds and cotyledons of commercially genotypes of ornamental red cabbage (*Brassica oleracea* cv. Kyoto red given) and ageratum (*Ageratum* sp.).

Obtaining of starting material for *in vitro* propagation

The seeds of commercial genotypes of both species after sterilization were inoculated on basal medium (1/2 MS solution, 3% sucrose, free of growth regulators) as 10 seeds in 10 Erlenmeyer flasks, or in total 100 seeds of each species. After seed germination, the meristem buds and cotyledons were isolated and they were used as starting explants in the research.



a b

Figure 1. a) Seed germination of ornamental red cabbage.
b) Fully developed seedlings of ornamental red cabbage before isolation of meristem buds and cotyledons.

Sterilization of seeds

The seeds were surface sterilized with:

- Submersing in 70% C_2H_5OH for 3 minutes,
- Submersing in 1,5% Izosan G for 10 minutes,
- Afterwards they were washed (x3 times) in sterile distilled water.

Regeneration of meristem buds and cotyledons on MS medium with different growth regulators

The meristem buds and cotyledons as starting explants of ornamental red cabbage and ageratum were inoculated on MS medium (Murashige and Skoog, 1962), supplemented with certain concentration of BA, GA_3, IAA and NAA. The development of the explants was followed during the experiment.

Ornamental red cabbage bud meristem and cotyledons were initially cultivated on MS medium enriched with the following growth regulators:

A: MS + 2 mg/l BA +0.1 mg/l IAA + 0.1 mg/l GA_3

B: MS + 2 mg/l BA +0.1 mg/l NAA

C: MS + 2 mg/l BA

D: MS + 5 mg/l BA + 5 mg/l NAA

Ageratum bud meristem and cotyledons were initially cultivated on MS medium enriched with the following growth regulators:

A: MS + 2 mg/l BA +0.1 mg/l IAA + 0.1 mg/l GA_3

B: MS + 2 mg/l BA +0.1 mg/l NAA

E: MS + 5 mg/l BA

F: MS + 3 mg/l BA + 1.5 mg/l NAA

Obtained shoots from the both species under study were subcultured on rooting medium MS + 0.5 mg/l IAA + 2.5 mg/l IBA.

The starting explants which after one month of cultivation did not resulted in formation of regenerant were subcultured on fresh MS medium supplemented with the same combination and concentration of growth regulators as in the starting cultivation.

Obtained shoots from the both species under study were subcultured on rooting medium MS + 0.5 mg/l IAA + 2.5 mg/l IBA.

Statistical analysis

Statistical analysis of variance was applied for the evaluation of each of the parameter among all tested combinations of growth regulators (One-Way ANOVA test) with IBM SPSP Statistics Software 19.0.

For evaluation of the difference between tested combinations of growth regulators Duncan's multiple range test is utilized for each trait in each experimental year on 0.05%

RESULTS AND DISCUSSION

In Table 1. the number and size of the meristem bud and cotyledons of ornamental red cabbage on MS medium supplemented with different growth regulators are presented. On the medium MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA_3 initially were cultivated 40 meristem buds with average width of 3.5 mm and average height of 8.8 mm and 48 cotyledons with average width 1.2 mm and length 2.5 mm. On MS + 2 mg/l BA + 0.1 mg/l NAA were inoculated 40 meristem buds with average width 4.7 mm and height 9.2 mm, and 55 cotyledons with average width 1.9 mm and length 2.1 mm. On MS + 2mg/l BA were initially cultivated 31 meristem buds with average width 1.15 mm and average height of 3.8 mm, and 47 cotyledons with average width 3.2 mm and length 1.4 mm. On MS + 5 mg/l BA + 5 mg/l NAA were inoculated 50 meristem buds with average width 2.8 mm and average height of 9.5 mm, and 50 cotyledons with average width 1.0 mm and average length 2.4 mm.

