UNIVERSITY OF PLOVDIV "PAISII HILENDARSKI" • FACULTY OF BIOLOGY
INSTITUTE OF BIODIVERSITY AND ECOSYSTEM RESEARCH -
BULGARIAN ACADEMY OF SCIENCES

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Kostadinova, S., Mollov, I., Dzhambazov, B.,
Naimov, S., Vassilev, K. & Georgiev, B. (Eds.)

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FOREWORD

Dear Colleagues,

On behalf of the Organizing Committee, we are pleased to welcome you to the 5th Balkan Scientific Conference on Biology, held in Plovdiv, Bulgaria from April 15th to April 16th, 2021.

Plovdiv is one of the oldest cities in Europe. It is claimed that the city is a contemporary of Troy and Mycenae, but it is more ancient than Rome, Athens, and Constantinople. Plovdiv is the second-largest and important city in Bulgaria. Plovdiv is strategically important industrial, commercial, scientific, cultural and transportation-communications center on the Balkans region. The city is famous for the international fair, whose spring, autumn and other specialized exhibitions make it a center of economics and business. Plovdiv is a strategic railway junction and the airport "Plovdiv" recently established itself as an alternative to the airport "Sofia".

Unfortunately, in these times of the pandemic, even though, we do not have the opportunity to meet in person, we hope that, the Conference will offer a valuable platform for the exchange of ideas and experiences between researchers in the field of biology.

Plovdiv University is one of the leading higher-education institutions in Republic of Bulgaria. It is the largest university in southern Bulgaria as well the second biggest in Bulgaria.

Faculty of Biology at the Plovdiv University is located in the cultural reserve Old Town in Plovdiv, next to the Ancient Theatre. Nowadays the Faculty of Biology has more than 50 years of history. Its development is associated with the changes in modern socio-economic conditions of transition to market economy and the process of democratization of society, the integration of Bulgaria into the European structures and the adoption of the achievements of European and world educational and scientific experience by providing resources for the new economy and society in the spirit of European cultural values.

After the success of the four previous Balkan conferences in Plovdiv (2005, 2010, 2014 and 2017), we hope that this edition will, once again, offer a dynamic and friendly environment for stimulating exchanges and discussions.

Official organizing partner for BalkanBio'2020 is the Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences.

Yours sincerely,

Sonya Kostadinova (Dean of the Faculty of Biology)

Gana Gecheva (Chair of the Organizing Committee)

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Preliminary Study on the Effect of LED Light and Cytokinin on the Growth of Pear Plants In Vitro

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Abstract. In the past two decades, light emitting diodes (LED) have become an alternative source of light for plant tissue culture, due to their low energy consumption, low heat emission, specific wavelength irradiation etc. The effect of three LED lights (white, blue and mixed) and two cytokinins (6-benzylaminopurine or *meta*-topolin) on the growth of pear (*Pyrus communis* L. 'OHF 333') *in vitro* was studied. The plantlets were cultivated in microboxes on a modified MS solid medium supplemented with 2.5 μM 6-benzylaminopurine (BAP) or *meta*-Topolin (mT). The plantlets were grown in controlled room using Philips GreenPower LED research module. Three groups of LEDs emitting in white (W), blue (B), mixed (W:R:B:far-red=1:1:1:1) (BR) lights were applied. Biometric parameters, content of photosynthetic pigments and gas-exchange analysis of the plants were measured after three four weeks passages under corresponding light/cytokinin treatment. The results obtained indicated that different LEDs and cytokinin specifically affected the growth and development of *in vitro* cultured pear plants. The highest fresh and dry mass distinguished the plants grown under white LED light with both cytokinins studied. The maximum values for plant height was achieved in plants grown under white LED light with BAP and blue LED light with mT. The leaf sizes of plants grown on mT enriched medium were larger than those grown on BAP enriched medium, regardless of light and the largest were the leaves of plants grown under white LED light. Also, plants grown with mT in the nutrient medium showed more intensive photosynthesis, with the difference between the white and mixed LED light being insignificant.

Key words: micropropagation, shoot culture, *in vitro*, light quality, blue light, photosynthetic pigments, *meta*-Topolin.

Introduction

One of the most important factors influencing the growth and morphogenesis of plant cells *in vitro* is light and the nutrient medium, as well as phytohormones composition.

Fluorescent lamps (FL) are the most commonly used light source in *in vitro* cultivation of plant cells and tissues. But in the past two decades, light emitting diodes (LED) have become an alternative source of light for plant tissue culture, due to their low energy consumption, low heat emission, specific wavelength irradiation etc. (Bourget, 2008; Morrow, 2008). Many authors reported the successful applications of LEDs in promoting *in vitro* growth and morphogenesis from various plant species (Gupta & Jatothu, 2013). Under various LED treatments improvement in shoot organogenesis, *ex vitro* survival rate and biomass yield have been demonstrated (Hahn et al., 2000; Nhut et al., 2003; Jao et al., 2005; Shin et al., 2008; Li et al., 2010; Gupta & Sahoo, 2015). According to Muneer et al. (2018) red and blue LEDs play a significant role in overcoming hyperhydricity in carnation often observed under fluorescent light.

Several authors reported better *in vitro* responses of different species when a combination of red and blue LED light was used (Nhut et al., 2003; Azmi et al., 2014; Ferreira et al., 2017). However, the spectral composition and photosynthetic photon flux density (PPFD) specifically affect the growth and morphogenesis of different plant species. Furthermore, the research with woody species and in particular fruit species are too limited - *Populus* (Kwon et al., 2015), *Castanea* (Park & Kim, 2010) as the studies mainly refer to somatic embryogenesis - *Pinus* (Merkle et al., 2005; Kim & Moon, 2014), coffee (Mai et al., 2016). The stimulating effect of red LED light on the length of the shoots and leaf area in pear multiplication has recently been reported (Lotfi et al., 2019). The growth and development

of plant cells in *in vitro* culture is largely determined by the plant growth regulators used, in particular cytokinins (CKs) due to their importance for cell division and cell expansion (Howell et al. 2003, Aremu et al. 2012). Currently N^6-benzyladenine (BAP) is the most widely used aromatic cytokinin in the micropropagation industry because of its effectiveness and affordability (Bairu et al., 2007; Aremu et al., 2012). But at a high concentration (BAP) has disadvantages such as hyperhydricity (Leshem et al. 1988, Teramoto et al. 1993, Magyar-Tabori et al. 2010), stunted growth, epigenetic and somaclonal variation in some crops (Harrar et al. 2003, Werbrouck, 2010, Smulders & De Klerk 2011).

Recently monomethoxy derivatives of 6-benzyladenine and 6-benzyladenosine were also isolated and identified from several different plant sources and their high cytokinin activity has been confirmed (Tarkowska et al. 2003). Podwyszyńska et al. (2012) reported that *meta*-methoxytopolin (MemT) and its riboside (MemTR), improved the micropropagation and shoot quality of smoke bush (*Cotinus coggygria* Scop.).

In our previous study, a good multiplication rate and high quality shoots were found at 6-9 μM mT treatment of pear rootstock OHF 333 (Dimitrova et al., 2016). The use of *meta*-topolin resulted in improvement of the leaf gas exchange and low content of phenols, as well as in the total antioxidant activity. These results indicate that the slight structural difference between BAP and mT could have a profound impact on plants during micropropagation and *meta*-methoxytopolins could be considered an alternative to other commonly used cytokinins in micropropagation of recalcitrant species. In this study, the effect of three LED lights (white, blue and mixed) and two cytokinins (6-benzylaminopurine or meta-Topolin) on the *in vitro* growth of pear (*Pyrus communis* L. 'OHF 333') was studied.

Materials and Methods

Plant material and experimental conditions

The experiment was carried out on pear rootstock (*Pyrus communis* L. 'Old Home' x 'Farmingdale' 333), which was characterized by good compatibility with most of the pear cultivars, high yields and a moderate degree of resistance to fire blight (Lombard and Westwood, 1987; Wertheim, 2002). *In vitro* culture was maintained at 3-week subculture intervals as described previously (Nacheva et al., 2009). Briefly, shoots were grown in microboxes with green filter ($SacO_2$, Belgium) on a modified MS (Murashige & Skoog, 1962) solid medium with ½ concentration of NH_4NO_3 and $CaCl_2$ and 1000 mg L^{-1} $Ca(NO_3)_2$, supplemented with 2.5 μM 6-benzylaminopurine (BAP), 0.05 μM IBA, 30 g L^{-1} sucrose, 6.5 g L^{-1} Phyto agar (Duchefa, The Netherlands). The medium (pH 5.6) was autoclaved at 121°C for 20 min. In each container on 100 mL of culture medium 10 shoot tips with a length of 7-8 mm with two leaves were set. The cultures were incubated in the growth room at an air temperature of 22±2°C with 16/8 h hours photoperiod supplied by cool-white fluorescent lamps (OSRAM 40W; 50 μmol $m^{-2}s^{-1}$ PPFD).

For the purpose of the present experiment the plantlets were cultivated *in vitro* on above mentioned basal nutrient medium, supplemented with 2.5 μM 6-benzylaminopurine (BAP) or 2.5 μM *meta*-Topolin (mT) at 22±2°C using an illumination system based on Philips GreenPower LED research module (16-h photoperiod with 80-95 μmol m^{-2} s^{-1} PPFD). Three groups of LEDs emitting in white (W), blue (B), mixed (1:1:1:1 far-red) lights (BR) were applied.

Growth parameters

Data on fresh (FW) and dry (DW) mass, number and length of shoots, leaf characteristics (the length and width of the first fully developed leaf), content of photosynthetic pigments was evaluated in six passages of three weeks of culture.

Photosynthetic pigments content

The photosynthetic pigment (chlorophyll *a*, chlorophyll *b* and total carotenoids) content in plantlets was determined. For pigment extraction, 250 mg of fresh material was extracted in the dark with 10 ml 85% acetone cooled up to 4°C. The pigment content was determined spectrophotometrically, and calculated according to the formulae of Lichtenthaler & Wellburn (1983).

Gas-exchange analysis

Gas-exchange analysis was performed on the all ten plants in one vessel. Measurements were taken with a LCpro + portable gas exchange system (ADC, UK) at a light intensity of about 180 µmol m^{-2} s^{-1} PPFD and a temperature of 25°C. Net photosynthesis rate (A, µmol CO_2 m^{-2} s^{-1}), transpiration intensity (E, mmol H_2O m^{-2} s^{-1}) were determined.

Statistical analysis

For each light treatment three replications, each containing ten shoots was tested and the experiment was repeated three times. Statistical analysis of physiological parameters was performed using a one-way ANOVA using the Tukey test to validate the different significance at $p \leq 0.05$.

Results and Discussion

The results obtained in this study indicated that LED sources with different light and cytokinin applied specifically affect the growth and development of *in vitro* cultured pear plants (Fig. 1, Table. 1). Due to the relatively low concentration of applied cytokinin (2,5), no significant differences were observed between the studied variants in the number of newly formed shoots. The plantlets grown in blue light with BAP had the longest shoots, followed by those in white light with BAP and mixed light (BR) with mT, but the differences between them were not statistically proven.

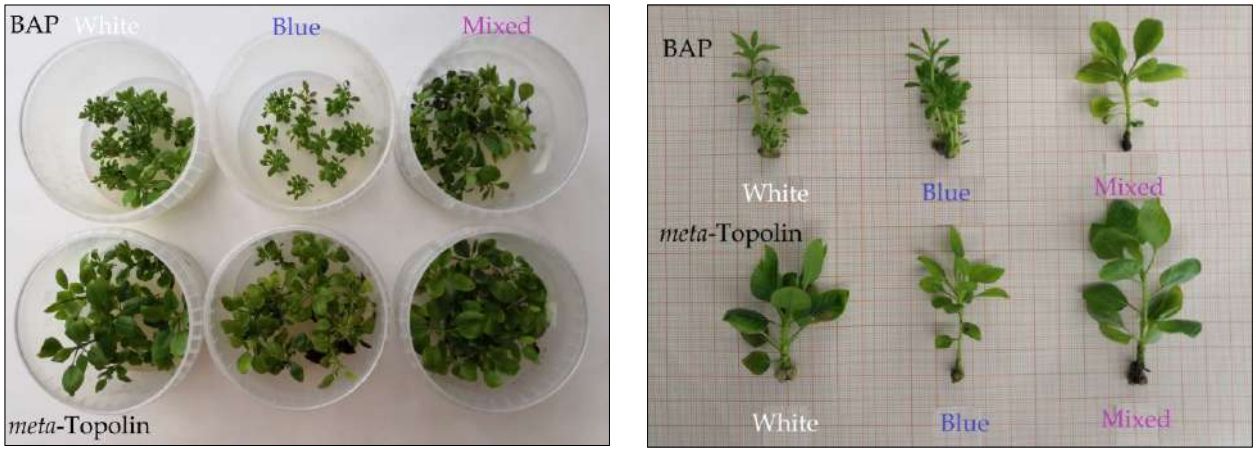

Fig. 1. Appearance of pear plantlets grown with BAP or mT in the nutrient medium at different light treatments. **W** white LEDs, **B** – blue LEDs, **BR** – mixed LEDs.

The highest number of leaves was reported in plants cultured in mixed light in the presence of BAP in nutrient medium (10.4) and the lowest – with mT under blue light (5.7). Despite some fluctuations in the fresh mass (FW) of the plants, there was no significant difference between the different treatments. However, the highest values of DW were reported in white LED light in both types of cytokinins used.

Table 1. Growth indices measured in pear plants grown with BAP or mT in the nutrient medium at different light treatments. **W** white LEDs, **B** – blue LEDs, **BR** – mixed LEDs. For each column, different letters indicate significant differences at $p \leq 0.05$.

LED Light	Cytokinin	Shoot length /mm/	Number of shoots	Number of leaves	FW /g/	DW /g/
White	BAP	20.76 [a]	1.2 [a]	7.3 [ab]	1.20 [a]	0.34 [ab]
BR	BAP	11.98 [c]	1.6 [a]	10.4 [a]	0.67 [a]	0.15 [b]
Blue	BAP	21.96 [a]	1.6 [a]	9.1 [ab]	1.17 [a]	0.29 [ab]
White	mT	15.96 [b]	1.0 [a]	9.2 [ab]	1.55 [a]	0.47 [a]
BR	mT	20.58 [a]	1.0 [a]	7.5 [ab]	0.98 [a]	0.18 [b]
Blue	mT	15.90 [b]	1.1 [a]	5.7 [b]	1.28 [a]	0.34 [ab]

The plants at mixed light, regardless of the cytokinin applied in the nutrient medium, had the lowest dry biomass. The leaves of plantlets, grown with mT in nutrient medium, were larger regardless of the type of light (Fig. 2, 3). The combination of the four types of light (BR) favorably affected the growth of leaf lamina and is a prerequisite for more intense photosynthesis.

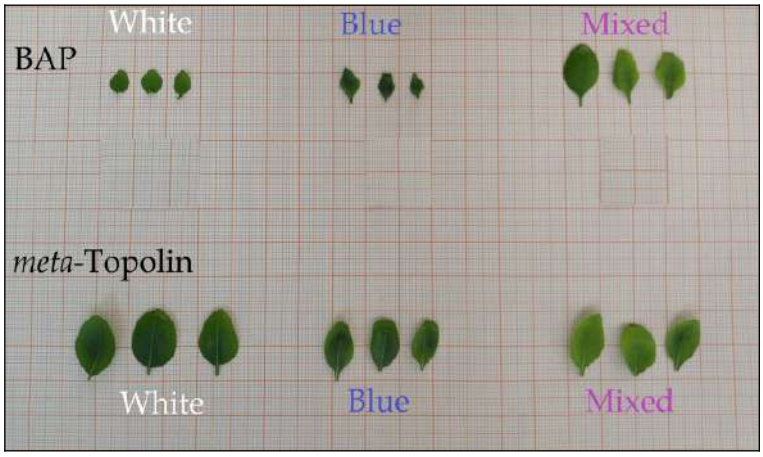

Fig. 2. Leaves of pear plantlets (*Pyrus communis* L. OHF 333) grown with BAP or mT in the nutrient medium at different light treatments. **W** white LEDs, **B** – blue LEDs, **BR** – mixed LEDs.

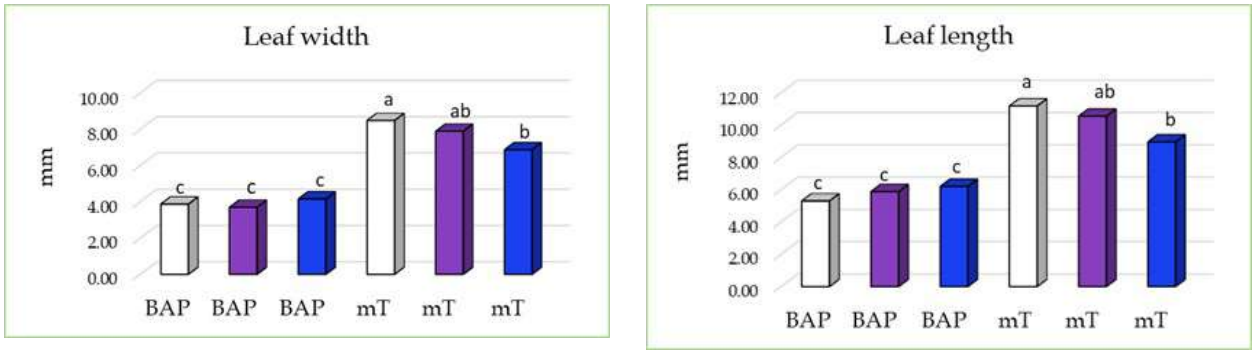

Fig. 3. The length and width of the first fully developed leaf of pear plantlets, grown with BAP or mT in the nutrient medium at different light treatments. **W** white LEDs, **B** – blue LEDs, **BR** – mixed LEDs.

Table 2. The content of photosynthetic pigments (mg g^{-1} FW) in the pear plantlets cultivated with BAP or mT in the nutrient medium at different light treatments. **W** white LEDs, **B** – blue LEDs, **BR** – mixed LEDs. For each column, different letters indicate significant differences at $p \leq 0.05$.

LED Light	Cytokinin	Chl *a*	Chl *b*	Chl (*a*+*b*)	Car	Chl *a*/*b*	Chl(a+b)/Car
White	BAP	0.52 ^{a}	0.17 ^{a}	0.69 ^{a}	0.23 ^{a}	3.05 ^{ab}	2.95 ^{b}
BR	BAP	0.25 ^{cd}	0.09 ^{bc}	0.34 ^{c}	0.15 ^{b}	2.58 ^{c}	2.31 ^{d}
Blue	BAP	0.39 ^{b}	0.12 ^{b}	0.51 ^{b}	0.16 ^{b}	3.15 ^{ab}	3.26 ^{a}
White	mT	0.26 ^{cd}	0.09 ^{bc}	0.35 ^{c}	0.14 ^{b}	2.96 ^{b}	2.58 ^{c}
BR	mT	0.21 ^{d}	0.08 ^{c}	0.30 ^{c}	0.11 ^{b}	2.61 ^{c}	2.74 ^{bc}
Blue	mT	0.32 ^{bc}	0.10 ^{bc}	0.42 ^{bc}	0.16 ^{b}	3.29 ^{a}	2.63 ^{b}

The highest content of chlorophyll *a*, *b* and carotenoids was reported in plants cultivated with BAP in the nutrient medium under white LED light, and the lowest in plants grown with mT under mixed LED light (Table 2). The lowest values of total chlorophyll content were reported under mixed LED light in both cytokinin tested. No deviations from the norm in the ratios between the photosynthetic pigments were found. Our results are similar to results with *Lippia filifolia* reported by Chaves et al. (2020) that white LEDs increased chlorophylls and carotenoids contents.

Our data clearly showed that plants grown in plastic vessels with mT had more efficient photosynthesis, with the difference between the white and mixed light treatments being insignificant (55 µmol $vessel^{-1}$ s^{-1} and 64 µmol $vessel^{-1}$ s^{-1}, respectively) – (Fig. 4).

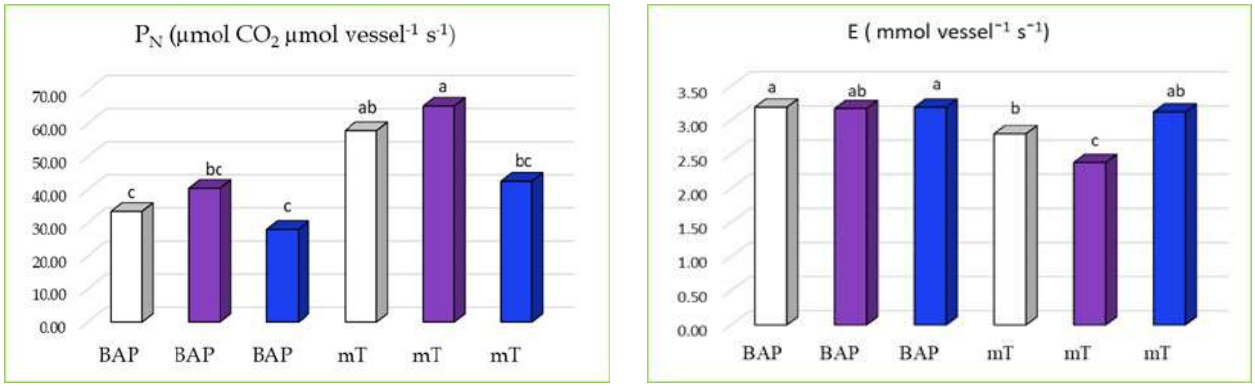

Fig. 4. Effect of the light and cytokinin on the net photosynthetic rate (µmol CO_2 m^{-2} s^{-1}) and transpiration intensity (E, mmol H_2O m^{-2} s^{-1}) of pear plantlets; Different letters within column indicated difference at ($p<0.05$).

In regard to transpiration rate, the lowest values were reported in the treatment with mT under mixed LED light (Fig. 4). Comparing these results with the content of the photosynthetic pigments, it can be assumed that the changes found in the rate of CO_2 assimilation were not affected by the pigment content.

Few studies are reported on *in vitro* cultivation of plants under LED lamps (Batista et al., 2018). The results show that the effects of spectral quality on photosynthetic competence vary according to the plant species, with LED lights being more efficient compared to fluorescent lights in *in vitro* culture. In addition, the combination of different color LEDs can overcome the limitations of individual colors.

More and more scientists are paying attention to combining red and blue LED lights in different ratios. According to Nhut et al. (2002), the banana plantlets *in vitro* were enhanced under 80% red + 20% blue LED. In the absence of blue LEDs, plantlets were abnormal. Normal plantlet growth is clearly related to the presence of blue LEDs and plant quality is a function of the amount of blue LEDs. (Nhut et al., 2002). The combination of red and blue lights using LED lamps resulted in a higher number of shoots per explant in *Acer saccharum* (Singh et al., 2017) and *Eucalyptus urophylla* Souza et al. (2020) indicating the importance of this light combination for *in vitro* culture.

There is extensive data on the effect of light quality on *in vitro* herbaceous species, while there is less data concerning woody species (Batista et al., 2018) . One reason may be the reduced effects of light quality on woody species compared to that of herbaceous species (Morini & Muleo, 2003).

Conclusions

Pear plantlets grown under white LED light had higher values of fresh (FW) and dry (DW) biomass regardless of the cytokinin applied. Plants grown on the nutrient medium enriched with mT in polypropylene vessels with green gas permeable filter under white light had the largest fresh and dry mass, large leaf size and maximum net photosynthesis rate. The combination of the four types of LED light (BR) favorably affected the growth of leaf lamina and is a prerequisite for more intense photosynthesis. In addition, the better performance of plants grown under mixed LED light in terms of leaf size and photosynthetic performance showed that it is necessary to optimize the *in vitro* growing conditions of the pear rootstock OHF 333 by appropriately combining the light regime and cytokinin in the nutrient medium.

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In Vitro Increased Wheat Stalk Growth by Highly Diluted Agitated Preparations of a Commercial Fertilizer

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Abstracts. Drugs at ultra-high dilutions close to the Avogadro´s number (theoretical 0-molarity is 10^{-24}) and without apparently containing any drug molecule, have been used in homeopathy therapy for about two centuries. Nowadays, homeopathic procedures involving the controversial phenomenon called "water memory", have regained their use in different application areas. In the present study, 10^{-7}, 10^{-14}, and 10^{-36} dilutions of a commercial fertilizer were evaluated in a 7-d wheat growth bioassay. Test dilutions were prepared following a standardized protocol, according to the method of stepwise dilution and succession, as derived from traditional homeopathy. Experiments were performed on herbicides- and pesticides-free wheat grains (*Triticum durum* Rafi C97 variety). Treatments were compared with negative controls including; a) pure bi-distilled water and b) sham-treated pure bi-distilled water, following the same protocol as used for fertilizer dilutions with 200 strokes and the same steps of dilutions. A 2% of commercial fertilizer was used as a positive control. The observed results of three independent bioassays, showed an increased wheat stalk growth after treatment with diluted and agitated fertilizer solutions, as compared with negative controls ($p < 0.05$). Positive control showed the highest stalk growth, but it was not significantly different from that of diluted fertilizer. These results suggest that there was an influence of highly diluted commercial fertilizer on wheat seedling development.

Key words: Wheat growth, Sustainable agriculture, Fertilizers.

Introduction

Samuel Hahnemann (1755-1843) introduced homeopathy as a therapeutic system in his book "*Organon of the rationale System of Medicine*". Nowadays, basic research on homeopathy involves several aspects; however, investigations have been mainly focused on the potentization or dynamization principle (Mathie, 2019), and evidence has been mounting for substance homeopathic ultra-dilutions specific effects (Endler et al., 2010; Pfleger et al., 2011; Betti, et al., 2013; Munshi et al., 2019). In this regard, medical applications and clinical studies on therapeutic and prophylactic effects have been reported (Sukul & Sukul, 2006; Bracho et al., 2010). On the other hand, agricultural use of homeopathy for plant growth and germination stimulation (Brizzi et al., 2005) and against plant pests (Betti et al., 2013; Wyss et al., 2010) has significantly increased.

In view of this relevant issue involving biological effects of homeopathic preparations, mainly based in the stepwise dilution and agitated succussion method, as shown by Hahnemann, the present study aimed to evaluate a highly diluted commercial fertilizer on *in vitro* wheat stalk growth.

Materials and Methods

Homeopathic preparations

Generally, water and ethanol are proper ingredients to generate homeopathic potentized drugs. However, it is well recognized that water does not retain the efficacy of a potentized drug (Boyd, 1954), for which, in this study, highly diluted and agitated preparations were immediately used after succussions. To prepare test dilutions, a 2% stock solution of the liquid commercial fertilizer Vita-Plant Nutrition®, purchased from a local supplier and containing a complex mixture of nutrients and

salts, was prepared and considered as "mother substance"; this concentration was selected based in our previous bioassays (data not shown). Next, 1 mL of the mother substance was added to 9 mL of bi-distilled water in a glass bottle. The dilution was vigorously agitated against an elastic surface (200 strokes of vigorous turbulent shaking were made for each step of dilution) and 7, 14, and 36 steps of agitated dilutions were performed to obtain 10^{-7}, 10^{-14}, and 10^{-36} dilution treatments.

Bioassays

As previously described (Pfleger et al., 2011), a 7-d wheat growth bioassay was used to evaluate the biological effect of homeopathic dilutions. Herbicide- and pesticide-free wheat grains (*Triticum durum*; Rafi C97 variety harvested in 2018) were purchased at a local supplier and allocated in disposable Petri dishes; 120 grains were equally divided in 12 plates, containing 2 layers of filter paper (Whatman, cellulose, 90 mm, sort 2). Plates were then covered and placed in alternating rows according to a random model (stratified randomization) in a bio-climatic chamber at 27 ± 0.3 °C and 45% of relative humidity, with a photoperiod of 12 h darkness/12 h light. Wheat grains had not been soaked prior to treatment. During the bioassay, grains were watered every 24 h by adding pure bi-distilled water or treatments. Stalks were cut off to individual measurements after 7 days of treatment. The total stalk length was evaluated by using an electronic Vernier instrument and expressed as total length (cm). Experimental groups were harvested in the same sequence as they were planted. Measurements were performed blindly. In this study, we followed recommendations provided by Stock-Schröer et al. (2009) in order to present objective information and avoid speculations.

Experimental design

We used diluted (10^{-7}, 10^{-14}, and 10^{-36}) and agitated conditions of the original substance, as explained above. The following controls were matched to every experiment: negative controls consisting of a) pure bi-distilled water and b) sham-treated pure bi-distilled water, following the same protocol as used for fertilizer dilutions with 200 strokes and the same steps of dilutions, and 2% of commercial fertilizer as a positive control. Three independent experiments, in a period of 18 months, were performed and every experiment included the same set of dilutions and controls, analyzing 360 grains for every treatment and controls, which were encoded and blindly applied and evaluated. At the end of the experiment, codes were revealed and data statistical analysis performed.

Statistical Analysis

Statistical differences among groups were determined by analysis of variance for normal distributions and the correspondent Tukey test for establishing individual differences. Data normality was calculated by the Kolmogorov-Smirnov test ($p<0.05$). When no-normal distributions were found, a Kruskal-Wallis test was applied and the correspondent non-parametric Tukey test, using the SPSS v.15.0 package. Results were expressed as mean ± SD of triplicate determinations.

Results

In the present study, the effect of 10^{-7}, 10^{-14}, and 10^{-36} dilutions of a commercial fertilizer on an *in vitro* model of stalk wheat growth was evaluated. As shown in Figures 1 to 3, high dilution and positive control (2% commercial fertilizer) groups presented higher wheat stalk lengths, as compared with negative and sham-treated controls ($p<0.05$). Although positive control induced the highest ($p<0.05$) wheat stalk growth, it was no different from that of highly diluted agitated treatments.

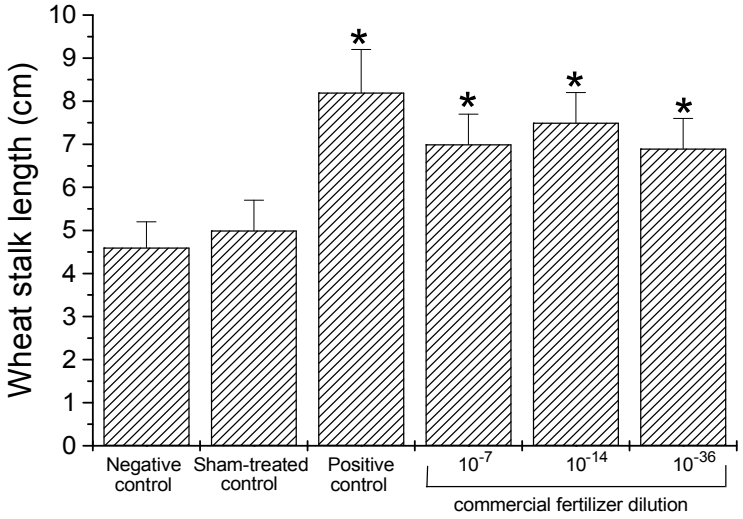

Fig. 1. Mean wheat stalk length (cm) under the influence of highly diluted-agitated fertilizer. One hundred and twenty wheat grains were used for treatments and controls. **Bioassay 1.** The negative control included only the vehicle (untreated pure bi-distilled water). Sham-treated control was processed following the same protocol as used for fertilizer dilutions, as explained in the text. A 2% of the original commercial fertilizer was used as a positive control. Bars represent mean ± SD of triplicate determinations. *$p < 0.05$ as compared with negative and sham-treated controls.

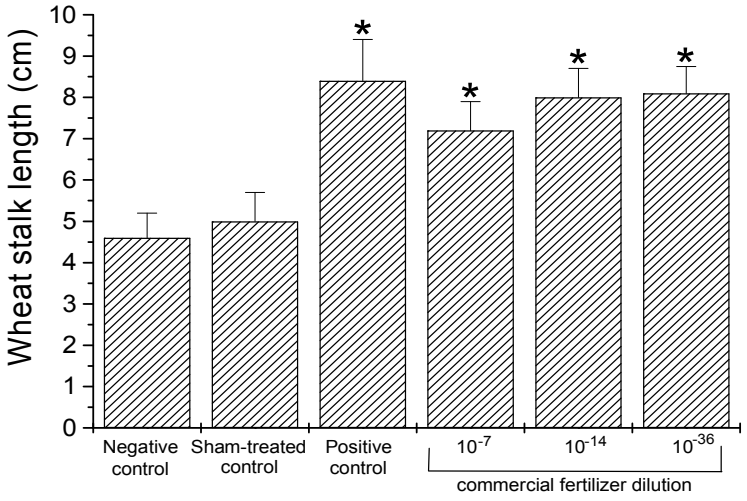

Fig. 2. Mean wheat stalk length (cm) under the influence of highly diluted-agitated fertilizer. One hundred and twenty wheat grains were used for treatments and controls. **Bioassay 2**. The negative control included only the vehicle (untreated pure bi-distilled water). Sham-treated control was processed following the same protocol as used for fertilizer dilutions, as explained in the text. A 2% of the original commercial fertilizer was used as a positive control. Bars represent mean ± SD of triplicate determinations. *$p < 0.05$ as compared with negative and sham-treated controls.

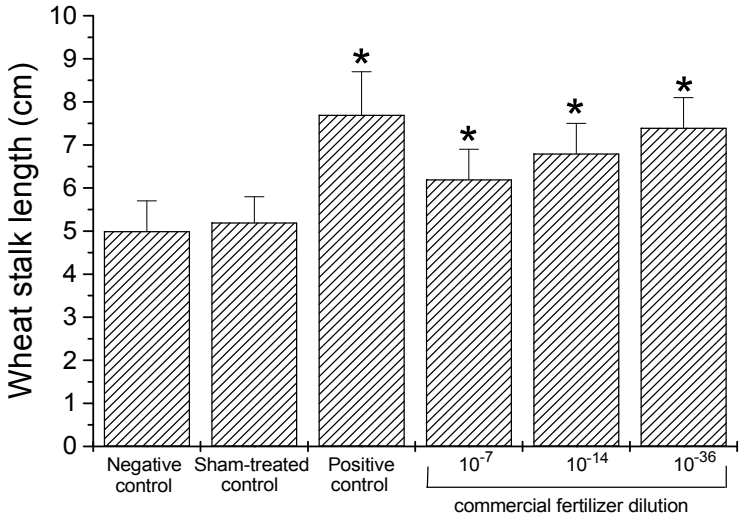

Fig. 3. Mean wheat stalk length (cm) under the influence of highly diluted-agitated fertilizer. One hundred and twenty wheat grains were used for treatments and controls. **Bioassay 2**. The negative control included only the vehicle (untreated pure bi-distilled water). Sham-treated control was processed following the same protocol as used for fertilizer dilutions, as explained in the text. A 2% of the original commercial fertilizer was used as a positive control. Bars represent mean ± SD of triplicate determinations. *$p < 0.05$ as compared with negative and sham-treated controls.

Discussion

There is a trend towards the use of homeopathic procedures in several areas of science and applications in a diversity of fields. Considering this trend and the lack of consensus on the effectiveness of the techniques involving ultra-diluted chemical compounds, it is of considerable interest to examine the potential biological effects of ultra-diluted solutions. In the present study, homeopathic preparations made according to the traditional method of stepwise dilution and succussion, as derived from traditional homeopathy, were tested by using a previously described and validated wheat growth bioassay (Pfleger et al., 2011).

It has been proposed that the phenomenon known as "water memory" is involved in the high dilution process (Thomas, 2007). However, this issue is controversial and some investigations claim that any interpretation calling for memory effects in pure water is totally excluded (Teixeira, 2007) or at least is an elusive phenomenon (Colic & Morse, 1999). Regarding this controversial phenomenon of water memory, we have previously demonstrated that water mimics the behavior of a chemical substance by electronic transmission of the original substance to water samples, via an electronic amplifier. We tested the biological effect of pure water samples treated in a bioresonance instrument to inhibit the growth of *Entamoeba histolytica* and *Trichomonas vaginalis* (Heredia-Rojas et al., 2011), fungus of clinical importance (Heredia-Rojas et al., 2012), and bacteria (Heredia-Rojas et al., 2015) by electro-transferring metronidazole, fungizone and vancomycin to water, respectively. We have hypothesized that water possesses memory based on biological activity of such water samples transferred with electronic information from the original compound (Norman et al., 2016). Moreover, this controversial phenomenon of water memory has been gaining scientific credibility, either through direct empirical findings such as results presented in this work or by theoretical considerations (Ruzic et al., 2008).

In compliance with the Avogadro number, the potency of 10^{-36} used in the present study contains only the molecules of the diluent medium, in this case, water. As observed in all Figures, we assumed that wheat stalks growth was stimulated by influence of diluted fertilizer, as compared with negative control and sham-treated water samples. These results agreed with previous reports indicating biological activity of drugs at high dilutions, even those that exceeded the Avogadro's limit (Boujedaini et al., 2012; Seker et al., 2018).

Regarding the mode of action or behavior of the homeopathic potencies, attempts have been made in order to explain the physical basis of this phenomenon. Sukul & Sukul (2006) using nuclear magnetic resonance spectroscopy indicated that potentized drugs differ from each other and from the medium (vehicle) with respect to the spin-lattice relaxation time; infrared spectra of potentized drugs showed variation in the vibrational frequencies of O-H, C-O and C-H bands. Furthermore, Fourier transform infrared of potentized drugs demonstrated marked variation in O-H bending vibration. Moreover, electronic and fluorescence spectra of homeopathic preparations presented variation with regard to spectral pattern, peaks and absorbance or intensities. All of these findings are indicative of the variation in hydrogen bonding and H-bonding strength among the potencies (Sukul & Sukul, 2006). By using thermoluminescence techniques, Rey (2002) reported that lithium chloride and sodium chloride ultra-high dilutions showed changes among them. By the way, Demangeat (2015), suggested that gas nanobubbles and aqueous nanostructures play a critical role in the dynamization process. Taken together, in the present study we selected to follow the traditional diluted and agitated procedure to prepare homeopathic dilutions. Thus, by applying 200 strokes of vigorous turbulent shaking to dilutions, it was possible to create enough of such nanobubbles by mechanical action.

On the other hand, it is well known that increasing organic matter content in soil due to application of chemical fertilizers is of ecotoxicological concern, because potentially toxic elements accumulate and become available for crops (Iglesias et al., 2018). Thus, it is relevant to develop approaches such as the one presented in this study, to avoid the use of large quantities of chemicals in soils.

We did not dilute a specific substance, but a complex mix of several elements that according to the fertilizer manufacturer, act as a growth stimulator for crops and other plants. In contrast, most studies testing high-diluted substances use specific and isolated chemical compounds (Teixeria & Carneiro, 2017).

In conclusion, we demonstrated that 10^{-7}, 10^{-14}, and 10^{-36} dilutions of a commercial fertilizer significantly stimulated *in vitro* wheat stalk growth. These findings indicated that "fertilizer information" remained in the diluted sample, even at 10^{-36}, which is beyond the Avogadro´s number. The potential use of high-diluted fertilizers for agricultural purposes is discussed. However, further studies at larger scales are required to confirm these results.

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Influence of Biostimulators Regoplant and Charkor on Growth and Development of Micropropagated Pear Plants at Acclimatization Stage

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Abstract. In recent years, there has been a growing interest in biostimulants as an alternative to chemicals for safe and sustainable agriculture. The aim of this study was to analyze the effect of biostimulators Regoplant and Charkor (Agrobiotech, Ukraine) on growth and development of micropropagated pear plants (*Pyrus communis* L. 'Old Home' x 'Farmingdale') at acclimatization stage. In vitro propagated and rooted plantlets from pear rootstock OHF 333 were acclimatized in a floating system with 100 µl l^{-1} Regoplant or Charkor. Plantlets with no additional treatments served as control. Data on growth parameters, chlorophyll a fluorescence (OJIP test) and antioxidant activity were collected 45 days after transplanting to *ex vitro* conditions. Enrichment of the nutrient solution with biostimulator Charkor (100 µl l^{-1}) in floating system led to the highest survival rate (82.5%) of pear plants, the greatest stem length, number, fresh and dry mass of leaves. Combining innovative approaches such as a floating system and biostimulators would significantly improve the acclimatization and the overall process of micropropagation of fruit plants.

Key words: *in vitro* micropropagation, *ex vitro* acclimatization, floating system, nutrient solutions, chlorophyll a fluorescence, OJIP test.

Introduction

For the last few decades *in vitro* culture methods have successfully produced disease-free woody fruit plants but some limitations still remain. *In vitro*, plants grow in a special environment and rely on sugars in the nutrient medium, while in the process of adaptation to *ex vitro* conditions they must switch to autotrophic nutrition. The ability of in vitro derived propagules to withstand transplanting stress very often determines the success or failure of tissue culture operations (Nowak & Pruski, 2004). Therefore, the acclimatization is a key stage of the micropropagation process. Different approaches have been developed to increase the plant survival rate after transplanting from in vitro conditions to a greenhouse. Several authors reported that float hydroculture could be successfully applied in acclimatisation of in vitro produced plantlets, such as *Solanum tuberosum* L. (Nhut et al., 2006), *Grammatophyllum speciosum* Blume (Sutthinon et al., 2015), *Lycium barbarum* L. and cherry rootstocks (Clapa et al., 2013; Dimitrova et al., 2020). In this system, on the surface of the nutritive solution, there are floats made of polystyrene or other materials that sustain the plants (Sheikh, 2006). Floating systems are intensively used for greenhouse production of fresh-cut leafy vegetables and for the cultivation of medicinal plants (Dorais et al., 2001). However, there has been little information about an efficient method for acclimatization of in vitro pear plantlets using a hydroponic system.

In recent years, there has been a growing interest in biostimulants as an eco-friendly alternative to chemicals for boosting the growth of plants in stress conditions. Regoplant and Charkor are a part of a new generation of plant growth biostimulators (Agrobiotech,Ukraine, http://www.agrobiotech.com.ua) and contain metabolism products of in vitro cultivation of endophyte micromycetes of ginseng roots. Regoplant also contains aversectin – biological product with antiparasitic activity. Charkor contains a complex of amino acids, fatty acids, sugars, macro- and microelements and analogs of phytohormones. According to the authors, Charkor is more effective than indolyl-acetic and indolyl-butyric acid in rooting cuttings of a number of ornamental trees and shrubs (Ponomarenko et al., 2010). It was successfully applied for rooting of micropropagated magnolia plantlets (Gercheva et al., 2015). According to our previous results,

Charkor and Regoplant stimulate growth and improve acclimatization of micropropagated pear plantlets (Dimitrova et al., 2017; Dimitrova, et al., 2019). It was found that soaking the pear plantlets for 10 minutes in a solution of Regoplant (50 µl l^{-1}) before planting them in a soil substrate led to a significant increase in their biomass (Dimitrova et al., 2017). Also, enriching the agar nutrient medium for rooting with 0.5 ml l^{-1} Charkor stimulated rooting and had a long-term positive effect on plants growth during acclimatization (Dimitrova, et al., 2019).

The aim of this study was to analyze the effect of biostimulators Regoplant and Charkor on growth and development of micropropagated pear plants during ex vitro acclimatization in a floating system.

Materials and Methods

Plant material and experimental conditions

The experiment was carried out on micropropagated pear rootstock (*Pyrus communis* L. 'Old Home' x 'Farmingdale' 333).

The research was done in September – October, 2019 in the greenhouse at the Fruit Growing Institute – Plovdiv, Bulgaria.

A preliminary study with a floating system (unpublished data) showed that Knopp's nutrient solution (1865) had a better effect on the growth of pear plants than Hellriegel's (1898) and Pryanishnikov 's (1976) nutrient solutions. Therefore, Knop's nutrient solution was chosen in this experiment.

Well-rooted plantlets were potted in styrofoam form pads (528x308x60 mm) filled with peat-perlite 1:1 (v:v). The pads were placed in a plastic tank containing 5 l Knopp's nutrient solution (1865), supplemented with 36.7 mg l^{-1} iron sodium ethylenediaminotetraacetate (FeNaEDTA). Regoplant and Charkor at concentration 100 µl l^{-1} were added to the nutrient solution. Plants, potted in the same way, but without nutrient solution, served as a control (conventional ex vitro acclimatization). Thus, four treatments were formed:

1. Control (C) – conventional acclimatization in peat-perlite 1:1 (v:v), without nutrient solution;
2. Acclimatization in a floating system with Knopp's nutrient solution (K);
3. Acclimatization in a floating system with Knopp's nutrient solution, supplemented with 100 µl l^{-1} Regoplant (R);
4. Acclimatization in a floating system with Knopp's nutrient solution, supplemented with 100 µl l^{-1} Charkor (CH).

In order to prevent nutrient depletion and the development of pathogens the nutrient solutions were renewed weekly.

Growth parameters

The survival rate (%) and growth analyses were made on the 45th day after transplantation of plants to ex vitro conditions. The fresh weight (FW) of leaves, stems and roots as well as the leaf area were determined immediately after removing the plants from the soil. The dry weight (DW) of the corresponding botanical organs was measured after drying the material at 80° C (±5°C) for 48 h (Beadle, 1993). The leaves were scanned and the leaf area was calculated using the software program Wisegeek.

Physiological and biochemical parameters

Chlorophyll a fluorescence

Chlorophyll *a* fluorescence analysis was performed on the youngest native fully developed leaves of 5 representative plants of the respective variant. The basic parameters of the rapid chlorophyll *a* fluorescence were made with a HandyPEA portable fluorimeter (Hansatech

Instruments, UK). The measured spots of the leaves were dark adapted for 40 minutes with special clips. Induction curves of the rapid chlorophyll a fluorescence (JIP test) were recorded for 1 s with 3000 μmol m^{-2} s^{-1} PPFD. The primary data processing was done using the PEA Plus Software (V1.10, Hansatech Instruments Ltd., UK). The parameters measured and calculated using this OJIP test (Table 1.) were interpreted and normalised according to Strasser & Strasser (1995) and Goltsev (2016).

Table 1. Definitions of measured and calculated chlorophyll a fluorescence parameters used in the experiment (Based on Strasser and Strasser, (1995) and Goltsev et al., (2016)

Chlorophyll Fluorescence Parameter	Description
Measured parameters and basic JIP-test parameters derived from the OJIP transient	
$F_O \sim F20\mu s$	Minimum fluorescence, when all PSII reaction centres (RCs) are open; Fluorescence intensity at 20 μs
F_J	Fluorescence at the J-step (2 ms) of the O-J-I-P transient
F_I	Fluorescence at the I-step (30 ms) of the O-J-I-P transient
$F_M = F_P$	Maximum fluorescence at the P-step when all RCs are closed
$V_J = (F_J - F_O)/(F_M - F_O)$	Relative variable fluorescence at the J-step
$F_V = F_M - F_O$	Variable fluorescence
Quantum yields and probabilities	
$\psi_{EO} = 1 - V_J$	Probability (at t = 0) that a trapped exciton moves an electron into the electron transport chain beyond QA-
$\varphi_{EO} = (1 - F_J/F_M)$	Quantum yield (at t = 0) for electron transport from QA- to plastoquinone
$\delta R_O = (1 - V_I)/(1 - V_J)$	Efficiency/ probability (at t = 0) with which an electron from the intersystem carriers moves to reduce end electron acceptors at the PSI acceptor side
Performance indexes	
PI_{ABS}	Performance index of PSII based on absorption
$PI_{total} = PI_{ABS} \times \delta R_o/(1 - \delta R_o)$	Performance index of electron flux to the final PSI electron acceptors, i.e., of both PSII and PSI

Leaf chlorophyll content

Non-destructive measurement of leaf chlorophyll content was made using a portable chlorophyll meter (CL-01, Hansatech, UK). This device determines the relative chlorophyll content using dual-wavelength optical absorbance (620nm and 940nm wavelength) measurements from leaf and allows to estimate the Chl content in relative units - the Chl index (CI). Measurements were made on the youngest fully developed leaf without detaching from the plants. The same leaves, on which the chlorophyll fluorescence was measured, were used.