Very often the cells react differently in different developmental phases, when interaction between signal paths of auxins and cytokinins can occur (Shi et al., 1994). BA alone or in combination with auxin is proven as optimal for regeneration and multiplication of different Brassica species (Metz et al., 1995; Munshi et al., 2007). Gerszberg et al. (2015) tested cotyledon and hypocotyl of eight Brassica cultivars on five types of media, where MS + 8.88 μM 6-benzyloaminopurine (BAP) + 0.53 μM α-naphthylacetic acid (NAA) have been identified as most effective for shoot regeneration. Cogbill et al. (2010) reported that *Brassica rapa* L. five-day-old cotyledonary explants produced shoots on a MS medium containing 1.5 mg/L thiadiazuron (TDZ) and 0.5 mg/l 1-naphthaleneacetic acid (NAA) at a mean rate of 8.8%. This rate was increased to 14.8% in explants placed on 1.5 mg/L TDZ and 0.5 mg/l NAA medium supplemented with 5.0 mg/l silver nitrate ($AgNO_3$).

Table 1. Initial cultivation of meristem buds and cotyledons from ornamental red cabbage (*Brassica oleracea* cv. Kyoto red given) on MS medium supplemented with different combination and concentrations of growth regulators.

Medium + growth regulators	Number of meristem buds	Width (mm)	Height (mm)	Number of cotyledons	Width (mm)	Length (mm)
A	40.0	3.5a	8.8a	48.0	1.2b	2.5a
B	40.0	4.7b	9.2a	55.0	1.9b	2.1ab
C	31.0	1.15c	3.8b	47.0	3.2a	1.4b
D	50.0	2.8a	9.5a	50.0	1.0c	2.4a

Means within each column having different letters are significantly different according to Duncan's test at $p<0,05$.

In Table 2. the number and size of the meristem bud and cotyledons of ageratum on MS medium supplemented with different growth regulators are presented. On the medium MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA_3 were inoculated 6 meristem buds with average width and height of 1.7 mm and 12 cotyledons with average width 2.2 mm and length 1.4 mm. On MS + 2 mg/l BA + 0.1 mg/l NAA were cultured 6 meristem buds with average width 1.2 mm and height 1.0 mm, and 12 cotyledons with average width 1.6 mm and length 1.1 mm. On MS + 5 mg/l BAP were inoculated 6 meristem buds with average width 1.3 mm and average height of 1.2 mm, and 12 cotyledons with average width 2.2 mm and length 1.0 mm. On MS + 3 mg/l BA + 1.5 mg/l NAA were cultured 6 meristem buds with average width 1.7 mm and average height 1.8 mm, and 12 cotyledons with average width 1.4 mm and average length 1.2 mm.

Table 2. Initial cultivation of meristem buds from ageratum *(Ageratum* sp.) on MS medium supplemented with different combination and concentrations of growth regulators.

Medium + growth regulators	Number of meristem buds	Width (mm)	Height (mm)	Number of cotyledons	Width (mm)	Length (mm)
A	6.0	1.7a	1.7a	12.0	2.2a	1.4a
B	6.0	1.2a	1.0a	12.0	1.6a	1.1a
E	6.0	1.3a	1.2a	12.0	2.2a	1.0a
F	6.0	1.7a	1.8a	12.0	1.4a	1.2a

Means within each column having different letters are significantly different according to Duncan's test at $p<0,05$.

Meristem buds and cotyledons from ornamental red cabbage resulted in shoot formation without exception, but different combination of growth regulators stimulated different frequency of shoot formation (Figure 2).

The percentage of shoot formation from meristem buds stimulated by the growth regulators ranged from the lowest frequency of 64% (MS + 5 mg/l BA + 5 mg/l NAA) to the highest 70% (MS + 2 mg/l BA +0.1 mg/l IAA + 0.1 mg/l GA_3 and MS + 2 mg/l BA) (Table 3, Figure 3a).

After one month of cultivation of cotyledons, the medium MS + 2 mg/l BA + 0.1 mg/l NAA showed the best stimulation effect with significantly the highest 56% shoot formation, while MS + 2 mg/l BA +0.1 mg/l IAA + 0.1 mg/l GA_3 and MS +2 mg/l BA gave significantly the lowest frequency of shoot formation from cotyledons. The cotyledons of the fourth used combination of growth regulators were lost due to narcotization in the course of the experiment, thus, without response to the applied growth regulators in one month period (Table 3, Figure 3b).

The presence of BAP in the medium significantly increase the number of produced explants in *in vitro* cultures of *Brassica oleracea* L. (Sretenović-Rajičić et al., 2007). Genus *Brassica* has shown increased regeneration of meristem buds in media supplemented with BAP and NAA. In different *Brassica* species, the regeneration depends on explants age, where younger explants always give better regeneration ratio (Maheshwari et al., 2011). Cheng et al. (2001) reported a high-frequency shoot regeneration obtained with BA or TZD (thidiazuron)-supplemented media. Pavlović et al. (2010) results showed a satisfactory frequency of shoot regeneration from hypocotyl explants and multiplication of shoots on media containing 1 mgl^{-1} BA alone or in combination with IBA in the four investigated *B. oleracea* varieties.

Table 3. Effects of MS medium and different growth regulators on meristem buds and cotyledons of ornamental red cabbage one month after the initial cultivation.

Medium + growth regulators	Number of meristem buds	Width (mm)	Height (mm)	Regenerants	% of frequency of shoot formation	Number of cotyledons	Width (mm)	Height (mm)	% of frequency of shoot formation	Regenerants
A	18	4.1c	1.9a	shoot	70a	18	10.0b	6.0a	37.5b	shoot
B	26	3.1b	2.5a	shoot	65b	31	9.6b	17.0a	56.0a	shoot
C	21	4.4c	2.5a	shoot	70a	31	17.5b	13.1b	38.0b	shoot
D	32	1.3a	2.2a	shoot	64b	Cotyledons lost due to necrotization				shoot

Means within each column having different letters are significantly different according to Duncan's test at $p<0,05$.

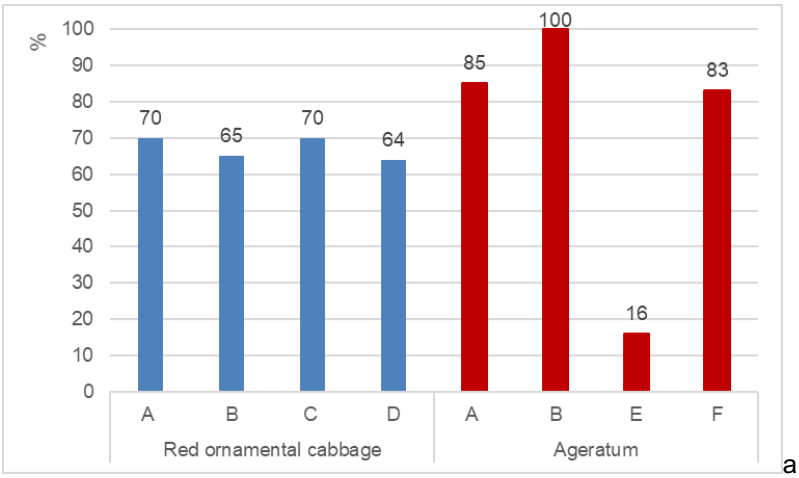
Figure 2. Shoot regenerants as a result of the effect of MS medium and different growth regulators on meristem buds of ornamental red cabbage one month after the initial cultivation.

Ageratum meristem buds on all MS media supplied with certain growth combinations resulted in shoot formation. MS suppled with 2 mg/l BA + 0.1 mg/l NAA was significantly the best combination for initiation of ageratum meristem buds in shoots, therefore 100% of the meristem buds responded with shoot formation. Meristem buds cultivated on MS supplied with 5 mg/l BA resulted in significantly the lowest shoot formation frequency (16%). One month after the initial cultivation, the ageratum cotyledons on all media under research resulted in narcotization, thus, no response to the applied growth regulators in one month period (Table 4, Figure 3a).