Determination of antioxidant activity

Antioxidant activity was determined according to the method of Yen & Chen (1995) and DPPH percent inhibition was calculated according to Rossi et al. (2003). Briefly, fresh leaves (0.1 g) were extracted with 50 ml of methanol (HPLC grade) in the ultrasonic bath for 15 minutes. The

extract was centrifuged at 10 000 RPM for 5 minutes at 10°C. One ml of the plant extract was mixed with 1,5 ml freshly prepared solution of DPPH in methanol (0.3 M) and 3.5 ml methanol. The samples were kept in the dark for 15 minutes at room temperature. The absorbance was measured at 517 nm with spectrophotometer.

DPPH percent inhibition was calculated using the following formula:

$$\%DPPH = 100 - [(AT / AR) \times 100],$$

where: AT – absorbance of tested sample (test solution); AR – absorbance of blank sample (reference solution).

Statistics of analysis

Data was analyzed with SPSS program (13 for Window), using the analysis of variance and least significant difference means separation of Duncan test to compare for each treatment. All analyses were done at the 95% confidence level. All analyses were done in triplicate.

Results and Discussion

In the beginning of all treatments the pear plants grew vigorously, but the difference between the plants grown with the biostimulators Regoplant and Charkor were visible two weeks after transplanting to ex vitro conditions. A relatively low survival rate (35%) was reported in the floating system without biostimulators (Table 2). In the other three treatments, significantly higher values were found in the survival rate - between 72.5 and 82.5%. Enrichment of the nutrient solution with biostimulators Regoplant and Charkor led to a higher survival rate of pear plants (75% and 82.5%, respectively).

The enrichment of the nutrient solution with biostimulators led to an increase in the number, fresh and dry biomass of the leaves, as well as in stem length (Table 1, Fig. 1). The highest values of these indicators were reported in the treatment with Charkor, although the differences were statistically proven only with the plants acclimatized in a floating system without added biostimulators. There was a tendency for a larger leaf area of plants acclimatized in a floating system compared to the control, but there were no statistically proven differences.

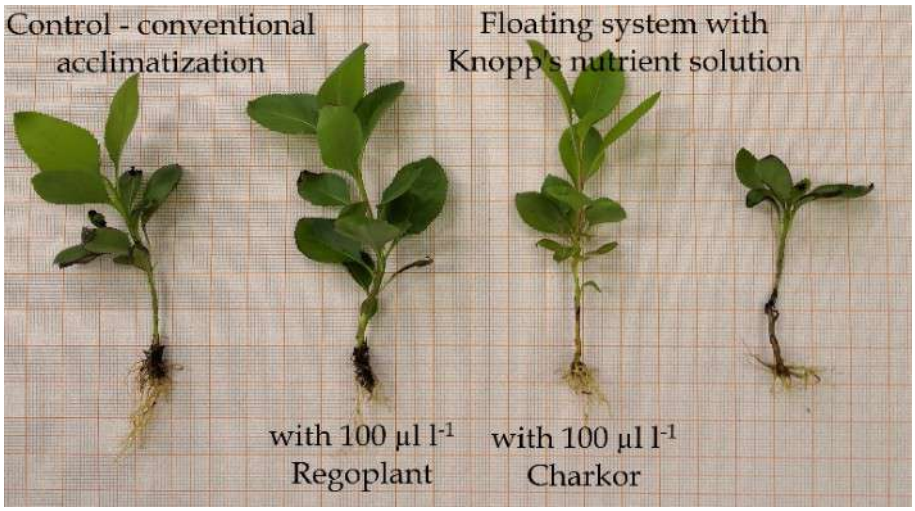

Fig. 1. The pear plants (*Pyrus communis* L. 'OHF 333') on the 45th day after transplanting for acclimatization to ex vitro conditions in a floating system. Control – conventional acclimatization in peat-perlite 1:1 (v:v), without nutrient solution.

Table 2. Growth parameters, survival rate (%) and antioxidant activity (% DPPH) of pear plants 45 days after acclimatization on floating system with biostimulators Regoplant or Charkor. Control – conventional acclimatization in peat-perlite, without nutrient solution. For each column, different letters indicate significant differences at $p \leq 0.05$.

Plant Growth Parameters	Control	Knopp	Knopp + Regoplant	Knopp + Charkor
Survival rate (%)	72.5	35	75	82.5
Number of leaves	13.4 ± 2.97 ^{ab}	9.4 ± 2.40 ^{b}	14.8 ± 1.30 ^{a}	15.8 ± 1.79 ^{a}
Leaf area (dm^2)	1.62 ± 0.54 ^{a}	1.68 ± 0.36 ^{a}	1.72 ± 0.34 ^{a}	1.87 ± 0.36 ^{a}
Stem stem length (mm)	34.08±5.51^{b}	33.61±3.14^{b}	42.55±1.95 ^{ab}	44.2±3.07 ^{a}
FW leaves (g $plant^{-1}$)	0.059 ± 0.015 ^{ab}	0.035 ± 0.014 ^{b}	0.072 ± 0.025 ^{a}	0.082 ± 0.026 ^{a}
FW roots (g $plant^{-1}$)	0.022 ± 0.010 ^{a}	0.011 ± 0.001 ^{a}	0.012 ± 0.005 ^{a}	0.013 ± 0.011 ^{a}
FW stem (g $plant^{-1}$)	0.021 ± 0.005 ^{a}	0.017 ± 0.007 ^{a}	0.027 ± 0.010 ^{a}	0.026 ± 0.007 ^{a}
DW leaves (g $plant^{-1}$)	0.053 ± 0.014 ^{ab}	0.031 ± 0.014 ^{b}	0.058 ± 0.021 ^{ab}	0.069 ± 0.023 ^{a}
DW roots (g $plant^{-1}$)	0.016 ± 0.009 ^{a}	0.008 ± 0.001 ^{a}	0.008 ± 0.005 ^{a}	0.008 ± 0.009 ^{a}
DW stem (g $plant^{-1}$)	0.016 ± 0.006 ^{a}	0.012 ± 0.005 ^{a}	0.019 ± 0.010 ^{a}	0.019 ± 0.008 ^{a}
Chlorophyll (CI)	3.78 ^{b}	3.28 ^{b}	5.89 ^{a}	6.61 ^{a}
DPPH (%)	55.58 ^{c}	58.88 ^{c}	78.92 ^{b}	92.24 ^{a}

Antioxidant activity, determined as DPPH percent inhibition, in plants treated with biostimulators was higher than the control plants and these from floating system with Knopp's solutions without any supplements. Significant differences were reported in this indicator between plants treated with different biostimulants - 78.92% in Regoplant and 92.24% in Charkor, which could correspond to a higher resistance of plants to transplant stress. Along with better plant growth, higher values of DPPH- radical scavenging activity could be an indicator of the protective role of the two biostimulators. DPPH- radical scavenging activity is a measure of non-enzymatic antioxidant activity (Kang and Saltveit, 2002a) and higher levels of DPPH- radical scavenging activity have been correlated with enhanced stress tolerance in rice and cucumber seedlings (Kang & Saltveit, 2002a; b).

Higher chlorophyll content was measured in the leaves of plants treated with the biostimulators used, with a maximum value reported for Charkor treatment (Table 2). This could be a prerequisite for better functioning of the photosynthetic apparatus in these plants.

Chlorophyll *a* fluorescence is another nondestructive method to evaluate the functional activity of the photosynthetic apparatus of plants. The analysis of the induction curves of rapid chlorophyll fluorescence (OJIP test) links the structure and functionality of the photosynthetic apparatus and allows for rapid assessment of plant viability, especially in stress conditions (Strasser et al., 2000, 2004). In the four variants studied, the rapid chlorophyll fluorescence curves had a typical OJIP shape from Fo to F_M level with clearly separated J and I phases (Fig. 2), indicating that the pear plants, included in the experiment, were photosynthetically active (Yusuf et al., 2010). No significant differences in the minimum (Fo), maximum (F_M) and variable (Fv) fluorescence in plants were found from the studied variants (Table 3).

The quantum yield (Yield = Fv/F_M), reflecting the potential of photochemical activity of PS II, ranges from 0.794-0.809 and corresponds to normal (0.750- 0.830) in healthy, unstressed leaves (Bolharnordenkampfh & Oquist, 1993). This indicates that in all four variants studied, the photosynthetic apparatus was functioning normally.

No significant differences were observed between the treatments in the other three important indicators of the OJIP test - φ_{EO}, ψ_{EO}, PI_{ABS} and PI_{total}. The parameter ψ_{EO} reflects the probability of electron transport outside QA. The performance index (PI_{ABS}) shows the functional activity of the PSII relative to the energy absorbed, and the total performance index (PI total) reflects the functional activity of the PS II,

PS I and the electron transport chain between them. PI_{total} is closely related to the overall plant growth and survival rate under stress and is considered to be a very sensitive indicator of the JIP test.

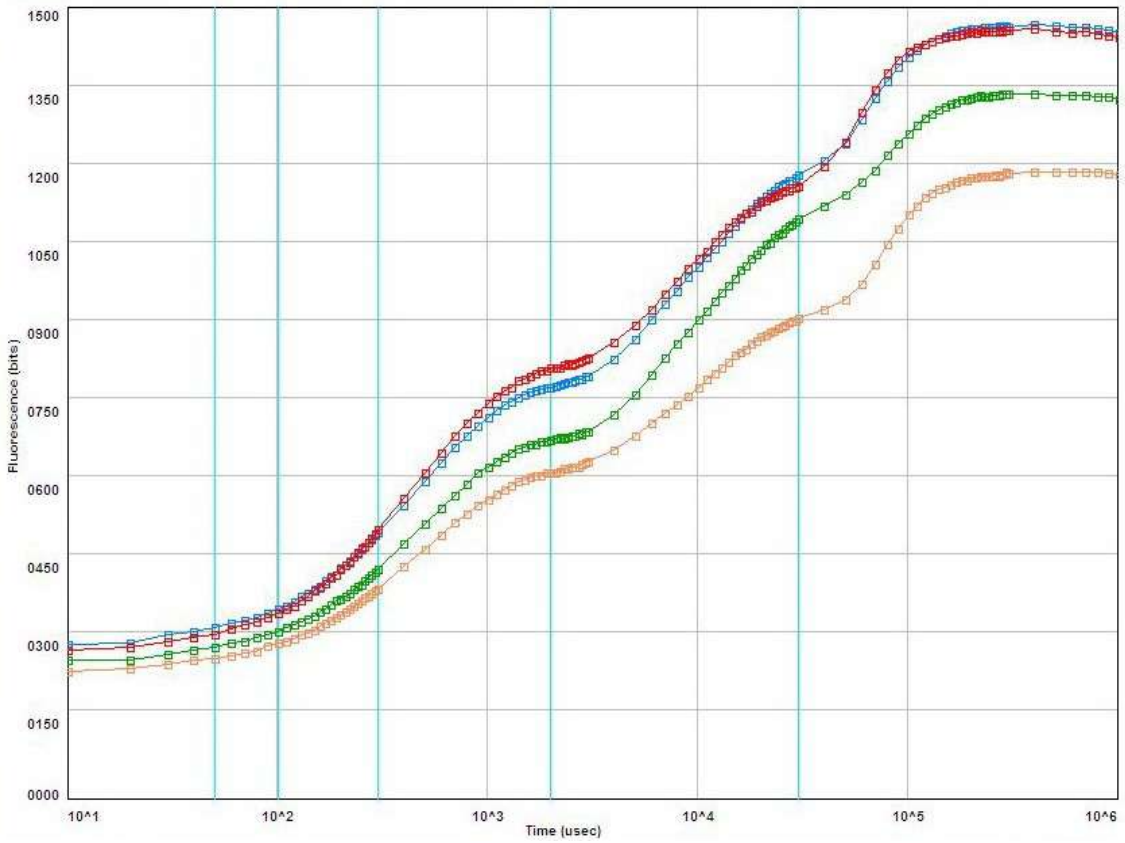

Fig. 2. Induction curves of rapid chlorophyll fluorescence (OJIP test) of pear plants acclimatized in a floating system - (--) Control, conventional acclimatization in peat-perlit; (--) Knopp's nutrient solution; (--) Knopp's nutrient solution, supplemented with 100 µl l^{-1} Charkor; (--) Knopp's nutrient solution, supplemented with 100 µl l^{-1} Regoplant.

Table 3. Chlorophyll fluorescence parameters (OJIP test) of the pear plants 45 days after acclimatization on floating system with biostimulators Regoplant or Charkor. Control – conventional acclimatization in peat-perlite, without nutrient solution. For each column, different letters indicate significant differences at $p \leq 0.05$.

Basic parameters	Control	Knopp	Knopp + Regoplant	Knopp + Charkor
F_O	291 ± 9 ^{a}	243 ± 65 ^{a}	260 ± 5 ^{a}	276 ± 5 ^{a}
F_M	1465 ± 51 ^{a}	1190 ± 356 ^{a}	1338 ± 129 ^{a}	1460 ± 135 ^{a}
Fv	1173 ± 49 ^{a}	947 ± 291 ^{a}	1078 ± 124 ^{a}	1183 ± 139 ^{a}
Fv/F_M	0.801 ± 0.007 ^{a}	0.794 ± 0.010 ^{a}	0.805 ± 0.016 ^{a}	0.809 ± 0.020 ^{a}
φ_{EO}	0.465 ± 0.006 ^{a}	0.479 ± 0.008 ^{a}	0.491 ± 0.029 ^{a}	0.437 ± 0.045 ^{a}
ψ_{EO}	0.581 ± 0.013 ^{a}	0.604 ± 0.017 ^{a}	0.611 ± 0.042 ^{a}	0.539 ± 0.042 ^{a}
δR_O	0.411 ± 0.0373 ^{ab}	0.483 ± 0.031 ^{a}	0.362 ± 0.007 ^{b}	0.469 ± 0.046 ^{ab}
PI_{ABS}	3.052 ± 0.243 ^{a}	3.364 ± 0.215 ^{a}	3.774 ± 0.857 ^{a}	2.894 ± 1.160 ^{a}
PI_{total}	2.164 ± 0.468 ^{a}	3.149 ± 0.413 ^{a}	2.149 ± 0.526 ^{a}	2.480 ± 0.554 ^{a}

At a first glance, the established higher values of this indicator contradict the weaker growth of plants acclimatized to *ex vitro* conditions in a floating system with Knopp's solution alone. The plants from the control variants in conventional acclimatization and those from the floating system enriched with biostimulators continue to grow actively. Therefore, the first fully developed leaves from the top (on which the fluorescence was measured) are physiologically younger and less mature than the top leaves in the treatment of the floating system without additives. This could explain that despite the greater increase in height and number of leaves, the PI_{total} values of plants grown with Knopp's solution alone were higher than those of the other treatments.

The results of this study confirmed the beneficial effect of the floating system in the acclimatization of micropropagated OHF 333 rootstock, but in which the Regoplant biostimulator (100 μl l^{-1}) had a better effect.

Rooting and acclimatization of pear plants are difficult (Chevreau et al., 1992). Resumption of plant growth at acclimatization, which is often difficult, can be improved by foliar sprays of gibberellic acid (100–200 ppm).

The low survival rate of plants when they are removed from *in vitro* culture is associated with poor stomatal functioning and excessive water loss (Brainerd and Fuchigami, 1982). During *ex vitro* acclimatization, many changes can occur to the morphological and physiological state as well as to the photosynthesis due to differences in the environmental conditions (Shin et al., 2014). The floating system provides easier maintenance of the air humidity, especially in the conditions of autumn acclimatization and is a valuable approach, especially in the conditions of hot and dry climate.

Biostimulants have been a popular subject of interest in sustainable agriculture because their application activates several physiological processes that enhance nutrient use efficiency, stimulating plant development and allowing the reduction of fertilizer consumption (Kunicki et al. 2010). Many biostimulants are also able to counteract the effects of biotic and abiotic stresses, enhancing quality and crop yield by stimulating plant physiological processes (Ziosi et al. 2013). But, the effect of the biostimulants is not always consistent among the plant species.

Combining innovative approaches as a floating system and biostimulators could help plants overcome the stress of transfer provides an alternative for acclimatizing *in vitro* propagated plants in a clean, convenient and water-saving way.

Conclusions

The results of this study demonstrate that the enrichment of the nutrient solution with biostimulator Charkor (100 μl l^{-1}) in a floating system could improve the acclimatization of *in vitro* micropropagated pear plantlets to *ex vitro* conditions. The beneficial effect of Charkor, expressed in better survival rate and better growth could support the acclimatization of other woody species in floating conditions.

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Effect of Polysaccharides Obtained from Plantago major L. leaves on Lactobacillus bulgaricus L14 in an In Vitro Model System of the Gastrointestinal Tract

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Abstract. There is evidence that prebiotics can impart a range of health benefits if consumed on a regular basis. With a growing market for prebiotic-containing foods there is increasing interest in understanding how prebiotics function at the molecular level. Advances in the genomics of lactobacilli and bifidobacteria have enabled modeling of transport and catabolic pathways for prebiotic utilization. The goal of this study was to develop a physiological model of the upper gastrointestinal tract to investigate the metabolic profile of probiotic strain *Lactobacillus bulgaricus* L14 in present of 0.75% polysaccharides obtained from *Plantago major* L. leaves. The polysaccharide degradation and utilization from probiotic strain L14 using *in vitro* GIT system including gastric phase and intestinal phase simulated digestion in humans was developed. As a results of hydrolytic processes in gastric phase after 2h, polysaccharides obtained from *Plantago major* L. leaves were partially hydrolyzed to monosaccharides- mainly galactose and oligosaccharides with degree of polymerization higher than 6. In the conditions simulating intestinal phase, the growth of *Lactobacillus bulgaricus* L14 from $1x10^5$ CFU/ml on the 0h to $4x10^5$ CFU/ml on the 4th hour was observed. D-lactate (15.69 mmol/L) and L-lactate (3.07 mmol/L) were detected after 4h cultivation in conditions simulating intestinal phase. In addition, an *iv vitro* model induced hyperglycemia (100mM glucose) in human erythrocytes, treated 48h with metabolites simulating intestinal phase from 4th hour, showed 41% reduction of catalase and 26% incising of superoxide dismutase activity.

Key words: polysaccharides, *Lactobacillus bulgaricus* L14*, Plantago major, in vitro.*

Introduction

Polysaccharides are type of natural macromolecules usually consist of more than 10 monosaccharides linked through glyosidic bonds in linear or branched chains (Xie et al., 2016). Although plant derived polysaccharides are one of major source of energy, they have a lot of beneficial effects related with reducing the risk factors for some chronic diseases, including diabetes, cardiovascular diseases, hyperlipidemia, certain type of cancer (Lovegrove et al., 2017; Wu et al., 2019), but also have antioxidant, hepatoprotective, anti-inflammatory and immunomodulatory effects (Ren et al., 2017; Zeng et al., 2016; Li et al., 2017).

Plant polysaccharides can evade the action of human digestive enzymes from saliva, stomach and small intestine (Wang et al., 2015; Di et al., 2018), but when they reach large intestine, they can be utilized and broken down by gut microbiota (Di et al., 2018; Chen at al., 2018). As a consequence of this, some health-promoting bacteria's such as *Lactobacillus*, *Bifidobacterium* increased, and production of short-chain fatty acids (SCFAs) in the colon are enhanced (Fernández et al., 2016; Chen at al., 2018). Lukova and colleagues, prove that pectin type water-extractable polysaccharides from *Plantago major* L. leaves and their lower molecular weight hydrolysates stimulate the growth of four *Lactobacillus* strains: *L. acidophilus* N, *L. plantarum* S30, *L. sakei* S16 and *L. brevis* S27 (Lukova et al., 2020). *P. major* L. leaves have been used in traditional medicine in treatment of a different diseases related to the respiratory organs, skin, digestive organs, against

infections and so on. Those effects are owing to variety of biologically active ingredients like polysaccharides, lipids, derivatives of caffeic acid, flavonoids, iridoid glycosides and terpenoids (Samuelsen, 2000).

Studies about food digestion should be performed in vivo but sometimes this is not possible by ethical, technical or financial reasons (Li et al., 2020) Development of *in vitro* digestion methods, static or dynamic, stimulate the complex physiological conditions of the gastrointestinal tract. Static models use a constant ratio of food to enzymes and electrolytes, pH is constant in each digestive phase- oral, gastric and intestinal. Previous studies show that static *in vitro* digestion models could be used for the prediction of the outcomes of *in vivo* digestion (Bohn et al., 2018; Sanchón el al., 2018; Brodkorb el al., 2019). The goal of this study was to develop a physiological model of the upper gastrointestinal tract to investigate the metabolic profile of probiotic strain *Lactobacillus bulgaricus* L14 in present of 0.75% polysaccharides obtained from *Plantago major* L. leaves. To aim this, an *in vitro* model including gastric phase and intestinal phase simulated digestion in humans was performed. In addition, an *in vitro* model induced hyperglycemia on human erythrocytes, treated with metabolites produced in intestinal phase, was developed. Enzymatic activities of catalase and superoxide dismutase was measured in order to verify potential antioxidant activity of polysaccharides obtained from *P. major* L. leaves.

Materials and Methods

Materials and reagent

Acid-extractable polysaccharides from *P. major* L. leaves were obtained by Lukova and colleagues (Lukov et al., 2017). The probiotic strain of *L. bulgaricus* L14 was received from the bacterial culture collection of the department of Biochemistry and Microbiology, Plovdiv University, Bulgaria. Catalase and superoxide dismutase assay kits were purchased from Arbor Assays, Michigan, USA. Hemoglobin assay kit was purchase from Human, Wiesbaden, Germany.

Static in vitro digestion method

Simulated *in vitro* digestion was carried out according to the INFOGEST protocol set up by Minekus et al. (2014)vwith some modification. Gastric and intestinal phase were simulated. For 400mL simulated gastric fluid were needed electrolytes, which were prepared by mixing of 2.76 ml 0.5 M KCl, 0.76 ml 0.5 M KH_2PO_4, 5 ml 1 M $NaHCO_3$, 4.72 ml 2 M NaCl, 0.16 ml 0.15 M $MgCl_2$ x $(H_2O)_6$ and 0.2 ml 0.5 M $(NH_4)_2CO_3$. Three grams of polysaccharides and 0.2 g pepsin were separately dissolved in 50 ml sterilized H_2O. After that, H_2O was added to final volume of 400 ml and pH was adjusted to 3 by 1 M HCl solution. The simulated gastric fluid was incubated for 120 min with shaking (100 rpm) at 37 °C. At 0h and 2h of digestion, 30mL of samples are taken out.

After 2h of digestion, pH was adjusted to 7 by 1 M NaOH solution and in the simulated gastric fluid were added: 2g peptone, 2g yeast extract, 0.168g bail salts and 0.8g trypsin. The probiotic strain of *L. bulgaricus* L14 was cultivated overnight in MRS medium at 37°C. Overnight cells were washed twice in 0.9% NaCl solution and after that were added. The simulated intestinal fluid was incubated for 360 min with shaking (100 r/min) at 37°C. At 0h, 1h, 2h, 3h and 4h of digestion, 30 ml of samples are taken out.

Analytical assays

Bacterial growth

Bacterial growth was measured by a turbidimetric method at 600nm against a cell dry weight standard curve using a UV/Vis spectrophotometer (Beckman Coulter DU 800, USA). Furthermore, 50 µl of taken samples were used to inoculate MRS agar medium (Eddy jet 2W) for period of 24h at 37°C for examination of CFU/ml (Sphere Flash).

After that, samples were centrifuged at 9000 rpm, 20 min, 4 °C and cells were collected. The separated supernatants were used for further analysis of metabolites.

Analysis of metabolites

L-Lactic acid, D-Lactic acid, acetic acid, propionic acid and butyric acid were determined with HPLC system Konik-Tech, with UV Detector (Konik-tech, λ=210nm) and Aminex HPX-87H 5μm (250 x 4,6 mm) column, mobile phase 0.005 M H_2SO_4, flow rate 0.6 ml/min, temperature 40°C. The registered peaks of the samples were evaluated using reference short chain fatty acids standards of: L-Lactic acid, D-Lactic acid, acetic acid, propionic acid and butyric acid.

Analysis of carbohydrates and proteins

The oligosaccharides and residual sugars were analyzed by HPLC system Shimadzu (Japan) coupled with autosampler Nexera X2, SIL-30AC; column over – CTO-20AC and detector RID-20A, Shimadzu (Japan). Ten μl of the sample was injected in the system and eluted into a guard Tracer carbohydrate 5μm 15 x 0.46 column (Tecknohroma, Spain) with mobile phase of 65:35 acetonitrile: water, flow rate 0.78mL/min and temperature 35°C. Results were analyzed with LabSolution, Nexera- XR-RF software using the standard monosaccharides.

Protein content in the samples were measured by the method of Bradford (Bradford, 1976).

Enzymatic activity

The collected bacterial cells were washed twice with 50 mM sodium acetate buffer pH 7.5 and suspended in 2 ml cold lysis buffer which contain 50 mM sodium acetate buffer pH 7.5, 2% glycerol and 30 mM NaCl. The sonication of cells was executed by Tech-pan Ultasonic Disintegrator UD – 20, Warsaw, Poland for 15 cycles and 50% amplitude. Duration of each cycle was 5 sec and 2 min break between them on ice. Thereafter, the lysate cells were centrifuged at 12000 rpm, 10min, 4 °C and supernatants were collected for measure of α-galactoidase, β-galactosidase, α-glucosidase, β-glucosidase and β-xylosidase activity. Alpha-glucosidase activity was determined by the method of Dewi et al. with some modifications, as the amount of p-nitrophenol (pNP) released by the degradation of the pNP-α-D-glucopyranoside substrate (Sigma-Aldrich). The reaction mixture contained 250 μl of 5 mM pNP-α-D-glucopyranoside substrate in 50 mM KH_2PO_4 at pH 6.8, 150 μl of water, and 100 μl of the bacterial lysate. The mixture was incubated for 10 min at 37 °C. The reaction was stopped by the addition of 2 mL of 1 M Na_2CO_3. The absorbance of the released pNP was measured at 405 nm. Alpha-galactosidase activity of *L. plantarum* strains was determined by the method of Petek et al. as the amount of pNP released by the degradation of pNP-α-D-galactopyranoside substrate (Sigma-Aldrich). The reaction mixture was composed by 100 μl 9.9 mM PNP-α-D-galactopyranoside substrate in 0.1M KH_2PO_4 buffer and 100 μl of the bacterial lysate. The total volume was brought up to 500 μl with water and incubated for 5 min at 37°C. The reaction was stopped by the addition of 1 mL of borate buffer (pH 9.8). The amount of the released pNP was measured at 405 nm. Beta-xylosidase activity was determined by the method of Lasrado and Gudipati as the amount of pNP released by substrate degradation of pNP-β-D-xylopyranoside (Sigma-Aldrich). The reaction mixture contained 900 μl 5 mM pNP-β-D-xylopyranoside in 50 mM KH_2PO_4 (pH 5.7), 100 μl water, and 100 μl of the bacterial lysate. The mixture was incubated for 30 min at 30 °C. The reaction was stopped by the addition of 100 μl saturated sodium tetraborate solution. The amount of pNP was measured at 410 nm. 2.4. *In vitro* model induced hyperglycemia.

Erythrocytes were isolated from blood of three healthy young man. First of all, the blood was centrifuged at 3000 rpm, 20 min, 4 °C. Plasma and leukocyte buffy coat were aspirated and the erythrocyte pellet was washed three times with 4 volumes of PBS buffer, pH 7.4. Washed erythrocytes were suspended to a final hematocrit of 50% in PBS containing 5 mM, 50 mM and 100 mM of glucose. To 250 μl of corresponding hematocrit was added 150 μl of sample taken out on 4^{th}h of intestinal phase. The samples were incubated at 37 °C for 24h and 48h with continuous mixing. The samples suspended with 5 mM glucose represented a control group and they simulated the physiological conditions in erythrocytes.

The erythrocytes were lysed by adding ice-cold water in ratio 1:20 (Ferreira et al., 1999). They were centrifuged at 7000 rpm, 10 min. The supernatant was used for measuring activity of catalase and superoxide dismutase and concentration of hemoglobin.

Results and Discussion

As a results of hydrolytic processes in gastric phase after 2h, polysaccharide obtained from *Plantago major* L. leaves were partially hydrolyzed to monosaccharides- mainly galactose (RT- 5.429) and oligosaccharide (RT- 6.666) with degree of polymerization (DP) higher than 6 (Fig. 1A).

After 4h digestion in conditions simulating intestine, hydrolysis of polysaccharide (RT 2.729) and production of monosaccharides continue. The content of monosaccharides probably were: galacturonic acid, galactose and rhamnose. This is proved in study by Lukova and colleagues (Lukova et al., 2017). Oligosaccharides were also cleaved from polysaccharide (RT- 6.788 and RT- 7.029) (Fig. 1B).

In their study, Hu et al. Observed that polysaccharides from seeds of *P. asiatica* L. could be degraded into smaller unites by gastric fluid because of breakdown of glyosidic bonds (Hu et al., 2014).

On the Fig. 2 was presented the growth kinetics of *L. bulgaricus* L14 in terms of OD 600 nm during 4h digestion.

In the conditions simulating intestinal phase in the presence of polysaccharide, the growth of *L. bulgaricus* L14 increased 4 time during process of fermentation - $1x10^5$ CFU/ml on the 0h up to $4x10^5$ CFU/ml on the 4th hour.

Concentration of SCFAs were presented on Fig. 3. Our study show that D-Lactic acid and L-Lactic acid were primary fermentation products. Their concentrations were increased rapidly after one hour of fermentation and reach the highest values at the 4th hour (D-Lactate 15.59 mmol/L and L-Lactate 3.07 mmol/L). Butyric acid was detected in trace (0.23 mmol/L) at 1st hour. Acetic acid and propionic acid weren't produced by *L. bulgaricus* L14 after 4h of fermentation.

Some plant polysaccharides as a result of fermentation by distinct group of beneficial bacterial species in gastrointestinal tract like *Lactobacillus, Bifidobacterium, Faecalibacterium,* etc. produced SCFAs mainly lactic acid, acetic acid, propionic acid and butyric acid (Fernández et al.,2016). Acetic acid is important energy source for muscles, brain but also is involved in lipogenesis, gluconeogenesis and cholesterol synthesis (Mateos-Aparicio et al., 2016). It also could protect from food allergy via induction of CD103+ DCs and Treg cell response (Ding et al., 2017). Butyrate act as an anti-tumor compound for tumor colonocytes via promoting pro-apoptotic routes (Fernández et al., 2016). SCFAs are associated with reducing risk factors for development of some diseases including the irritable bowel syndrome, inflammatory bowel disease, cardiovascular disease and cancer. SCFAs perform their effects in different cell types by different mechanisms. Binding with specific G protein-coupled receptors (GPR) such as GPR41, GPR43, and GPR109a may mediate SCFAs activities (Offermanns, 2014; Iraporda et al., 2015).

In our study, the activity of β-galactosidase and α-glucosidase were detected during fermentation of polysaccharide. The highest activity of β-galactosidase was measured after 4h of fermentation (0.08 U/mg protein). The same tendency was preserved in the activity of α-glucosidase (0.06 U/mg protein) (Fig. 4). The α-galactosidase, β-glucosidase and β-xylosidase activity weren't detected. This indicate that *L. bulgaricus* L14 strain can produce a enzymes which are able to hydrolyze glyosidic bonds in the plant polysaccharides. The same conclusion but for different *Lactobacillus* strains was proved in study by Lukova and colleagues (Lukova et al., 2020).

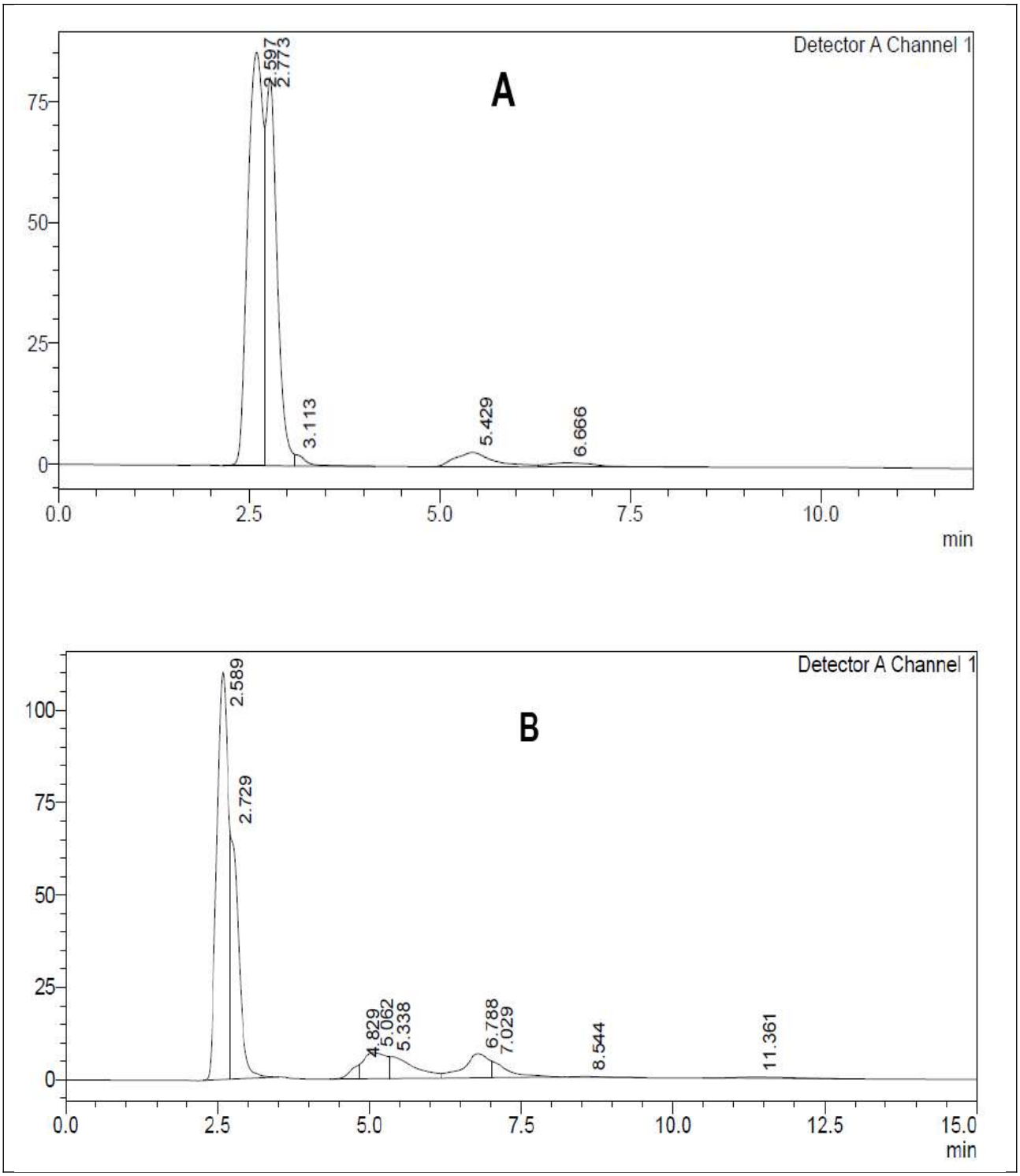

Fig. 1. HPLC profile of polysaccharide hydrolysis products in simulated gastrointestinal tract: (A) gastric phase 2nd hour, (B) intestinal phase 4th hour.

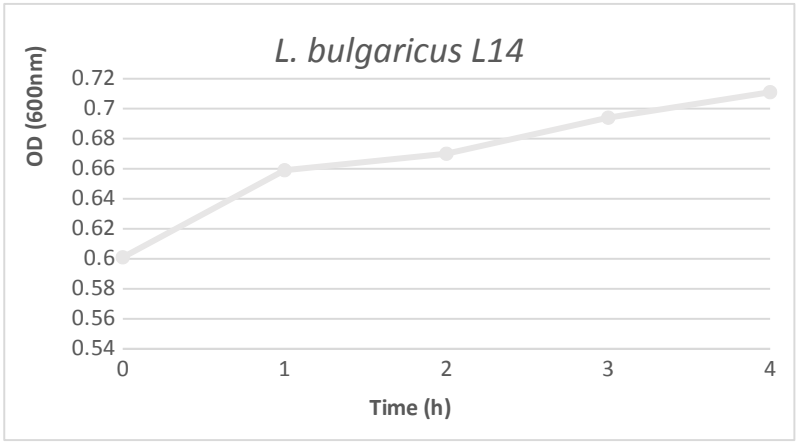

Fig. 2. Optical density value (OD) of *L. bulgaricus* L14 strain in the intestinal phase.

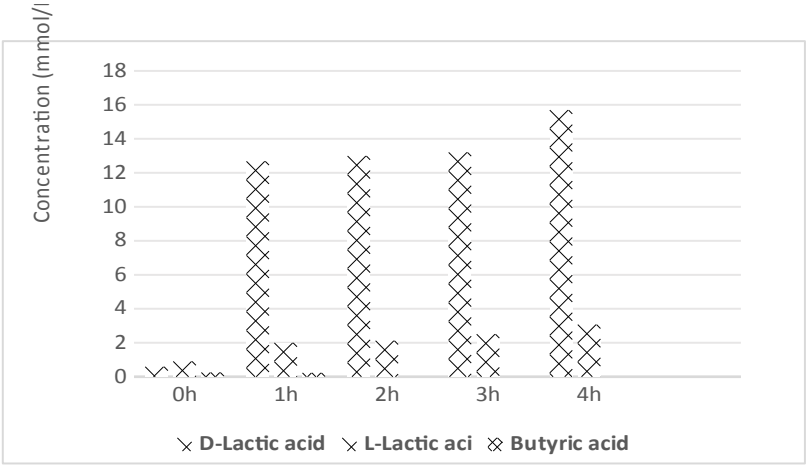

Fig. 3. Concentration of SCFAs at different time points of fermentation in intestinal phase.

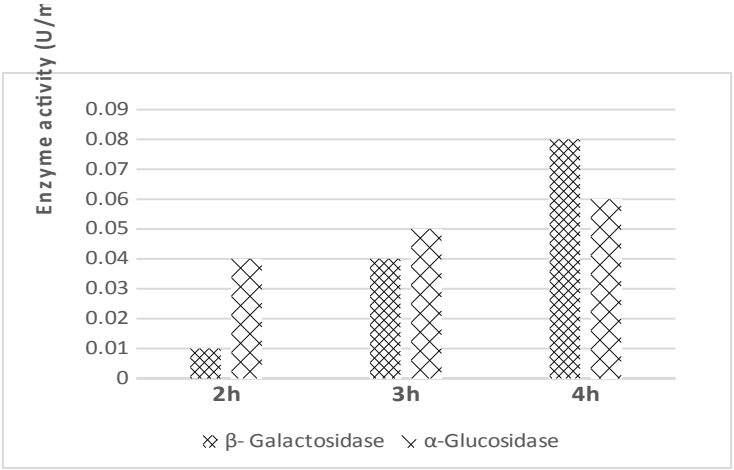

Fig. 4. β-galactosidase and α-glucosidase activity of *L. bulgaricus* L14 strain involved in utilization of polysaccharide.

Lactobacillus and *Bifidobacterium* species are the typical groups of probiotic microbes (Hill et al. 2014). They have genes that encode carbohydrate active enzymes (Flint et al., 2012). *Lactobacillus* species us Embden-Meyerhof pathway and phosphoketolase pathway for utilization of monosaccharides. The depolarization of complex prebiotics by probiotic bacteria involve collaboration between glycosyl hydrolases (GHs) localized intra or extracellular and transport system for hydrolyzed substrates. The genes encoding the transporter components and the related catabolic enzymes for the same substrates are clustered and coregulated as single operons (Goh et al., 2015).

The activity of antioxidant enzymes in protection of erythrocytes from ROS is very important. Our date show that activity of catalase was reduced 41% in erythrocytes treated 48h with metabolites simulating intestinal phase (Fig. 5A). This could be as a result of glycosylation of intracellular proteins caused by high glucose levels.

On the other hand, the activity of superoxide dismutase in experimental groups after treatment 48h in conditions simulating hyperglycemia (50mM glucose and 100 mM glucose) was higher, significantly in the group treated with 100mM glucose, around 26% (Fig. 5B). We can consider that, fermentation product from polysaccharides obtained from *Plantago major* L. leaves activate protective systems in erythrocytes in conditions of high oxidative stress induced by savior hyperglycemia.

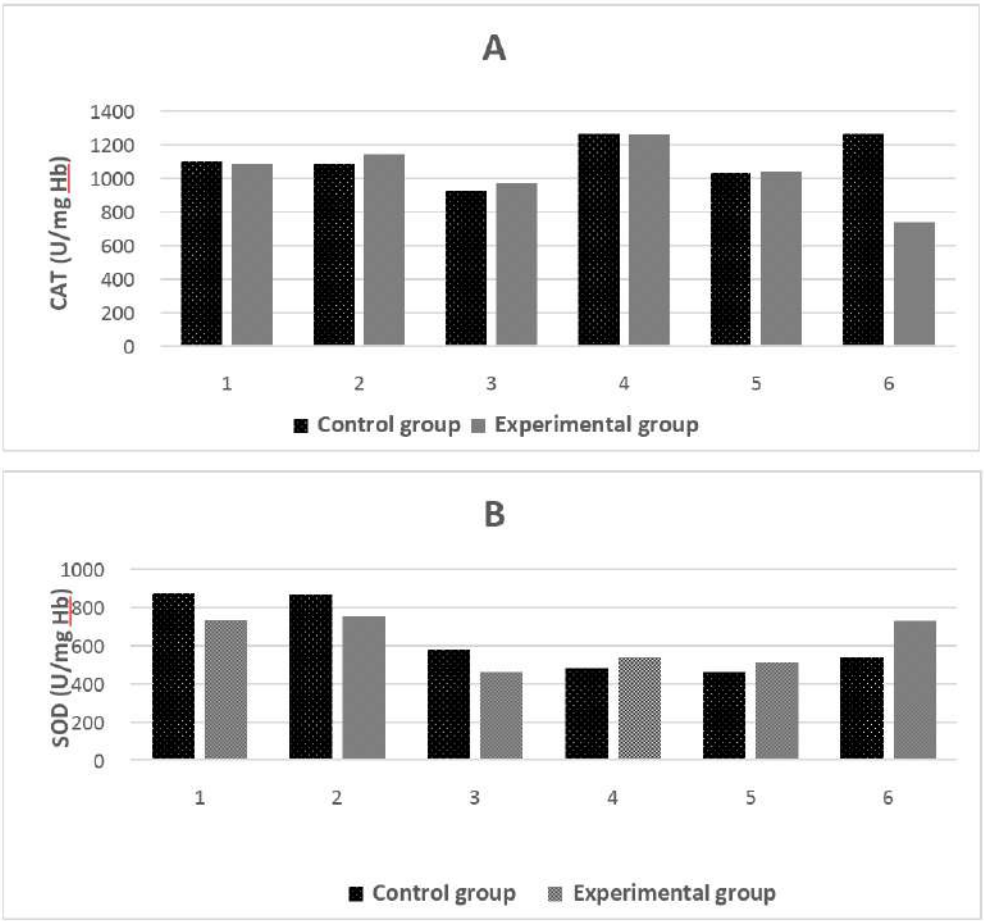

Fig. 5. Catalase and superoxide dismutase activity in erythrocytes treated with metabolites from intestinal phase.

Hyperglycemia is the reason for various changes in different tissues and cells. Erythrocytes as the most abundant cells in human body couldn't escape from those changes. The structure, the functions and the longevity of erythrocytes are impaired. This could be due to high production of reactive oxygen species (ROS) and induction of oxidative stress which lead to lipid peroxidation and disruption of erythrocytes membrane phospholipid bilayer (Pazzini et al., 2015).

Conclusions

The impact of non-digestible plant derived carbohydrates on human microbiota depends on the complex relationship between their chemical composition, bacteria strain specificity and metabolism. The ability of pectin type water-extractable polysaccharides from *Plantago major* L. leaves, as well as their lower molecular weight hydrolysates, to stimulate the growth of probiotic strain *Lactobacillus bulgaricus* L14 strains *in vitro* GIT system was proven. The obtained high amount of lactic acid and the secretion of some glycohydrolases in condition of *in vitro* GIT system are a prerequisite for an in-depth study of the prebiotic capacity of *P. major*. Furthermore, substantial in vivo investigations on the correlation between the probiotic properties of lactobacilli and the prebiotic activity of *P. major* carbohydrates fractions could reveal their potential application as functional food with synbiotic characteristics.

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Spermatozoa Morphology Abnormalities in Men with Reproductive Problems Influenced by Various Environmental and Lifestyle Factors

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Abstract. Totally 1304 men were included in conventional analysis to determine their spermatozoa morphology. Deviations from the normal morphological status were found in 16.5% of them. The identified morphological abnormalities were analyzed by groups and their frequency of occurrence was determined, both individually and in various combinations. The established anomalies, in descending order according to their frequency, were as follows: tapered heads (95.2%); neck defects (88%); amorphous heads (87.2%); microcephalic – with small heads (79.2%); excess residual cytoplasm (48.8%); macrocephalic – with large heads (42.4%); tail defects (39.2%), double heads (25.6%), acephalic (<0.08%). In the studied individuals there were various complexes of morphological anomalies, which are discussed in detail. The information concerning environmental and lifestyle factors was collected on the base of voluntarily completed questionnaire. The results obtained showed that about 21% of the participants were undergoing occupational hazards, 22% were smokers, 42% alcohol consumers, 13% – drug users, 7% taking anabolic steroids, 31% – taking medications and 5% work or live under stress. Some of the men surveyed were influenced by multiple of the mentioned factors. Statistical analysis demonstrated differences in the clarity of the relationship between the established abnormal spermatozoa morphology and the studied environmental and lifestyle factors. The results of the study showed the presence of statistical significance in the relations "harmfulness – spermatozoa morphology" ($P<0.017$) and "occupations – spermatozoa morphology" ($P<0.003$).

Key words: spermatozoa morphology, environment and lifestyle, male reproductive health.

Introduction

Infertility in human populations is a worldwide problem affecting about 15% of couples of reproductive age. Approximately, half of these cases are related to the male factor (Yu et al., 2015). Studies show that at the root of this global puzzle is the deteriorating quality of sperm, and many researchers are turning their attention to the causes of increasingly global negative trends. There are evidences that male fertility is influenced by a variety of environmental factors, lifestyle and bad habits. Among the possible risk factors for male infertility are alcohol, cigarettes, androgenic anabolic steroids, different medications, occupational hazards, socio-psychological instability and stress (Martini et al., 2004; Brezina et al., 2012; Yu et al., 2015). Strict assessment of spermatozoa morphology correlates with fertilization capacity and has prognostic value in assisted reproduction. Spermatozoa pathology is related to their structural and functional defects. Its study helps to elucidate the mechanisms associated with the fertilization inefficiency and to identify the genetic causes for that. Abnormal morphology is associated with various changes in the organization and function of the sperm chromatin, the perinuclear cell wall, the acrosome, and the cytoskeleton.

Chemes & Alvarez Sedo (2012) pay attention to the fact that the correct identification of spermatozoa pathologies is associated with a more reliable determination of the fertility potential and with expected better results in the assisted reproduction. On this basis, it is possible to assess the genetic risk on a case-by-case basis.

The present study analyzes different abnormalities in spermatozoa morphology in men with reproductive problems who are influenced by various environmental and lifestyle factors, searching potential dependencies between spermatozoa morphology and these factors.