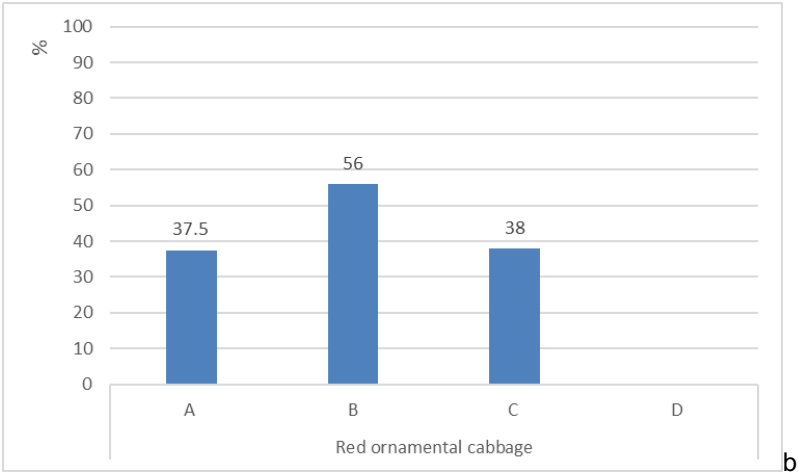
Table 4. Effects of MS medium and different growth regulators on meristem buds and cotyledons of ageratum one month after the initial cultivation.

Medium + growth regulators	Number of meristem buds	Width (mm)	Height (mm)	Regenerants	% of frequency of shoot formation	Number of cotyledons
A	7	11.2ab	19.4a	Shoot	85b	Cotyledons lost due to narcotization
B	6	12.0ab	9.6a	Shoot	100a	
E	2	5.0b	10.0a	Shoot	16c	
F	6	13.1a	14.2a	Shoot	83b	

Means within each column having different letters are significantly different according to Duncan's test at $p<0,05$.



a



b

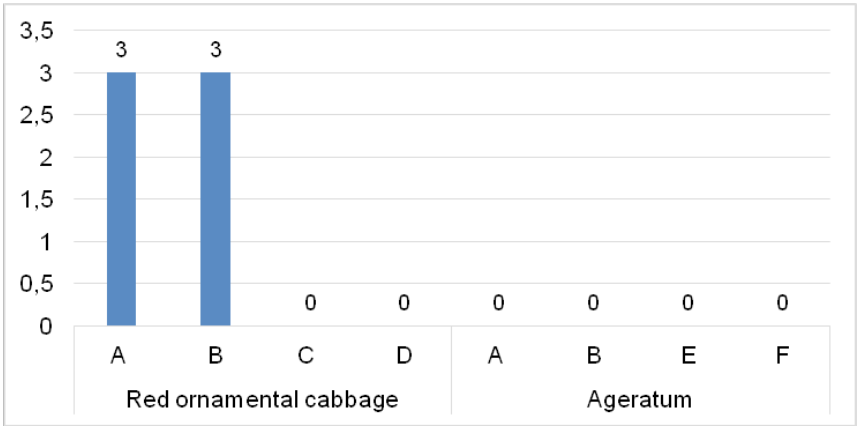
Legend:
A: MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA_3
B: MS + 2 mg/l BA + 0.1 mg/l NAA
C: MS + 2 mg/l BA
D: MS + 5 mg/l BA + 5 mg/l NAA
E: MS + 5 mg/l BA
F: MS + 3 mg/l BA + 1.5 mg/l NAA

Figure 3. a) Frequency of shoot formation from meristem buds of ornamental red cabbage and ageratum on MS medium supplied different growth regulators one month from the initial cultivation.

b) Frequency of shoot formation from cotyledons of ornamental red cabbage on MS medium supplied different growth regulators one month from the initial cultivation.

From the available literature, little-to-none report on tissue culture research has been done on ageratum. Certainly, it is not enough to produce a regeneration method or transformation protocol. If and when genetic engineering is successful with ageratum, caution would be warranted because *A. houstonianum* readily establishes itself as a weed and transgenic races could easily escape cultivation, especially in the tropics (Stephens, 2007). According to Laxmikant (2008) maximum number of multiple shoots of ageratum propagated *in vitro* were developed in plant medium fortified with 3.0 mg/l concentration of IAA-BAP combinations. In this study, IAA-BAP combinations proved as best effective for inducing multiple shooting and roots were formed at 2.0 mg/l and 3.0 mg/l rather than IAA-KN combinations.