Material and Methods

This investigation was accepted by the Institutional Ethical Committee by a Certificate N 2/16.01.2019. Accordingly to the ethical principles, an informed consent was obtained from each patient. Men with varicocele, cryptorchidism, parotitis, azoospermia, genital trauma, infections and other genitourinary diseases were not included in this study. Totally 1304 individuals were involved in the conventional analysis to determine the spermatozoa morphology. The information concerning environmental (such as: hazardous chemicals, harmful fumes, Roentgen rays, high temperatures, radiation therapy, etc.) and lifestyle (such as: different professions - engineers, IT professionals and office workers, drivers, car mechanics and service workers, farmers, builders and carpenters, police officers, firefighters, military and sportsmen, cooks and kitchen workers; users of cigarettes, alcohol, drugs, anabolic steroids, medications; work or living under stress) factors was collected on the base of voluntarily completed questionnaire. All men, included in the study provided a standardized semen sample. The morphological abnormalities were calculated as % of all spermatozoa cells analyzed per individual (Stanislavov & Nikolova, 2013; WHO, 1999; 2010). For each individual, standard sperm smears were prepared on two slides. Totally 200 cells per individual were analyzed. Staining was performed with SpermBlue®.

The results obtained were characterized and compared by the usage of descriptive statistics. Differences between the groups compared were analyzed by χ^2 and t-test. Statistical significance was defined as $P<0.02$ and $P<0.005$.

Results

Spermatozoa morphology

The data from the conducted sperm analysis show that in 16.5% of the examined individuals there are abnormalities in the spermatozoa morphology. These are all cases in which cells with abnormal morphology are more than 14% (according to Stanislavov & Nikolova, 2013).

The identified morphological defects were analyzed in detail by groups and their frequency of occurrence was determined, both individually and in various combinations. The data on the types of morphological abnormalities in the spermatozoa and their frequency in the studied men are presented in Fig. 1 and in Table 1.

The established anomalies, in descending order according to their frequency, were as follows (Table 1): tapered heads (95.2%); neck defects (88%); amorphous heads (87.2%); microcephalic – with small heads (79.2%); excess residual cytoplasm (48.8%); macrocephalic – with large heads (42.4%); tail defects (39.2%), double heads (25.6%), acephalic (<0.08%). In the studied individuals there were various complexes of morphological anomalies, which are discussed in detail.

The analysis of the obtained data shows that in the individuals there are complexes of morphological anomalies (Table 1), as follows: in 32.8% - spermatozoa with tapered heads and macrocephalic spermatozoa in combination with neck defects; in 27.2% - sperm with amorphous and tapered heads in combination with neck defects; in 20.8% - spermatozoa with amorphous heads and macrocephalic spermatozoa in combination with neck defects and excess residual cytoplasm; in 14.4% - all reported defects except for neck defects; in 13.6% - spermatozoa with amorphous and tapered heads, microcephalic spermatozoa, tail defects and excess residual cytoplasm; in 11.2% - spermatozoa with amorphous and double heads, microcephalic spermatozoa and neck defects and in 7.2% - tapered heads, microcephalic and macrocephalic spermatozoa combined with neck defects (Table 1, Fig. 1).

Environmental and lifestyle factors

The results obtained show that about 21% of the participants in the present investigation were undergoing occupational hazards (presence of hazardous chemicals, harmful fumes, Roentgen rays, high temperatures, radiation therapy, etc.), 22% were smokers, 42% alcohol consumers, 13% – drug users, 7% using anabolic steroids, 31% – taking medications and 5% working or living under stress. Some of the men surveyed are influenced by multiple of the mentioned factors.

Table 1. Frequencies of different types of abnormal spermatozoa morphology and combinations of them in men with teratozoospermia: % – percentage of manifestation; (+) – presence. The percentage of men with morphological abnormalities in spermatozoa exceeds 100 due to the manifestation of different defects in the same individual

Types of abnormal spermatozoa morphology								**Frequency**
Amorphous heads	Tapered heads	Microcephalic	Macrocephalic	Double heads	Tail defects	Neck defects	Excess residual cytoplasm	**Total %**
+	+	+			+		+	13.6
	+	+				+		32.8
+		+		+	+			11.2
+			+			+	+	20.8
	+	+	+			+		7.2
+	+					+		27.2
+	+	+	+	+	+		+	14.4
87.2	**95.2**	**79.2**	**42.4**	**25.6**	**39.2**	**88**	**48.8**	**Total %**

Established statistical relationships

Based on the data from the investigation done, the effect of various environmental and lifestyle factors on the spermatozoa morphology was analyzed, and the found relationships between these factors and the spermatozoa morphologic features were characterized.

The results of the study show the presence of statistical significance in the dependences "harmfulness – spermatozoa morphology" ($P<0.017$) and "occupations – spermatozoa morphology" ($P<0.003$). Regarding the relationship between the other studied environmental and lifestyle factors and the available defects in spermatozoa morphology, the statistical data were not sufficiently definite ($P\geq0.1$).

Discussion

The results of the present study demonstrate that teratozoospermia is a heterogeneous condition involving a variety of changes in the morphology of different spermatozoa components.

Chemes & Alvarez Sedo (2012) emphasize the fact that the morphological abnormalities are combined with various functional disorders and genetic abnormalities in spermatozoa, which in a complex worsens male reproductive health. A number of researchers draw attention to the fact that the spermatozoa morphology features are related to the fertility capacity of the individual, and that their assessment has prognostic value in assisted reproduction (Chemes & Rawe, 2003, 2010; Chemes & Alvarez Sedo, 2012).

The results obtained in the present study show an alarming trend among the analyzed men - 16.5% of them have defects in sperm morphology. In parallel, among the analyzed sample of men with reproductive problems a significant part are exposed to various negative environmental and lifestyle influences.

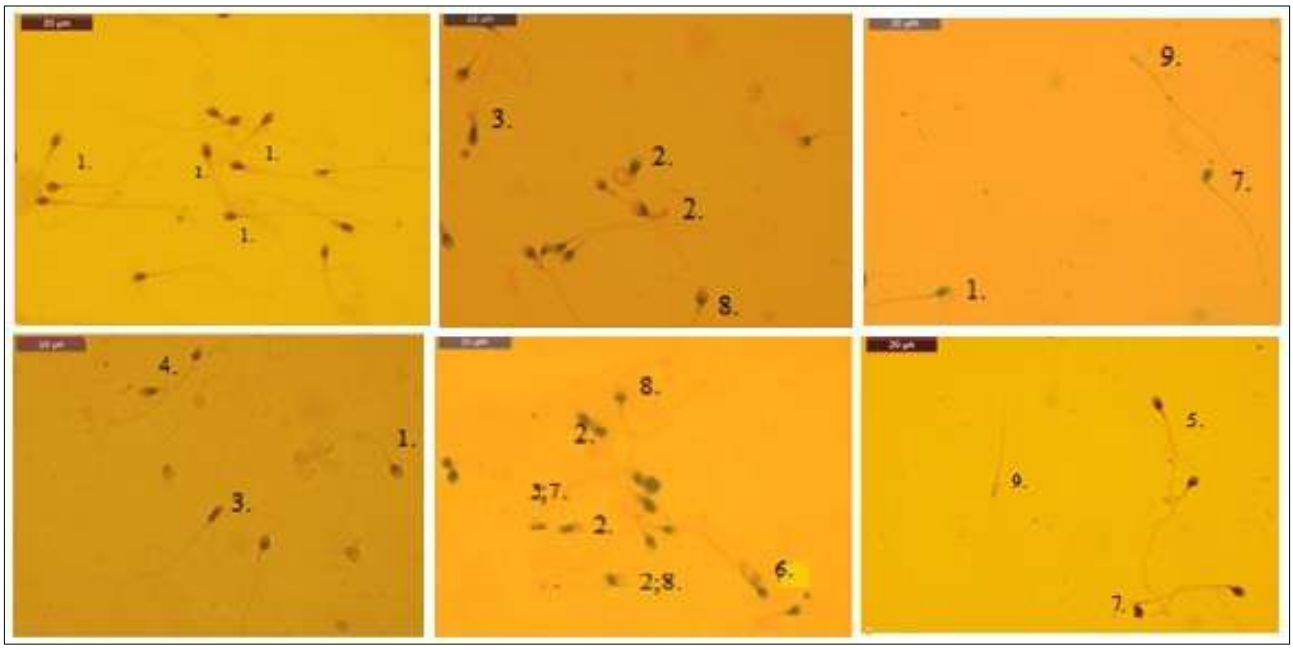

Fig. 1. Types of spermatozoa morphology anomalies: 1. normal morphology; 2. tail defects; 3. tapered heads; 4. amorphous heads; 5. microcephalic spermatozoa; 6. cytoplasmic residue; 7. defects in the neck and middle part; 8. macrocephalic spermatozoa; 9. acephalic sperm.

Statistical analysis on the collected data demonstrates differences in the clarity of the relationship between the established abnormalities of the spermatozoa morphology and the studied environmental and lifestyle factors.

The obtained results and their analysis lead to the conclusion that the harmful environment (presence of hazardous chemicals, harmful fumes, Roentgen rays, high temperatures, radiation therapy, etc.) and various professions (engineers, IT professionals and office workers, drivers, car mechanics and service workers, farmers, builders and carpenters, police officers, firefighters, military and sportsmen, cooks and kitchen workers), including unemployment (probably due to the stress impact), have high risk potential for the quality of sperm morphology.

The analyzes and conclusions of our study are similar to the findings of some other researchers (Hauser et al., 2007; 2015; de Souza et al., 2010; de Freitas et al., 2012), who note that toxic substances, affecting the reproductive processes include heavy metals, agricultural and industrial chemicals, petroleum products, different materials, separated from the light and the heavy

industry, such as dyes, varnishes, acetones, acids, alcohols, sulfur and nitrogen compounds, pesticides and others.

Janevic et al. (2014) and Nordkap et al. (2016) report the negative impact of stress from the work environment and lifestyle on spermatozoa morphology. This suggests that occupations with different harmful effects on sperm parameters are potentially risky for male reproductive health.

Although no statistically significant relationships were observed between spermatozoa morphology and alcohol, cigarette, androgenic anabolic steroid, drug, and medicamentation use in the present study, such dependences with high statistical significance were found in our earlier studies concerning other sperm parameters – ejaculate volume, spermatozoa concentrating and motility (Dzhoglov et al. 2021).

In addition, studies by a number of authors (Wong et al., 2000; Muthusami & Chinnaswamy, 2005; Hamad et al., 2014; Gundersen et al., 2015; Samplaski et al., 2015; Wdowiak et al., 2016; Christou et al., 2017; Jungwirth et al., 2018), prove the negative impact of these factors, both individually and in complex, on the male reproductive potential (including on sperm morphology). An exhaustive overview of the possible relationships between cigarette, alcohol and drug use and male reproductive health is also presented in the work of Sansone et al. (2018).

Knowledge about the spermatozoa morphology is essential in the detection (*in vitro* or *in vivo*) of abnormalities caused by various genotoxic factors. At the same time, the assessment of abnormalities in the spermatozoa head morphology is recommended by regulatory agencies as the main monitoring tool. In this aspect, the present study is pertinent and the described results and analysis could be useful when discussing measures for prevention and improvement of reproductive health in Bulgaria.

Conclusions

Different types of abnormalities in the spermatozoa morphology was found in 16.5% of the examined individuals. Dependencies in pairs "harmfulness – spermatozoa morphology" and "occupations – spermatozoa morphology" were found to be statistical significant which demonstrate that harmful environment and various professions have high risk potential for the quality of sperm morphology. The results obtained and conclusions done could be taken into consideration when discussing activities for prevention and improvement of male reproductive health in Bulgaria.

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Y Chromosome Microdeletions in men with Azoospermia and Oligoasthenoteratozoospermia

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Abstract. The causes of male infertility are different and significantly related to both genetic components and a variety of environmental factors. Approximately 5 to 10% of men with azoospermia or severe oligoasthenoteratozoospermia have microdeletions in the area of the azoospermic factor AZF - Yq11, which contains genes that control the processes of spermatogenesis. In the present study totally 48 men with azoospermia and oligoastenoteratozoospermia were included in the Y-microdeletion analysis by real time PCR. The set of primers used to detect microdeletions on the Y chromosome included: SRY (sY14) and ZFX / Y (short arm), sY84 and sY86 (for AZFa); sY127 and sY134 (for AZFb); sY254 and sY255 (for AZFc). No microdeletions in the Y chromosome were found in 29.2% of the men studied. Among all others, single microdeletion was found in the AZFa subregion of Yq (2.1%) and in the SRY zone of Yp (22.9%). For all other men included in the study, different combinations of microdeletions were found in two (AZFb + AZFc, AZFa + SRY, AZFb + SRY, AZFc + SRY and SRY + ZFX / Y), three (AZFa + AZFc + SRY, AZFb + AZFc + SRY and AZFb + SRY + ZFX / Y), four (AZFa + AZFb + AZFc + SRY, AZFa + AZFb + SRY + ZFX / Y, AZFa + AZFc + SRY + ZFX / Y and AZFb + AZFc + SRY + ZFX / Y) or five (AZFa + AZFb + AZFc + SRY + ZFX / Y) sections / subsections simultaneously. The obtained results support the opinion that interactions between different deletions in the AZF region of the Y-chromosome with other genes or gene groups are possible, which is a significant factor influencing spermatogenesis.

Key words: male infertility, Y chromosome, microdeletions, semen quality, reproductive health.

Introduction

Infertility is a complex medical problem affecting about 15% of couples in reproductive age worldwide. Approximately half of these cases are related to the male factor. Despite many studies in this field, the origin of infertility remains unknown in about 30% of individuals suffering from it (Poongothai et al., 2009). The causes of male infertility are different and significantly related to both genetic and environmental factors (Matzuk & Lamb, 2008). Changes in chromosome structure (e.g. Robertsonian translocation, originating from chromosomes 13 and 14; reciprocal translocations, etc.), in the number of sex chromosomes (such as Klinefelter syndrome - 47, XXY and 46, XX male syndrome) and autosomes, as well as gene mutations have been discussed as reasons for diminished reproductive potential in men (Poongothai et al., 2009). Such mutations typically affect genes responsible for the spermatogenesis course or associated with the differentiation of the male reproductive system (Matzuk & Lamb, 2008). Microdeletions in the Y chromosome associated with manifestation of azoospermia have been studied by a number of authors (Sadeghi-Nejad & Farrokhip, 2007; O'Flynn O'Brien et al., 2010). According to Ferlin et al. (2003; 2007) and Fernandes et al. (2006), they currently are one of the most common identified causes for severe spermatogenic damage. The Y chromosome contains genes associated with sex determination and differentiation. It has been established that the SRY region of the short arm of the Y chromosome is responsible for the maturation of Sertol cells and the normal course of spermatogenesis. The genetic changes in the SRY region are due to molecular genetic mechanisms associated with abnormalities in the testicular formation (Schumacher et al., 2008; Bradbury, 2017. This region is related

to the sex determination in the fetus, to the androgens' secretion and the activation of their receptors (Toncheva, 2010). In the short arm of the Y chromosome, there is also a ZFY sex-determining region homologous to that on the X chromosome (ZFX / Y), which encodes a zinc-finger protein (Page, 1988; Bradbury, 2017). In the AZF (azoospermia factor) region of the long arm of the Y chromosome, more than 20 genes involved in spermatogenesis have been identified (Navarro-Costa et al., 2010a, b). The small chromosomal deletions, spanning several neighboring genes, due to their size, cannot be detected by conventional chromosome analysis. Their frequency in men with azoospermia or severe oligozoospermia is high. Microdeletions in the long arm of the Y chromosome are specifically associated with abnormalities in spermatogenesis. Approximately 5 to 10% of men with azoospermia or severe oligozoospermia have deletions in the Yq11 AZF area, which contains genes that control the processes of spermatogenesis. Three different AZF areas, designated as "a", "b" and "c", are located in this region. Disorders of spermatogenesis in patients with deletions in the a, b, or c regions of AZF have been found to have different specificity and expression, suggesting that interactions between various deletions in AZF region and other genes (or gene groups) are possible, which also affects spermatogenesis (Navarro-Costa, 2010a, b). From a biomedical point of view, in Bulgaria this problem is insufficiently studied. Linev et al. (2017), Mitkovska et al. (2019) and Ivanova et al. (2021) emphasize the fact that different types of aberrations in gonosomes and autosomes are often in the root of reproductive problems in men, which determines the necessity for complex cytogenetic and molecular genetic approaches in the study of male reproductive health. One of the most commonly identified molecular genetic causes for male infertility are submicroscopic deletions in the long arm of the Y chromosome, and establishing the type and specificity of their manifestation are important to clarify the prospects for patients with azoospermia and oligozoospermia (Linev et al., 2017).

The aim of the present study was to analyze the specificity and frequency of occurrence of Y chromosome microdeletions in men with azoospermia and oligoasthenoteratozoospermia and to characterize the possible relations between the abnormalities found.

Material and Methods

This study was approved by the Institutional Ethical Committee with Certificate N 2 / 16.01.2019. Totally 48 men with azoospermia and oligoastenoteratozoospermia were included in the Y-microdeletion analysis. All study participants signed an informed consent form and voluntarily answered questions included in the survey, providing data on their medical history. Genomic DNA isolated from frozen (-20 ° C) semen was used for PCR analysis to identify deletions in the Y chromosome. Semen material from fertile men was used as a positive control. A complex of all components necessary for the reaction with the exception of the template DNA replaced with ddH2O, as well as a DNA sample from a female individual were used as negative controls. For detection of microdeletions in certain regions of the Y chromosome, Real time PCR was applied. The set of primers used to diagnose microdeletions on the Y chromosome includes: SRY (sY14) and ZFX / Y (short arm), sY84 and sY86 (for AZFa); sY127 and sY134 (for AZFb); sY254 and sY255 (for AZFc) – Table 1.

The lack of hybridization between the primers and their complementary regions of the template single-stranded DNA was graphically reported on the monitor and accepted as an evidence of a microdeletion in the relevant region of the Y chromosome.

Statistical analyzes were performed with the software package SPSS, version 22. Descriptive statistics were used to characterize the frequency of the compared groups. The differences (dependencies) between the compared groups were analyzed by the Pearson Chi-Square test ($\chi 2$) and the t-test. Statistical significance was defined as $P < 0.05$ for the different analysis schemes.

Table 1. Sequences of the primers used: F – forward; R – reverse.

Primers	Sequences
ZFX/Y – F	5′ – ACC R* CT GTA CTA CTG ACT GTG ATT ACA C – 3′
ZFX/Y – R	5′ – GCA C Y* T CTT TGG TAT C Y* G AGA AAG T – 3′
SRY – F	5′ – GAA TAT TCC CGC TCT CCG GA – 3′
SRY – R	5′ – GCT GGT GCT TTC TTG AG – 3′
sY86 – F	5′ – GTG ACA CAC AGA CTA TGC TTC – 3′
sY86 – R	5 ′ – ACA CAC AGA GGG ACA ACC CT – 3′
sY 127 – F	5′ – GGC TCA CAA ACG AAA AGA AA – 3′
sY 127 – R	5′ – CTG CAG GCA GTA ATA AGG GA – 3′
sY 254 – F	5′ – GGG TGT TAC CAG AAG GCA AA – 3′
sY 84 – F	5′ – AGA AGG GTC TGA AAG CAG GT – 3′
sY84 – R	5′ – GCC TAC TAC CTG GAG GCT TC – 3
sY134 – F	5′ – GTC TGC CTC ACC ATA AAA CG – 3′
sY134 – R	5′ – ACC ACT GCC AAA ACT TTC AA – 3′
sY255 – F	5′ – GTT ACA GGA TTC GGC GTG AT – 3′
sY255 – R	5′ – CTC GTC ATG TGC AGC CAC – 3′

Results

The results of the current study show the presence of microdeletions both – on the long and on the short arm of the Y chromosome. The data concerning microdeletions' frequencies calculated for the different subareas of the AZF region as well as concerning the SRY and ZFX / Y regions are presented in Table 2. Statistical information was presented in Table 3.

Table 2. Frequency of established microdeletions in the Y chromosome by regions in its short and long arm and combinations of them in the analyzed men with azoospermia and oligoasthenoteratozoospermia.

Microdeletions by regions and combinations of them	Number of individuals	Valid %
Absence	14	29.2
AZFa	1	2.1
AZFb+AZFc	1	2.1
SRY	11	22.9
AZFa+SRY	1	2.1
AZFb+SRY	1	2.1
AZFc + SRY	1	2.1
AZFa+AZFc+SRY	1	2.1
AZFb+AZFc+SRY	4	8.3
AZFa+AZFb+AZFc+SRY	2	4.2
SRY+ZFX/Y	2	4.2
AZFb+SRY+ZFX/Y	1	2.1
AZFa+AZFb+SRY+ZFX/Y	1	2.1
AZFa+AZFc+SRY+ZFX/Y	1	2.1
AZFb+AZFc+SRY+ZFX/Y	4	8.3
AZFa+AZFb+AZFc+SRY+ZFX/Y	3	6.3
Total	48	100.0

Table 3. Data concerning the relations in the groups compared.

Crosstab		AZFa		Total	Sig.
		none	yes		
SRY	none	15	1	16	0.07
	yes	23	9	32	
Total		38	10	48	
Crosstab		AZFb		Total	Sig.
		none	yes		
SRY	none	15	1	16	0.002
	yes	16	16	32	
Total		31	17	48	
Crosstab		AZFc		Total	Sig.
		none	yes		
SRY	none	15	1	16	
	yes	17	15	32	0.004
Total		32	16	48	
Crosstab		ZFX/Y		Total	Sig.
		none	yes		
SRY	none	16	0	16	
	yes	20	12	32	0.003
Total		36	12	48	

The data presented in Table 2 show that microdeletions in the Y chromosome were not found only in 29.2% of the studied men in this group. Single microdeletions were established in the AZFa subregion of Yq (2.1%) and in the SRY region of Yp (22.9%). For all other men included in this study, different combinations of microdeletions were detected in two (AZFb + AZFc, AZFa + SRY, AZFb + SRY, AZFc + SRY and SRY + ZFX / Y), three AZFc + SRY, AZFb + AZFc + SRY and AZFb + SRY + ZFX / Y), four (AZFa + AZFb + AZFc + SRY, AZFa + AZFb + SRY + ZFX / Y, AZFa + AZFc + SRY + ZFX / Y and AZF + AZFc + SRY + ZFX / Y) or five (AZFa + AZFb + AZFc + SRY + ZFX / Y) regions / subregions simultaneously (Table 2). The anomalies in the SRY region (alone and in combination) were a total of 66.7% in the examined men with azoospermia and oligoasthenoteratozoospermia.

Discussion

Submicroscopic deletions in the AZF zone of the long arm of the Y chromosome are one of the most common molecular genetic causes for male infertility (Harton & Tempest, 2012). It has been found that most of these microaberrations occur over again, as a result of interchromosomal homologous recombination within the region (Navarro-Costa, 2010a; b). They damage one or more of the genes and this results in different defects in spermatogenesis.

Genes in the AZF region have been suggested to have a regulatory function in the germ cell cycle and during meiosis. The diverse clinical manifestations in gametogenesis disorders are results of this complex regulation (Harton & Tempest, 2012).

Differences in the registered frequencies have been found when analyzing the literature data on microdeletions in the AZF subregions. Microdeletions in the Y chromosome were found in 12% of 100 men with azoospermia according to the study of Mirfakhraie et al. (2010). The authors found that 66.7% of these microdeletions were in the AZFb, 41.7% - in AZFc, 33.3% - in AZFd - and 8.3% - in AZFa region. Linev et al. (2017) reported the presence of microdeletions on the Y chromosome in approximately 22% of the 73 men studied with abnormalities in the quantitative and qualitative sperm parameters. The main percentage of microdeletions they found was in the AZFc region.

In the course of the present study, a high total percentage of microdeletions in the Y chromosome was found (Table 2), which is due to the fact that the analysis included patients with established azoospermia and oligoastenoteratozoospermia.

The results found in our study show that the highest percentage of microdeletions is in the AZFb subregion, but it is significantly lower than indicated by Mirfakhraie et al. (2003).

According to Navarro-Costa (2010a, b): deletions in the AZFa region are most often associated with azoospermia represented by Sertoli cell only syndrome (SCOS) and less frequently – with oligospermia; deletions in the AZFb region are associated with blocking spermatogenesis on spermatocyte level (Spc), and deletions in the AZFc region – with SCOS and fewer number of spermatogonia (Spg).

In the present study, the statistically significant dependences between the presence of microdeletions in the SRY region from Yp and the microdeletions in the other studied regions of the Y chromosome were analyzed (Table 3). The statistic data show that microstructural anomalies in the SRY region are statistically significantly associated with microdeletions in the ZFX / Y region of Yp and in the AZFa, AZFb and AZFc subregions of Yq (significance by $\chi 2$ test: 0.005; 0.079; 0.003; 0.005, respectively by Fisher's exact test: 0.003; 0.078; 0.002 and 0.004, respectively).

Conclusions

The obtained results support the opinion that interactions between different deletions in the AZF region of the Y-chromosome with other genes or gene groups are possible, which is a significant factor influencing spermatogenesis.

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Study on Diversity in Some Human Phenotypic Characteristics

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Abstract. The present study aimed to analyze the human phenotypic manifestation of AB0 and Rh blood groups, descriptive features cleft in chin, freckles, hairline, eyebrow shape, dimples, earlobes and the basic characteristics of personality extraversion, agreeableness, consciousness, emotional stability and intellect/imagination and the possible relations between them. About 950 individuals were included in the study. The frequencies of the investigated phenotypic groups were calculated by usage of descriptive statistics. The results obtained were analyzed by SPSS software. Statistically significant relationships were found between some of the studied human immunological, descriptive and behavior features as follows: lower levels of emotional stability in people with Rh+ blood groups ($P<0.05$); higher levels of agreeableness and consciousness in people without cleft in chin ($P=0.003$, $P=0.001$) and in persons with separeted eyebrows ($P=0.03$, $P=0.008$); higher levels of emotional stability in people with straight line of hair ($P<0.05$); higher levels of consciousness in people without dimples and higher levels of intellect/imagination in people with dimples ($P<0.05$); higher levels of agreeableness and intellect/imagination in people with a free ear lobe ($P=0.004$, $P=0.02$). No statistically significant differences were detected between blood groups of the AB0 system and the presence of freckles on the face from one side and the mean values of the personality characteristics by the other hand. The present study reveals interesting relationships between various human traits based on a complex approach. It could be used as an appropriate model for other future studies of human phenotypic diversity.

Key words: phenotypic variability, blood groups AB0 and Rh, human descriptive traits, human personality traits.

Introduction

Human genetics studies the heredity and variability which are characteristic of *Homo sapiens*. The subject of studies is a variety of human traits that are under the control of individual genes or are the result of complex interactions of genotype and environment (Mitkovska et al., 2019; Dzhoglov et al., 2021; Ivanova et al., 2021). The attention of scientists is focused mainly on the so-called pathological heredity and the studies dedicated to the inheritance of normal traits in human populations are less (Alexandrov, 2010; Ivanova et al., 2001; 2011). This interest can be explained by the growing role of medical genetics and its importance to the well-being of humanity. The human phenotypic diversity is the subject of studies for human population and formal genetics. The relationships between different, mainly monogenic, traits, and the more complicated phenotypic characteristics of the human personality are of particular interest to the researchers of the human behavior. This is the reason why the relations between well-studied components of the genetic constitution and significant phenotypic behavioral features are increasingly at the heart of interdisciplinary research (Borinskaya & Rogaev, 2000; Hill et al., 2002; Baker, 2004; Luchinin, 2005; Malykh et al., 2008; Alexandrov, 2010, etc.). From the psychology point of view, the basic characteristics of the personality, the approaches for their characterization and their frequency of manifestation in different human populations have been studied by a number of authors (McCrae & John, 1992; Pervin & John, 2001; Goldberg, 1990; 1992; 2001; Costa & McCrae, 1992a; b; 2008; Goldberg et al., 2006; Alexandrova-Karamanova, 2016; Magyar et al., 2017). Although there are data in the literature on some studies on candidate genes or descriptive characteristics in humans that could be associated with individual behavioral characteristics (Reedy et al., 1971; Wiedemann, 1990; Reiss, 1999; Bastiaens et al., 2001; Thibaut et al., 2005; Medland et al., 2009; Adhikari et al., 2016; Zaidi et al., 2018), in Bulgaria similar interdisciplinary research has hardly been done

(Ivanova et al., 2018a; b). This fact motivates the purpose of the present study: 1) to characterize the found phenotypic diversity in the group of participants in terms of some immunological, descriptive and behavior traits and 2) to look at possible relationships between them.

Material and Methods

Totally 945 individuals were studied in order to characterize the phenotypic diversity in the frequency of the human AB0 and Rh blood groups, some descriptive features and the basic characteristics of personality, as well as the possible relations between them. The persons included in the study (70.9% women and 29.1% men) were between 16 and 90 years old with average age 32.3 years. AB0 and Rh blood groups were reported by the participants themselves after having conclusions from a clinical laboratory. Using a self-reported questionnaire, data were collected about the following descriptive features: cleft in chin; freckles; hairline; eyebrow shape; dimples and earlobes. The basic characteristics of personality - extraversion, agreeableness, conscientiousness, emotional stability and intellect/imagination - were studied through the Goldberg's "Big-Five factor markers, International Personality Item Pool - IPIP" questionnaire (Goldberg, 2001, http://ipip.ori.org/), adapted for the Bulgarian culture (Alexandrova-Karamanova, 2016).

Data were analyzed through the IBM SPSS Statistics software package, version 22.0. Descriptive statistics analyses (frequencies, crosstabs) and the independent samples t-test were used.

Results

Concerning the AB0 blood groups' system, the results of our study showed that: 30.5% of the participants were with 0; 37% - with A; 18.7% - with B and 13.8% - with AB blood groups.

As for the Rh blood groups, the data show that 79.6% of the persons included in the study have Rh+ and 20.4% - Rh- factor (Fig. 1).

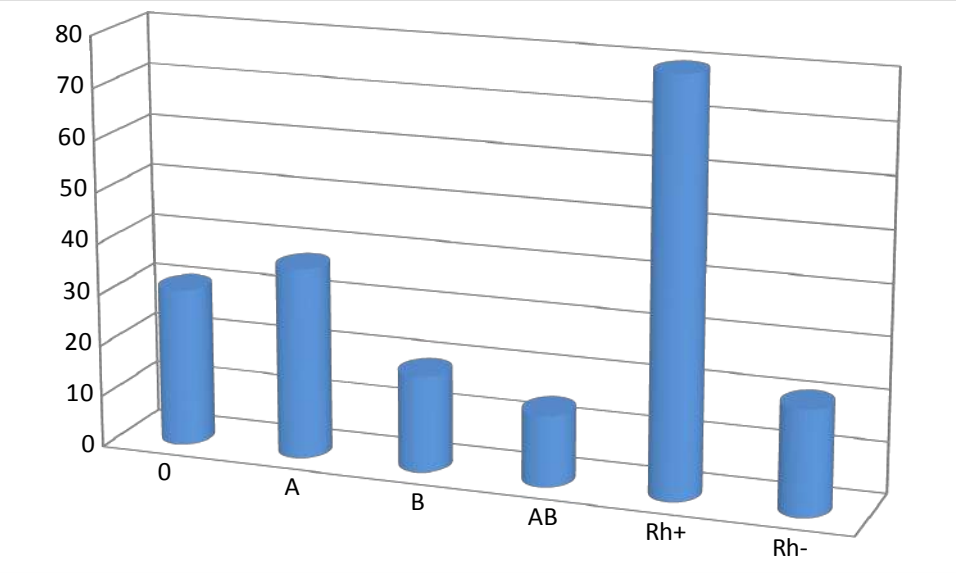

Fig. 1. Distribution of the AB0 and Rh blood groups within the studied individuals.

The data about the studied descriptive characteristics (cleft in chin, freckles, hairline, eyebrow shape, dimples, earlobes) and their frequencies in % is presented in Fig. 2. As could be seen from

the figure, the absence of cleft in chin (82.2%), freckles (85%), widow peak (64%) and dimples (64.1%), as well as the presence of separated eyebrows (88.9%) and free earlobes (71.8%) were with higher frequencies among the compared features pairs.

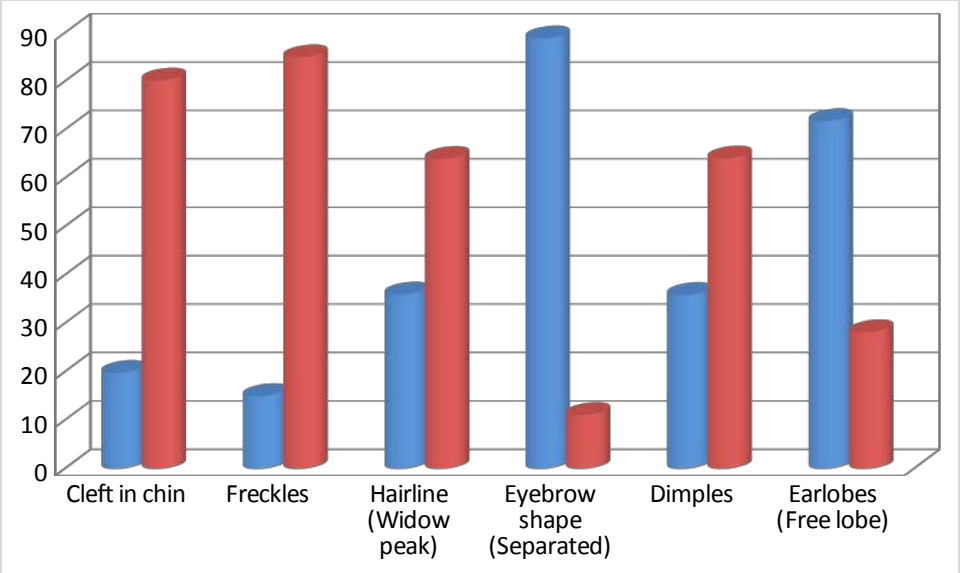

Fig. 2. Distribution (in %) of the studied descriptive characteristics cleft in chin, freckles, hairline, eyebrow shape, dimples, earlobes.

The Big-five personality traits' scales range from a minimum of 10 to a maximum 50 (Table 1). The mean values (M) found in our study concerning the extraversion, agreeableness, conscientiousness, emotional stability and intellect/imagination were as follows: 32.98 (SD=7.91); 40.25 (SD=6.35); 37.99 (SD=7.11); 29.15 (SD=9.17) and 37.58 (SD=6.35), respectively. According to the data obtained, agreeableness was most and emotional stability - lest pronounced (M=29.15) - Table 1.

Table 1. Number of individuals (N), minimum, maximum and mean values found concerning the studied personality traits.

		Extraversion	Agreeable-ness	Conscientio-usness	Emotional stability	Intellect/ Imagination
N	Valid	915	918	914	910	909
	Missing	30	27	31	35	36
Mean		32.98	40.25	37.99	29.15	37.58
Median		33.00	41.00	39.00	29.00	38.00
Mode		32	39	39	31	35
Std. Deviation		7.906	6.351	7.108	9.166	6.353
Minimum		10	11	15	10	10
Maximum		50	50	50	50	50

Also, individual results were classified into five groups (Fig. 3): individuals with low, somewhat low, average, somewhat high, and high expression of the respective personality trait. As could be seen from the figure, 45.2% of the participants in the study were agreeable (or somewhat agreeable); 64.1%

were conscientious (or somewhat conscious); 44.3% had high or somewhat high intellect/imagination; 21.4% were (somewhat) extraverted and 33.0% were (somewhat) emotionally stable (Fig. 3).

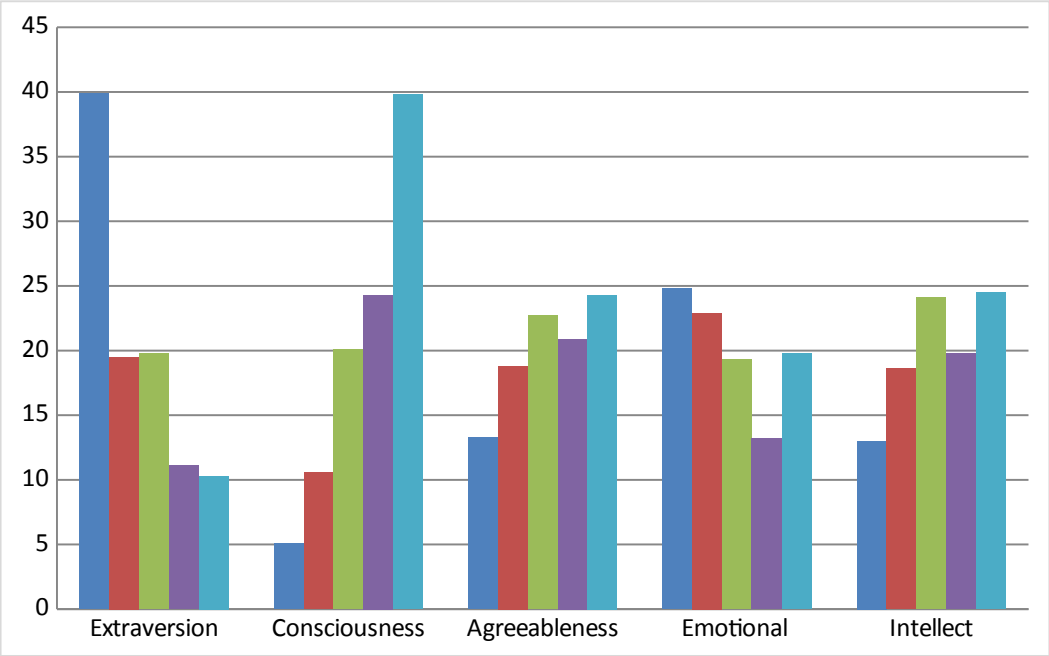

Fig. 3. Level of expression (% - valid percent) of the personality factors in the studied sample.

Statistically significant variations in the mean values of the personality factors studied were observed in relation to six of the studied traits, as follows: Rh factor - emotional stability; cleft in chin – agreeableness and consciousness; hairline - emotional stability; eyebrow shape - agreeableness and consciousness; dimples - consciousness and intellect/imagination; earlobes – agreeableness and intellect/imagination (Table 2).

Discussion

Phenotypic variations that occur in humans are a result of the gene expression, the complicated interactions between different genes and interactions between the genotype and environment.

The complexity of the relationships between many factors that have significant value for the expression of a particular phenotypic spectrum of immunological, descriptive and behavioral characteristics in humans is obvious. Investigations of their nature and mechanisms for their implementation would be useful in studying the phenotypic diversity in human populations and in the characterizing their anthropological and genetic profile.

From a clinical perspective, the AB0 is the most important blood groups antigen system. The results obtained in our study support and complement the pre-existing knowledge about the distribution of AB0 blood system among the Bulgarian population (Ivanova et al., 2001; Popov et al. 2012). Concerning the relationships with the studied personality factors, there were not found statistically significant relations in the present study between their mean values and the blood groups 0, A, B or AB. In contrast, data of our study showed lower levels of emotional stability in people with a positive Rh factor ($P<0.048$) – Fig. 1, Table 2.

The frequency of manifestation of the descriptive characteristics gives the information about the spectrum of the phenotypic diversity among the studied individuals. The available data show that most of the participants do not have as distinctive features cleft in chin, freckles, widow peak,

dimples, joined eyebrows and attached ear lobe (Fig. 2).

Table 2. Statistical associations between the human AB0 and Rh blood groups, the studied descriptive characteristics, and the Big-five personality factors.

Features studied	Personality trait	Type of manifestation	N	Mean	t (P)
Rh blood group	Emotional stability	Rh+	434	28.77	-1.983 (0.048)
		Rh-	110	30.65	
Cleft in chin	Agreeableness	Presence	174	38.98	-2.968 (0.003)
		Absence	709	40.58	
	Consciousness	Presence	176	36.49	-3.306 (0.001)
		Absence	704	38.34	
Hairline (Widow peak)	Emotional stability	Presence	316	28.25	-1.977 (0.048)
		Absence	566	29.51	
Eyebrow shape	Agreeableness	Separated	802	40.40	2.121 (0.034)
		Joined	99	38.96	
	Consciousness	Separated	795	38.16	2.657 (0.008)
		Joined	102	36.18	
Dimples	Consciousness	Presence	326	37.37	-1.991 (0.047)
		Absence	579	38.35	
	Intellect/ Imagination	Presence	321	38.18	2.044 (0.041)
		Absence	582	37.28	
Earlobes (Free lobe)	Agreeableness	Presence	641	40.61	2.887 (0.004)
		Absence	249	39.25	
	Intellect/ Imagination	Presence	643	37.86	2.312 (0.021)
		Absence	242	36.76	

The results obtained on the five basic personality traits among the studies persons show higher levels of agreeableness, conscientiousness and intellect/imagination and lower levels of extraversion and emotional stability. In our sample, the minimum values of agreeableness, conscientiousness and intellect/imagination are quite high. Possible explanation for this is that these are socially desirable characteristics, which leads to giving socially desirable answers. Based on the individuals results calculated, in the scales of most of the personality traits there is an asymmetry – higher agreeableness, higher conscientiousness, higher intellect/imagination, and lower extraversion are observed in the sample (Fig. 3.). In emotional stability factor, the high and low values are approximately equally distributed and the mean value is the lowest.

Statistically significant relations were found between the mean values of four of the studied personality factors and five of the studied descriptive features. The established dependencies lead to the following conclusions: 1) there are lower levels of agreeableness and consciousness in individuals with cleft in chin; 2) there are lower levels of emotional stability in individuals with widow peak; 3) there are higher levels of agreeableness and consciousness in people with separated eyebrow; 4) there are lower levels of consciousness and higher levels of intellect/imagination in people with dimples; 5) there are higher levels of agreeableness and intellect/imagination in persons with free earlobes (with a pendant of the earlobe).

Conclusions

The results of the study provide information on phenotypic diversity concerning some immunological, descriptive and personality characteristics in humans, as well as in the established relations between them. Among the studied sample of 945 individuals, the frequencies of the pairs of traits were established and analyzed together with the found frequencies of the five basic personality characteristics. Future investigations in this field would be useful in studying the phenotypic diversity in different human populations and in the characterizing their anthropogenetic profile.

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Assessment of Genetic Diversity of White Lupin (Lupinus albus L.) Accessions Based on Agro-morphological Traits

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Abstract. Large genetic diversity exists in *Lupinus albus* L. expressed by its morphological and agronomical traits. The aim of this study was to assess the genetic diversity of ten white lupin accessions regarding components of productivity and biological traits. Two years trial was carried out on the experimental field of Institute of plant genetic resources during the period 2018-2019. The traits were evaluated using the International *Lupinus albus* L. descriptor. The degree of earliness was also used. Based on agro-morphological traits the studied accessions were grouped into two clusters. The first cluster included only one accession. The second cluster was divided into two sub-clusters. The first sub-cluster included three genotypes which differed significantly from the remaining ones by its tall plants, high first formed pods and a big number of grains per plant. The second sub-cluster included six accessions possessing medium-high to short stem and medium-large to large grains. The studied traits were combined into two main clusters. The largest Euclidean distance had between vegetation period and other studied traits. According to the degree of earliness, the lupin genotypes were clustered into three groups - ultra-early, early and late. The BGR 3086 accession was selected also as an ultra-early variety with 1.00 coefficient of earliness together with two other genotypes - selected to the early group with a coefficient of earliness 1.60. Genetically distant genotypes will be involved in the breeding programs for effective combining the important features into a new genotype.

Key words: white lupin, genetic diversity, earliness, productivity.

Introduction

Lupinus is a relatively large genus and one of the most geographically widespread with a rich diversity of species. *Lupin* is an annual or perennial legume belonging to legume family, *Fabaceae*, one of the oldest crops. It is a major food legume in the Roman Empire and has a long history of cultivation in the Mediterranean basin, East Africa and the Atlantic islands of the northern hemisphere (Gladstones, 1998; Kurlovich, 2002). Among 300 *Lupinus* have been described, only five species are cultivated among which white lupin is the most important one (Hondelmann, 1984). It is probably domesticated in the Aegean region (Gladstones, 1998) and has increased in the recent years due to its high level of protein, oil and quality dietary fiber in seed's dry matter (Annicchiarico, 2008; Bhardwaj & Hamama, 2012). In our country lupin is not a traditional crop, but it has been used for a long time as a green manure source (Angelova, 2001).

The conservation and preservation of the white lupin genetic resources are of crucial importance for the breeding programs aiming the cultivar improvement (Raza & Jørnsgård, 2005; El-Sherif et al., 2014). In this respect, the success of any crop improvement programme essentially depends on the nature and magnitude of genetic variability (Hukumch & Parameshwarappa, 2019). Genetic divergence among parents is essential since the crossing programme involving genetically diverse parents is likely to produce high heterotic effects, successful combination of valuable genes and also more variability could be expected in the segregating generations (Sachan & Sharma, 1971; Kosev & Vasileva, 2020).

The aim of this study was to assess the genetic diversity of ten white lupin accessions regarding components of productivity and biological traits.

Material and Methods

The experiment was carried out on the experimental field in IPGR - Sadovo on cinnamon forest soil, after a precursor of wheat during the period 2018-2019. The area of Sadovo is characterized by a transitional continental climate with its frequent and prolonged droughts. During the period 2018-2019 mean temperature was 18.17ºC and mean precipitation was 73.84 mm. Ten white lupin genotypes were used for this study.

The field experiment was designed in Randomized Complete Block Design (RCBD) with three replications (Dimova & Marinkov, 1999). Experiment plot size was 5.0 m^2. Sowing was made by hand, in optimum sowing time, according to the technology of cultivation. At maturity, 10 plants were randomly taken from each plot to measure the following morphological traits: plant height (cm), height to the first pod (cm), number of productive branches, number of pods per plant, number of grains per plant, number of grains per pod, mass of grains per plant (g) and mass of 100 grains (g). Also several phenological traits were taken into account: beginning of flowering and plant life cycle. The phenological traits were assessment by coefficient of earliness (Kuzmova, 2002). For ultra early varieties the value of this coefficient was from 1.00 to 1.17, for the early varieties from 1.17 to 1.33, for middle-early ones from 1.34 to 1.66 and for the late varieties was greater than 1.66.

The studied agro-morphological traits were processed mathematically by analysis of variance. The means were compared by the three Least Significance Difference (LSD) - 0.05%; 0.01% and 0.001%. To investigate the hierarchical structure of genetic diversity between the studied lupin accessions was used Cluster analysis (Ward, 1963). All experimental data were processed statistically with using statistical package SPSS 19.0. for Windows (IBM SPSS Statistics 19 Product Version: 19.0.0) (IBM, 2019).

Results and Discussion

The clustering of the ten evaluated lupin accessions based on their morphological traits is presenting on Fig. 1. The traits data are given on Table 1. The accessions were divided into two main groups (clusters) giving an idea about quantitative traits diversity. The main trait that distinguished the accessions was the plant hight. Only the accession BGR 3080 was included in the first cluster. This genotype had large grains (40.80 g), big mass of grains per plant (18.91 g) and big number of grains per pod (4.90).

The second cluster was divided into two sub-clusters. The first sub-cluster included three genotypes (BGR 3086, BGR 6341 and BGR 3085), with tall plants, big number of productive branches and big mass of grains per plant. The BGR 3086 genotype differed significantly (LSD=0.05%) by plant height (68.43 cm). This accession also had a high number of grains per plant (52.86) and the shortest vegetation period (90 days). The accession BGR 6341 had significant differences (LSD=0.05%) by plant height (67.43 cm), number of productive branches (4.43), number of grains per plant (64.43) and mass of grains per plant (22.20 g). This accession characterized also by high values of number of pods per plant (15.14) and number of grains per pod (5.03). The BGR 3085 accession had significant differences (LSD=0.05%) by mass of grains per plant (22.14 g). The traits related to the number of pod and grains per plant, mass of the grains per plant are from significant economic importance for the leguminous forage crops, and this defines the genotype as a potential initial material for selection.