The starting explants which after one month of cultivation did not resulted in formation of regenerant were subcultured on fresh MS medium supplemented with the same combination and concentration of growth regulators as in the starting cultivation. After one month of the subculturing, there was root formation on few initial explants. Three initial explants of ornamental red cabbage resulted in formation of roots on MS + 2 mg/l BA +0.1 mg/l IAA + 0.1 mg/l GA_3 and 3 on MS + 2 mg/l BA + mg/l 0.1 NAA (Figure 4). The ageratum initial explants did not respond in root formation on media under this research.



Legend:
A: MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA_3
B: MS + 2 mg/l BA + 0.1 mg/l NAA
C: MS + 2 mg/l BA
D: MS + 5 mg/l BA + 5 mg/l NAA
E: MS + 5 mg/l BA
F: MS + 3 mg/l BA + 1.5 mg/l NAA

Figure 4. The effect of MS medium and different growth regulators on root formation on meristem buds from ornamental red cabbage and ageratum two months after the initial cultivation.

CONCLUDING REMARKS

In this research the influence of different combination of BA, GA_3, IAA and NAA on meristem buds and cotyledons of ornamental red cabbage and ageratum was studied. For ornamental red cabbage, the highest percentage of frequency of shoot formation was obtained on MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA_3 and MS + 2 mg/l BA. On the contrary, the meristem buds of Ageratum showed the best percentage of frequency of shoot formation on MS + 2 mg/l BA + 0.1 mg/l NAA. Cotyledons from ornamental red cabbage gave the highest frequency (56%) of shoot formation when initially cultivated on MS + 2 mg/l BA + 0.1 mg/l NAA, while the ageratum cotyledons responded with necrosis on all media utilized in the research.

Future micropropagation research extension for these two species will be focused on combination of growth regulators suitable for rooting of regenerants, which will lead to the development of complete protocol from micropropagation of ornamental red cabbage and ageratum in future.

Finally, the results presented in this paper are contribution to the limited research data conceding micropropagation of ornamental red cabbage and particularly ageratum.

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МИКРОПРОПАГАЦИЈА НА УКРАСНИТЕ ВИДОВИ *BRASSICA OLERACEA* CV. KYOTO RED GIVEN И *AGERATUM* SP.

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Резиме

Украсната црвена зелка (Brassica oleracea cv. Kyoto red given) и агератумот (Ageratum sp.) се важни украсни растенија кои вообичаено се одгледуваат на балкони, дворови, паркови и други места на отворено во летниот и во зимскиот период од годината. Околку 156 украсни видови се добиваат со in vitro култури во различни комерцијални лаборатории ширум светот. In vitro култура на растенија е една клучна алатка во растителната биотехнологија што ја користи тотипотентноста на растителната клетка.

Во ова истражување беа проучувани влијанијата на различните концентрации и комбинации на BA, GA3, IAA и NAA на меристемски пупки и котиледони како почетни експлантанти од украсна црвена зелка и агератум. Кај украсната црвена зелка највисокиот процент на честота на формирање изданоци од меритемиски пупки беше добиен на MS + 2 mg/l BA + 0.1 mg/l IAA + 0.1 mg/l GA3 и MS + 2 mg/l BA. Спротивно, меристемските пупки од агератум покажаа најдобар процент на честота на формирање на изданоци на MS + 2 mg/l BA + 0.1 mg/l NAA. Котиледоните од украсната црвена зелка покажаа најголема честота (56%) на формирање изданоци кога почетно беа култивирани на MS + 2 mg/l BA + 0.1 mg/l NAA, додека котиледоните од агератум реагираа со некроза на сите медиуми користени во текот на истражувањето. Ова истражување е основа за понатамошно проучување за подобрување на регенерацијата на различни експлантанти од различни економски важни растителни видови.

Клучни зборови: *украсна црвена зелка (Brassica oleracea cv. Kyoto red given), агератум (Ageratum sp.), in vitro, регулатори на раст, меристемска пупка, котиледон, изданок*