The second sub-cluster included six accessions (BGR 3078, BGR 6337, BGR 6339, BGR 3083, BGR 3084, BGR 3079) possessing medium-high to short stem and medium-large to large grains. The data showed that within this sub-cluster significant genetic distance was observed between the genotypes. The BGR 3078 genotype differed significantly (LSD=0.001%) by the height to the first pod (38.00 cm) and had tall plants (66.43 cm). The lowest formed first pod was observed in BGR 6337 accession at LSD=0.01%.

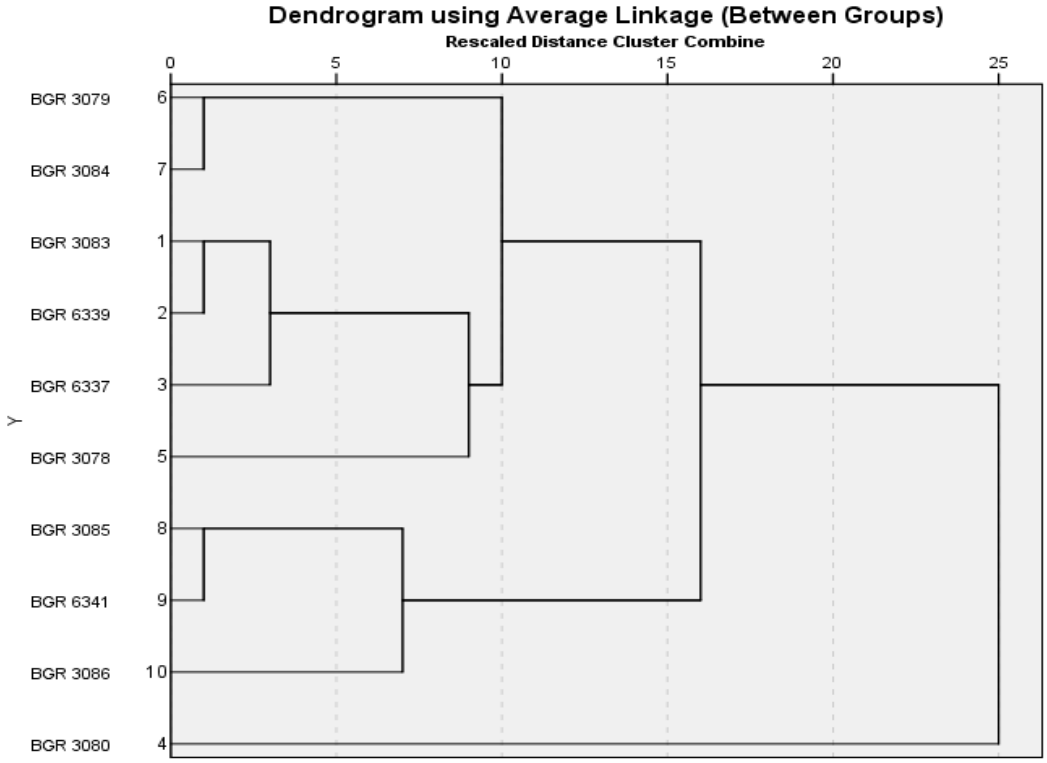

Fig. 1. Dendrogram of genotype grouping using cluster analysis during the period 2018-2019.

Table 1. Experimental data and grouping of the studied genotypes using cluster analysis during the period 2018-2019.

Accessions	Plant height, cm	Height to the first pod, cm	Number of productive branches	Number of pods per plant	Number of grains per plant	Numbe of grains per pod	Mass of grains per plant, g	100 grains mass, g
BGR 3083	53.29	28.00	2.14*	8.71	35.00	4.49	13.23	37.80
BGR 6339	51.5*	23.43*	3.14	10.14	40.29	4.77	14.84	37.05
BGR 6337	55.43	22.57**	3.57	11.57	45.43	4.80	14.43	32.45
BGR 3080	63.86	28.14	3.14	12.14	47.43	4.90	18.91	40.80
BGR 3078	66.43	38.00***	3.43	11.14	36.87	4.60	14.74	34.40
BGR 3079	56.57	30.86	2.14*	6.86	24.00*	3.91*	9.73	40.30
BGR 3084	54.71	32.57	2.71	6.14*	22.00*	3.84**	7.99*	35.30
BGR 3085	61.86	26.57	4.00	14.43	60.14	4.63	22.14*	37.75
BGR 6341	67.43*	28.14	4.43*	15.14	64.43*	5.03	22.20*	36.75
BGR 3086	68.43*	33.71	3.71	12.14	52.86	4.97	17.20	33.10
average St	59.96	29.20	3.24	10.84	42.84	4.59	15.54	36.57
min	51.57	22.57	2.14	6.14	22.00	3.84	7.99	32.45
max	68.43	38.00	4.43	15.14	64.43	5.03	22.20	40.80
LSD 0.05% *	**7.56**	**5.38**	**1.18**	**4.79**	**19.36**	**0.56**	**6.85**	**1.28**
LSD 0.01% **	**10.03**	**7.13**	**1.56**	**6.35**	**25.69**	**0.75**	**9.09**	**1.75**
LSD 0.001% ***	**12.99**	**9.24**	**2.02**	**8.23**	**33.28**	**0.97**	**11.77**	**2.35**

The clustering of the agro-morphological traits of the evaluated lupin accessions based on their similarity/differences is presented on Fig. 2. From the clustering of the traits we could concluded that they were combined into two main clusters. Only vegetation period was included in the first cluster. The second cluster was divided into two sub-clusters: the first sub-cluster included the following traits: plant height, number of grains per plant, germination-beginning of flowering, mass of 100 grains and height to the first pod united by the close value of the Euclidean distance. The remaining four traits (mass of grains per plant, number of pods per plant, number of grains per pod and number of productive branches) were combined into the second sub-cluster. The largest Euclidean distance had between traits - number of productive branches and vegetation period (281.98), vegetation period and number of grains per pod (277.76), vegetation period and number of pods per plant (257.68). Similar clustering of the traits were conducted with other grain legumes, as fababean where the traits plant height, vegetation period and mass of 100 grains were combined into one cluster, at a large distance in the factorial plane from other cluster (Velcheva & Petrova, 2020).

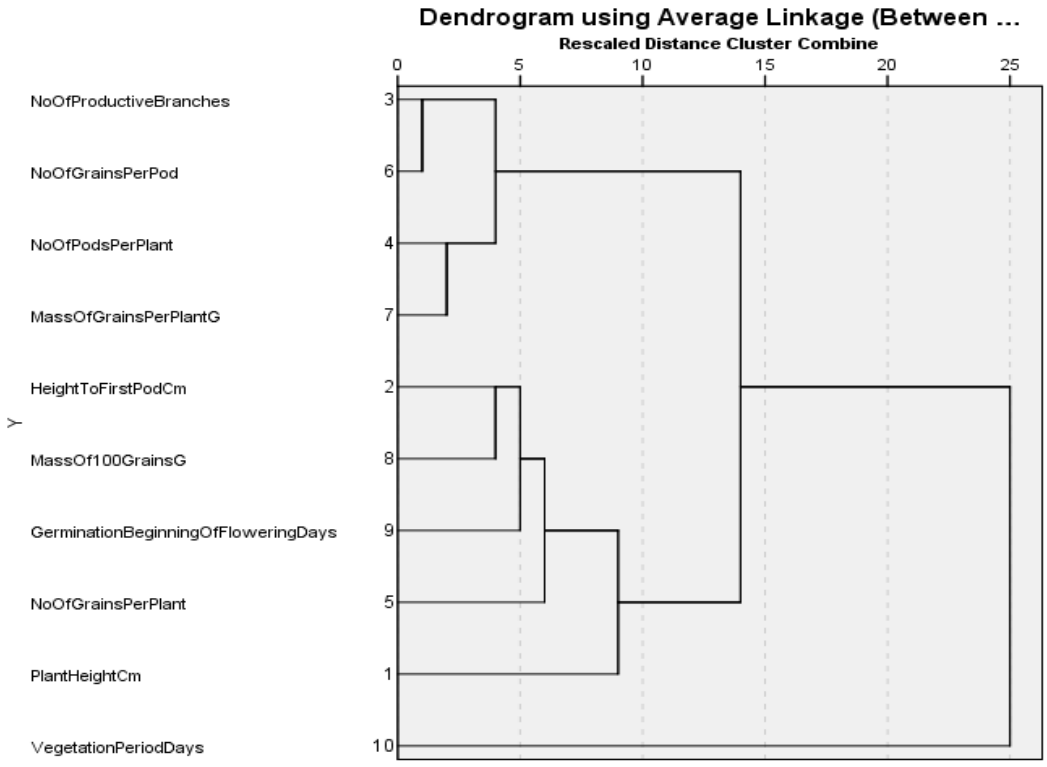

Fig. 2. Dendogram of studied traits grouping using cluster analysis during the period 2018-2019.

The phenological observations were performed in order to evaluate the earliness of the white lupin accessions (Table 2). The duration of the vegetation period for all genotypes varied between 90-99 days. The earliest blossom was observed in BGR 3086 accession (49 days), classified as ultra-early variety with a coefficient of earliness 1.00. Two accessions BGR 3084 and BGR 6341 with 52 days from sowing to beginning of flowering, were included in the group of early varieties with a coefficient of earliness 1.60. This period was slightly longer for the accession with BGR 3083 (53 days). All other accessions possessed coefficient of earliness greater than 1.66. The established phenological differences were preserved during the whole vegetation period. Similar grouping by the degree of earliness was done by Kosev & Vasileva (2019) in grass pea accessions.

Table 2. Phenological development of white lupin accessions during the period 2018-2019.

Accessions	BGR 3080	BGR 3085	BGR 3083	BGR 3079	BGR 3086	BGR 3078	BGR 6337	BGR 6339	BGR 3084	BGR 6341
Sowing-beginning of flowering, days	54	54	53	54	49	54	54	54	52	52
Sowing-maturity, days	97	98	99	93	90	97	97	99	95	95
Earliness coefficient	2.00	2.00	1.80	2.00	1.00	2.00	2.00	2.00	1.60	1.60

The established diversity in agro-morphological traits within the evaluated lupin accessions during the 2-years trial is an important initial step for every one breeding program aiming to select appropriate parents. Similar evaluations were performed by Julier et al. (1995), Raza & Jørnsgård (2005) and Lara-Rivera et al. (2017). Using cluster analysis the white lupin genotypes were grouped according to their yield potential, an important criteria for selection. It was used by some other researchers in evaluating lupin genotypes for yield and yield components (Berger et al., 2008; Abo-Hegazy et al., 2020). It will be useful to identify promising genotypes with high yield potential and quality trails direct as new cultivars. The main task of modern white lupin selection is the creation of varieties combining high grain yield with optimum duration of vegetation period (Naumkin et al., 2012).

Conclusions

Ten *Lupinus albus* L. accessions were characterized by their agro-morphological traits. The variability of the studied traits proved the genetic diversity within the accessions. The accessions were clustered in two groups by the economical important quantitative traits. On the other hand, the traits were combined into two main clusters that would facilitate the crop breeding programs. According to the coefficient of earliness, the white lupin accessions are grouped in three groups: ultra-early, early and late. BGR 3086, BGR 3084 and BGR 6341 were selected as early accessions and will be used as gene sources for improvement of vegetation period. The carried out assessment gave valuable data about important economical traits in tested accessions. This will increase opportunities for their use in different fields: indirectly in breeding programs, reproduction, recovery in the gene bank, international exchange, technology assessment and direct implementation open pollinated varieties.

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New Taxa for the Palaeoflora of Satovcha and Ustren (Rhodope Mts., Bulgaria)

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Abstract. Six new taxa for the Bulgarian palaeoflora are presented. The paleobotanical material was obtained during field activities funded by the Regional Natural History Museum – Plovdiv in 2019. Four taxa are represented from the early Oligocene flora of Ustren (E Rhodope Mts.). The new taxa established here are *Bauhinia* aff. *khasiana* Baker, *Juncus* sp., *Laurophyllum heeri* (Ettings.) Nemejc & Knobloch and *Vaccinium acheronticum* Unger. The nearest living relatives of these taxa are of different geographical origin. The recent species *Bauhinia khasiana* Baker is distributed in SE Asia while nearest living relatives of *Laurophyllum heeri* and *Vaccinium acheronticum*, respectively *Nectandra opositifolia* Nees et Mart. and *Vaccinium stamineum* L. are distributed in the Americas. The first one in tropical S America and the second – mainly in subtropical N America. Genus *Juncus* has a cosmopolitan distribution. The taxa *Lithocarpus* aff. *uvariifolius* (Hance) Rehder and *Oreopanax* aff. *anomalus* M.J. Cannon et Cannon have been identified from the middle Miocene flora of Satovcha (W Rhodope Mts.). The recent species *Lithocarpus uvariifolius* (Hance) Rehder is distributed in areas of SE Asia characterized by a subtropical climate while *Oreopanax anomalus* M.J. Cannon et Cannon is a tropical endemic species for Costa Rica (Central America).

Key words: early Oligocene, middle Miocene, Palaeoflora, Rhodope Mts., Satovcha, Ustren.

Introduction

Over the last decade, the Regional Museum of Natural History – Plovdiv has created its paleobotanical collection and increases it annually. In this presentation, six new taxa for the palaeoflora of Bulgaria are presented. They are found in the palaeobotanical materials obtained during field activities, which were funded by the museum in 2019.

Four taxa are represented from the area of the village of Ustren (E Rhodopes), Kardzhali District (Fig. 1). These are *Bauhinia* aff. *khasiana* Baker, *Juncus* sp., *Laurophyllum heeri* (Ettings.) Nemejc & Knobloch and *Vaccinium acheronticum* Unger. The early Oligocene flora of Ustren is poorly studied. It consists of about 30 taxa (in the collection of Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences), but so far only the species *Ficus palamarevii* Bozukov, Ivanov et Utescher has been published (Bozukov et al., 2013). The nearest living relatives (NLR) of these four new taxa are of different geographical origin. The recent species *Bauhinia khasiana* Baker is distributed in SE Asia. The Genus *Juncus* is cosmopolitan. Its representatives in the tropics are disseminated in the mountains. The NLR of *Laurophyllum heeri* - *Nectandra opositifolia* Nees et Mart. is distributed in tropical parts of Central and S America while NLR of *Vaccinium acheronticum*, respectively *V. stamineum* L. is widespread in Mexico and southeast parts of USA. Its area also covers Ontario to the north.

The taxa *Lithocarpus* aff. *uvariifolius* (Hance) Rehder and *Oreopanax* aff. *anomalus* M.J. Cannon et Cannon have been identified from the area of the village of Satovcha (W Rhodopes), Blagoevgrad District (Fig. 1). The Satovcha middle Miocene flora is characterized by its species diversity, which amounts to more than 120 fossil species (Bozukov, 2001; Bozukov & Ivanova, 2015; Bozukov et al., 2018). It is the richest local macro-palaeoflora in Bulgaria.

The recent species *Lithocarpus uvariifolius* (Hance) Rehder is distributed in areas of SE Asia while *Oreopanax anomalus* M.J. Cannon et Cannon is an endemic species for Costa Rica (Central America).

Fig. 1. An indicative map of the study area.

Materials and Methods

The studied material is stored in the palaeontological collection of Regional Natural History Museum – Plovdiv. It consists of leaf imprints on sedimentary rocks.

The flora-bearing rocks close to Village of Ustren (Fig. 2A) belong to the Limestone–Pyroclastic Formation [Limestone Pyroclastic Sequence after Boyanov & Goranov (2001)], which is a part of Chiflik Volcanic Subcomplex (Sarov et al., 2008). The Formation is composed of varied, predominantly massive to thick-bedded and subordinately thin- to medium-bedded acid tuffs, some of which contain well-preserved leaf imprints as we explore here. The early Oligocene age of pyroclastics situated north of our study area was determined by Moskovski et al. (2004) and Marchev et al. (2010) by K-Ar dating.

The studied material from Satovcha originates from the flora-bearing sediments (Fig. 2B) of the Sivik Formation (Vatsev & Pirumova, 1983), which is a part of the Satovcha Graben. These sediments are diatomites containing various admixtures. The middle Miocene age of these diatomites was proved by the analysis of diatoms (Vatsev & Pirumova, 1983), fossil macroflora (Bozukov, 2002) and fossil palynomorphs (Ivanov, 2004; 2013).

The determination of leaf types followed the scheme for leaf morphology of the angiosperms plants of Dilcher (1974). The arrangement of the corresponding taxa in the systematic part of the article follows the scheme for Magnoliophyta of Takhtajan (1987). The photos were taken with a digital camera Pentax Optio E70L.

Results

Systematics

Class Magnoliopsida

Family Lauraceae; Genus *Laurophyllum*

Laurophyllum heeri (Ettings.) Nemejc & Knobloch

1868. *Persea heeri* Eiitngshausen, p.9, Pl. 32, Fig, 17.

1973. *Laurophyllum heeri* (Ettings.) Nemejc & Knobloch, p. 711, Pl. 2, Figs 4-6.

2010. Lazarevic & Milivojevic, p. 144, Pl. 2, Fig. 33.

Material: Leaf imprint Ust.-20 (Fig. 2D).

Descriptions. The leaf margin is entire. The leaf lamina shape is elliptic. The apex is not preserved. The base is acute. The type of venation is camptodromous-brochidodromous. The first pair of secondary veins are arranged close to the leaf margin and traces its outline. The number of secondary veins is 7 pairs. They are curved and arranged at an angle of 40–60° to the midvein. Intersecondary veins are rarely observed. The tertiary veins are straight. Lamina dimensions – length of preserved part 3.5 cm, width 2.2 cm.

Remarks and comparisons. The fossil species is found close to Bulgarian geographical and stratigraphical location. It is described in the early Miocene flora of the Žagubica Basin (Serbia) (Lazarević & Milivojević, 2010). Nemejc & Knobloch (1973) consider *Nectandra opositifolia* Nees et Mart. to be a NLR of this fossil species. The recent species is common in the tropics of Central and South America. The genus *Laurophyllum* is represented in the Bulgarian palaeoflora by eight species. *L. acutimontanum* Mai is established by leaf imprint only. The rest are described by the cuticle. The stratigraphic distribution of these eight species covers the range from Upper Eocene to Upper Dacian (Palamarev et al., 2005).

Family Fagaceae; Genus *Lithocarpus*

Lithocarpus aff. *uvariifolius* (Hance) Rehder

Material: Leaf imprint Sat.-24 (Fig. 3E)

Descriptions. The leaf margin is entire. The leaf lamina shape is narrow elliptic. The apex is acuminate. The base is not preserved. The type of venation is camptodromous-brochidodromous. The number of secondary veins is 21 pairs on the preserved lamina part. They are curved and arranged at an angle of 60–70° to the midvein. Intersecondary veins are rarely observed. The tertiary veins are retroflexed. Lamina dimensions – length of preserved part 8.5 cm, width 2.5 cm.

Remarks and comparisons. The leaf lamina of the fossil material has very similar morphological features to those of the recent species *Lithocarpus uvariifolius* and especially to its variation *L. uvariifolius* var. *ellipticus* (Metcalf) Huang et Chang (Fig. 3F). This is the reason why we determine the fossil material in this way and accept the indicated recent species as its NLR. The distribution of this species covers SE China: SW Fujian, N and NE Guangdong, Guangxi. It forms a broadleaved evergreen forest, or in association with *Castanopsis*/*Quercus* (subgenus *Cyclobalanopsis)* or *Pinus massoniana Lamb.* on dry, hilly areas between 200 and 1000 m.a.s.l. (eFloras, 2008a). The genus *Lithocarpus* is present in the Bulgarian palaeoflora by three taxa, two of which are registered in the Rhodope region. The stratigraphic range of these taxa covers the Upper Eocene-Middle Miocene (Palamarev et al., 2005).

Family Ericaceae; Genus *Vaccinium*

Vaccinium acheronticum Unger

1850. Unger, p. 43, Pl. 24, Figs 1-17.

1958. Grangeon, p. 165, Tex-fig. 26, Fig. 4.

Material: Leaf imprint Ust.-20 (Fig. 2C).

Descriptions. The leaf margin is entire. The lamina shape is wide elliptic. The apex is acute. The base is acute-normal. The type of venation is camptodromous-brochidodromous. The midvein is much thicker than the secondary veins. The secondary veins themselves are four pairs, arcuate, sinuous and are connected to one another relatively away from the leaf margin. They are arranged at an angle of 70–80° to the midvein. The tertiary veins are percurrent forked. They form a fine network of tetragonal or pentagonal areolae. Lamina dimensions – length 4.5 cm, width 2.5 cm.

Remarks and comparisons. Palamarev (1964) reported the species *V. acheronticum* in the Middle Miocene flora from Chukurovo, which was subsequently revised as *Anagyris foetida* L. foss. (Palamarev & Petkova, 1987). In this situation, our find is the first for the Bulgarian palaeoflora. According to Grangeon (1958) the stratigraphic distribution of the species covers the Lower - Upper Miocene. The same author accepted the recent species *V. stamineum* L. as the most adequate for NLR of the fossil species mentioned here. *V. stamineum* is native to North America, including Ontario, the eastern and central United States, and parts of Mexico. It is most common in the southeastern United States. This plant usually grows in dry, rocky habitat types in forests and fields, but it sometimes occurs in moist areas such as bogs and swamps (Hill, 2002). This is probably the reason the species to be quite variable in its morphology.

Family Fabaceae; Genus *Bauchinia*

Bauhinia aff. *khasiana* Baker

Material: Leaf imprint Ust.-38 (Fig. 3A).

Descriptions. The preserved part of the leaf margin is entire. The lamina shape is probably wide ovate or very wide ovate. The apex is not preserved. The base is cordate. The type of venation is campylodromous. There are seven primary veins. The angle between them is about 50°. Secondary veins are not preserved. Probably they are on the upper lamina part which is missing. Tertiary veins are straight or convex. Dimensions of lamina preserved part – length of 3.0 cm, width 4.0 cm.

Remarks and comparisons. The preserved part of the fossil leaf lamina has very similar morphological features to those of the recent species *B. khasiana* and especially to its variation *B. khasiana* var. *khasiana* (Fig. 3A). This is the reason why we determine the fossil material in this way and accept the indicated recent species as its NLR. The species is distributed in mixed forests in SE China (Hainan Province), India, Laos, N Thailand, Vietnam (eFloras, 2008b). The Genus *Bauchinia* is new for the Bulgarian macro-palaeoflora.

Family Araliaceae; Genus *Oreopanax*

Oreopanax aff. *anomalus* M.J. Cannon et Cannon

Material: Leaf imprint Sat.-14a (Fig. 3C).

Descriptions. The leaf margin is entire. The lamina shape is narrow elliptic. The apex is not preserved. The base is acute-normal. The type of venation is camptodromous-brochidodromous. The midvein is slightly curved and much thicker than the secondary veins. The secondary veins are seven pairs, arcuate, sinuous. They are arranged at an angle of 60–70° to the midvein. The tertiary veins are percurrent forked. They form a fine network of tetragonal or pentagonal areolae. Lamina dimensions – length 7.0 cm, width 2.7 cm.

Remarks and comparisons. The genus *Oreopanax* is known in the Bulgarian palaeoflora from the same locality. The new taxon differs from the already published species *O. protomulticaulis* (Rasky) Hably (Bozukov, 2000) by the shape of the leaf lamina, and the entire leaf margin. The new

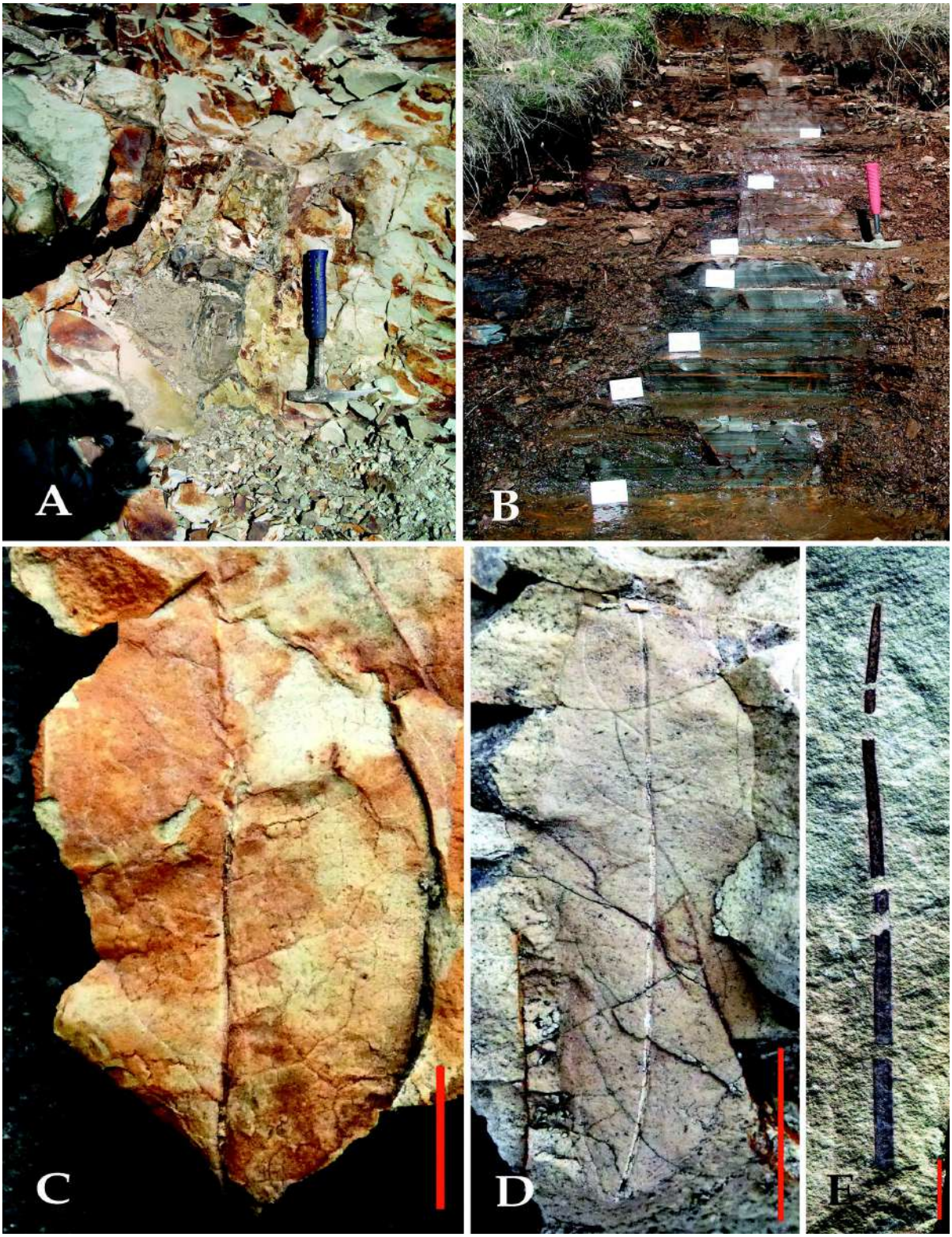

Fig. 2. A – Ustren flora-bearing rocks; B – Satovcha flora-bearing rocks; C – *Vaccinium acheronticum*; D – *Laurophyllum heeri*; E – *Juncus* sp. (measuring bar – 1 cm).

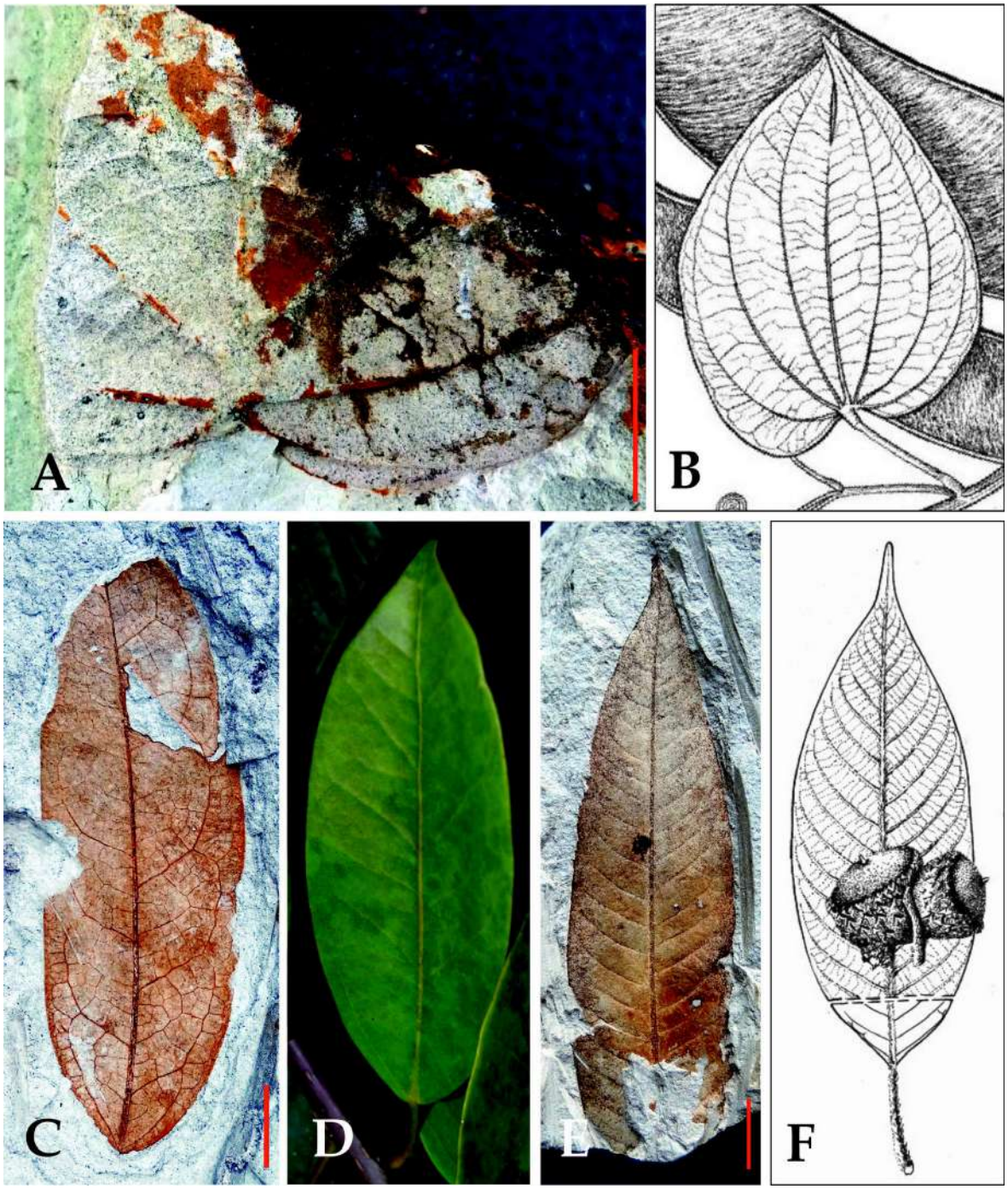

Fig. 3. A – *Bauhinia* aff. *khasiana*; B – *B. khasiana* var. *khasiana* (after eFloras, 2008b); C – *Oreopanax* aff. *anomalus*; D – *O. anomalus* (after Monro, 2019); E – *Lithocarpus* aff. *uvariifolius;* F – *L. uvariifolius* var. *ellipticus* (after eFloras, 2008a) (measuring bar – 1 cm).

fossil material has very similar morphological structure of the leaf lamina to those of the recent species *O. anomalus* M.J. Cannon et Cannon (Fig. 3D). This is the reason why we determine the fossil material in this way and accept the indicated recent species as its NLR. *O. anomalus* is a tropical species native to Costa Rica (Central America).

Class Liliopsida
Family Juncaceae; Genus *Juncus*
Juncus sp.
Material: Leaf imprint Ust.-8a,b (Fig. 2E).

Descriptions. The fossil material is an imprint of a needle-like leaf. The venation is paralelodromous. The veins are of the same type, regardless of thickness. Dimensions – length 9.5 cm, width 0.3 cm.

Remarks and comparisons. So far, the genus *Juncus* has been registered in the Bulgarian macro-palaeoflora only by fossil seeds material (Uzunova, 2001). Traces of other plant parts of the genus are expected as it is cosmopolitan.

Conclusions

The study of each local palaeoflora should not be limited in time. This study shows that both well-studied and poorly studied macroflora deposits can provide additional information in new research on them. Of course, it should be expected that new taxa are more likely to be found in poorly studied palaeoflora, as are the results of the present study. The new data will primarily enrich the composition of the palaeoflora, but at the same time will bring more information about the ecological conditions under which it existed. For example, information on the presence of biotopes with different ecological parameters in the vicinity of the plant deposition basin. It is also possible to get a clearer picture of the migration of individual taxa and the changes of flora and vegetation in palaeo-geographical aspect.

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Toxic Effects of the Insecticide "Actara WG" on the Allium cepa Root Meristem

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Abstract. The effect of the insecticide "Actara WG" on the cell division rate and on the chromosomal apparatus of *Allium cepa* root meristem cells was studied. By applying anaphase analysis and micronucleus mutagenicity test, a control sample (tap water) and experimental samples of insecticide solutions with different concentrations - 100%, 50% and 25% ("Actara WG" SS; "Actara WG" 50; "Actara WG" 25) of the recommended by the producer were compared. Approximately 2,000 cells per individual and five individuals per sample were analyzed. Comparative analysis of mitotic indices showed a negative effect of the tested pesticide in solution with the recommended concentration and in 50% solution of it on the rate of the cell division during the root germination for 48 hours. The genotoxic effect of the studied insecticide was analyzed. The chromosomal structural changes observed during the investigation are classified into 7 categories. Chromosomal abnormalities such as pulverized chromosomes, diagonal anaphases, chromosome fragments, anaphase and telophase bridges - alone and in combination with fragments, wandering and lagging chromosomes and micronuclei were detected in meristem cells after treatment with all the tested solutions of "Actara WG", but the highest percentage of aberrations was found after treatment with "Actara WG" 50%. Some chromosome aberrations were found in the control sample, but in a significantly lower percentage. It was concluded that the insecticide "Actara WG" negatively affects the cell division rate and has a genotoxic effect on the *Allium cepa* root meristem cells.

Key words: Actara, insecticide, neonicotinoids, *Allium cepa*, chromosomal aberration.

Introduction

Neonicotinoids are neurotoxic insecticides widely used in modern agriculture to control pests such as ants, aphids, whiteflies, beetles, and some Lepidoptera species (Goulson, 2013). Their negative impact on various natural components and nature in general has attracted the attention of researchers from around the world in recent decades. "Actara WG" is one of the most widely used agrochemicals with the active substance thiamethoxam. Thiamethoxam is a second generation (class III - moderately toxic) neonicotinoid insecticide (Maienfisch et al., 2001; Nauen et al., 2003). Compared to another neonicotinoid insecticide, imidacloprid, thiamethoxam has a lower affinity for nicotinic receptors. While imidacloprid is effective at nanomolar concentrations, thiamethoxam acts in millimolar concentrations (Wiesner & Kayser, 2000). Its binding to biological receptors *in vivo* can cause a series of biochemical reactions (Copeland, 2000). According to Motohiro & John (2005), neonicotinoids are easily absorbed by plants and kill insect pests at very low doses, but at the same time have low toxicity to vertebrates. In contrast, Su et al. (2021) in their study on the binding mechanism of thiamethoxam with three protein models report that it is an environmental pollutant and due to its accumulation in the ecosystem, poses potential risks to the health of mammals and even humans. This view is also supported by studies by other researchers who provided data on the presence of thiamethoxam and its metabolites in the human body (Wang et al., 2019; Zhu et al., 2019).

Cytogenetic markers such as chromosomal aberrations and micronuclei have been used in screening studies for neonicotinoid genotoxicity (Yadav & Kaushik, 2002; Zeljezic & Garaj-Vrhovac, 2004). De Morais et al. (2019) investigated and compared the genotoxic capacity of different concentrations of thiamethoxam, acetamiprid, imidacloprid and fipronil by a micronucleus

test using the *Tradescantia pallida* test object. The authors reported genotoxic activity in the study subject at the highest concentrations of the studied pesticides. The genetic test object *Allium cepa* is widely used in biomonitoring studies on the genotoxicity of various factors due to its proven high efficiency (Saxena, 2010). Using this test object, Verma & Srivastava (2018) investigated the morphotoxic and cytogenotoxic effects of the pesticide pendimethalin, developing a system of morphological and genotoxic biomarkers. Datta et al. (2018) also used *Allium cepa* to compare the genotoxicity of soils treated with pesticide and vermicompost and reported significant differences in the established mitotic indices and the frequency of the established chromosomal aberrations with respect to the studied samples.

The present study aims to monitor and characterize the effect of the pesticide "Actara WG" on the rate of cell division and to analyze its genotoxic potential using the *Allium cepa* test system.

Material and Methods

Three concentrations of the broad-spectrum insecticide "Actara WG" (active substance thiamethoxam, 250 g.kg-1) were tested in the present study. Stock solution ("Actara WG" SS 250 g.kg-1) was prepared according to the manufacturer's recommendations. Working solutions with a lower concentration were prepared from it - 50% ("Actara WG" 50 125 g.kg-1) and 25% ("Actara WG" 25 62.5 g.kg-1) of the basic solution. *Allium cepa* bulbs of the variety "Asenovgradska kaba 5" were used as a test object. The sprouted roots were fixed at the 48th hour of the treatment. Bulbs grown in tap water were used for control. Five bulbs from every concentration (and control) and five roots per a bulb were used in the analysis. A temporary microscopic slide was prepared from each root after washing with distilled water, treatment with 3N hydrochloric acid, 45% acetic acid and staining with acetocarmine. About 500 cells from a microscope slide were analyzed - a total of about 2000 cells per a sample.

The effect of the pesticide in solutions with the indicated concentrations on the rate of cell division was studied by calculating the mitotic index and phase indices. The mitotic index (IM) was determined as a percentage of the number of dividing cells and the total number of cells analyzed. Phase indices are calculated as percentages of the number of cells in a particular mitotic phase and the total number of dividing cells. To evaluate the cytogenetic effect of the experimental concentrations, the IM found in the analysis of the test samples was compared with the IM calculated for the control sample.

The genotoxic potential of the tested concentrations was investigated by anaphase analysis and micronucleus mutagenicity test. The observed chromosomal aberrations are summarized in seven categories as follows: 1) pulverized chromosomes in metaphase and anaphase; 2) wandering and lagging chromosomes; 3) fragments in metaphase, anaphase and telophase; 4) anaphase and telophase bridges with additional presence of fragments; 5) diagonal anaphases; 6) K mitosis and 7) micronuclei. For categories 1-6, the encounter frequencies were calculated as a percentage of the total number of cells and the number of dividing cells. For the seventh category, the frequency is calculated as the ratio between the number of cells with micronuclei and the total number of cells. The data obtained in the experimental and control samples were statistically compared. Student's t-test (Stangroom, 2018) was used to assess statistically significant differences between each test group and the control. Established differences at $P < 0.05$ were considered statistically significant.

Results

The results on the mitotic index and phase indices reported in the control and experimental samples are presented in Table 1.

Table 1. Mitotic index (IM) and phase indices in (%) in Allium cepa treated for 48 hours with different concentrations of "Actara WG". *Legend:* $p < 0.05$*; $p < 0.01$**; $p < 0.001$***

Samples	Mitotic index IM	Prophase index IPph	Metaphase index IMph	Anaphase index IAph	Telophase index ITph
Control	53.09 ± 3.88	87.92 ± 1.46	6.09 ± 1.00	2.94 ± 0.71	3.05 ± 0.64
"Actara WG" SS 250 g.kg-1 thiamethoxam	33.36 ± 7.24***	82.84 ± 1.56***	6.28 ± 1.61	6.36 ± 1.89**	4.53 ± 1.01*
"Actara WG" 50 125 g.kg-1 thiamethoxam	40.10 ± 6.68**	82.85 ± 2.43**	7.12 ± 1.78	5.94 ± 0.47***	4.09 ± 0.96*
"Actara WG" 25 62.5 g.kg-1 thiamethoxam	47.98 ± 8.31	85.87 ± 2.06	5.45 ± 0.90	4.61 ± 0.82**	4.07 ± 1.11

The mitotic index is an indicator of the rate of cell division. In the present study, the highest intensity of cell division was recorded with respect to the control sample (IM - 53.09%). Statistically significant differences compared to the control were found after the treatment with the recommended concentration of the pesticide - "Actara WG" SS (IM - 33.36%) and after the treatment with the sample "Actara WG" 50 (IM - 40.10%). Although not statistically significant, the data for IM showed a decrease even after treatment with "Actara WG" 25. When analyzing the phase indices, it was found that the cells in prophase have the highest frequency when treated with the control sample – 87.92%. This frequency was statistically significantly higher in comparison with those found for the tested samples of "Actara WG" SS and "Actara WG" 50 (82.84% and 82.85%, respectively). Statistically significant differences compared to the control were found with respect to the anaphase index in the three experimental samples with solutions of "Actara WG" SS, as well as with respect to the telophase index in the two samples with higher concentration (stock solution and 50% dilution) of the insecticide studied (Table 1).

Data on the established frequency of chromosomal aberrations in the root meristem of *Allium cepa* (control and experimental concentrations) are presented in Table 2.

During the study, chromosomal aberrations from the seven mentioned categories were found in the analyzed cells from the experimental samples (Figure 1). Some chromosomal aberrations were also detected in the control sample (0.53% in total), but in a statistically significantly lower percentage than all tested concentrations of the insecticide Aktara. The statistically significant differences found when comparing the results obtained in the control and experimental samples are shown in Table 2.

The highest percentage of structural chromosomal changes in dividing cells when testing the analyzed experimental samples was found after treatment with "Actara WG" 50 (5.57%) where the aberrations detected were as follows: wandering and lagging chromosomes (1.44%); single chromosome fragments (0.77%); anaphase and telophase bridges with fragments (0.63%); pulverized chromosomes and K mitosis (0.31%) and diagonal anaphases (0.26%). The frequencies of the detected chromosomal aberrations among the dividing cells in the other two tested solutions of the "Actara WG" (4.05% and 2.83%) were statistically significantly higher in comparison with the control (1.0%). The highest percentage of micronuclei in the total number of cells was found when treated with the solution "Actara WG" 50 - 0.67%. The data from the present study showed the highest pronounced genotoxic effect on meristem cells of *Allium cepa* with respect to the tested "Actara WG" 50 solution.

Table 2. Frequency of occurrence of different types of chromosomal aberrations analyzed by the Allium test: *Legend:* $p < 0.05$*; $p < 0.01$**; $p < 0.001$*** For each sample, the data in the first row are calculated as % relative to the total number of cells (N), and the data in the second row as % relative to the number of dividing cells (N').

Samples	Pulverized chromosomes in metaphase and anaphase	Wandering and lagging chromosomes	Fragments in metaphase, anaphase and telophase	Anaphase and telophase bridges + fragments	Diagonal anaphases	K mitosis	Micronuclei	Total
Control	-	0.33±0.16 0.60±0.25	-	0.04±0.04 0.07±0.07	-	0.08±0.07 0.14±0.14	0.09±0.08	0.53±0.11 1.00±0.17
"Actara WG" SS 250 g.kg-1 thiamethoxam	0.06±0.14 0.08±0.19	0.54±0.27 1.57±0.61**	0.05±0.06* 0.14±0.17	0.15±0.11* 0.43±0.38*	0.24±0.12** 0.78±0.56**	0.17±0.11 0.55±0.41*	0.14±0.21	1.34±0.46** 4.05±1.18***
"Actara WG" 50 125 g.kg-1 thiamethoxam	0.12±0.04*** 0.31±0.22**	0.71±0.03*** 1.44±0.33***	0.07±0.13 0.77±0.75*	0.12±0.05** 0.63±0.21***	0.13±0.01*** 0.26±0.09***	0.10±0.08 0.31±0.14*	0.67±0.55*	2.14±0.53*** 5.57±2.18***
"Actara WG" 25 62.5 g.kg-1 thiamethoxam	0.08±0.03*** 0.17±0.07***	0.46±0.09 0.97±0.18*	0.08±0.10 0.16±0.21	0.27±0.13** 0.57±0.27**	0.13±0.06*** 0.27±0.09***	0.24±0.15* 0.49±0.25*	0.11±0.23	1.38±0.43** 2.83±0.54***

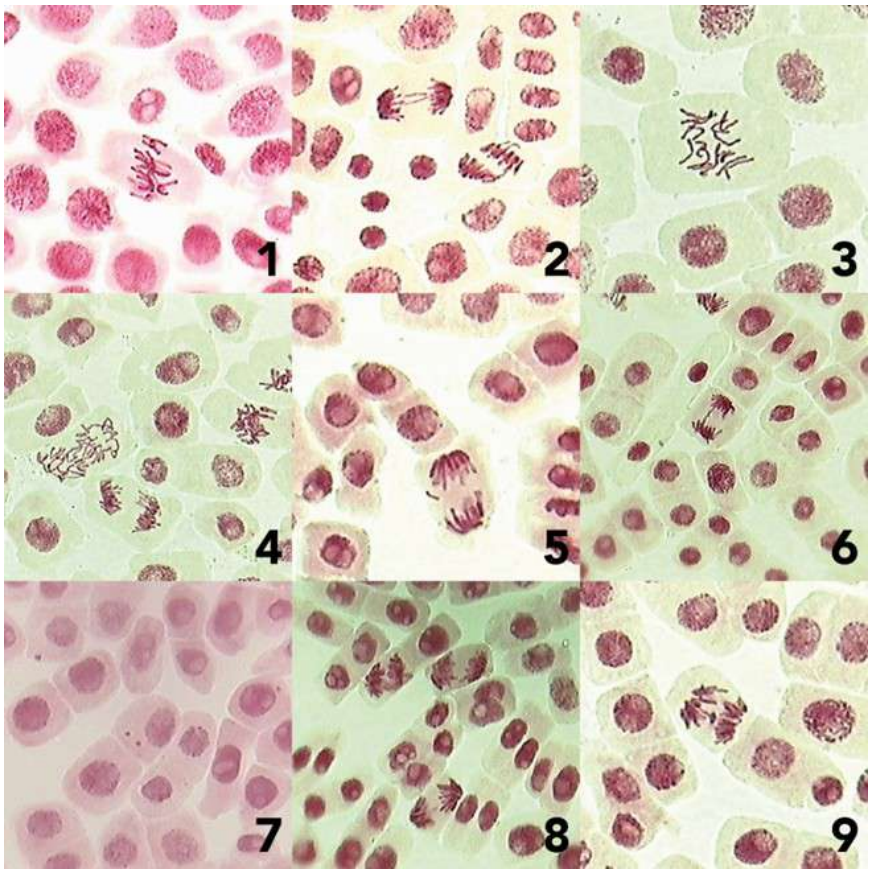

Fig. 1. Mutagenic effect of the pesticide "Actara WG" on the root meristem of *Allium cepa*. *Legend*: 1 - Wandering chromosomes; 2 - Anaphase and telophase bridges; 3 - K mitosis; 4 - Pulverized anaphase; 5 - Fragment in anaphase; 6 - Telophase bridge; 7 – Micronuclei; 8 - Diagonal anaphases; 9 - Fragment in anaphase.

Discussion

The negative impact of pesticides on the rate of cell division has been studied by many researchers (Singh, 2007; Liman et al., 2010; Asita & Mokhobo, 2013; Huan et al., 2016). There is evidence that treatment with certain agrochemicals adversely affects the course of the mitotic cycle, disrupting the regulation of its basic processes (Zabka et al., 2012). In accordance with these studies, the data from the present experiment showed reduced values of IM in meristem cells after treatment with the studied solutions in all of the three concentrations (Table 1). These results are an indication for the mitosis-depressive effect of the insecticide "Actara WG". Studies on the problem show that cell division can be negatively affected by various reasons – blocking the mitotic cycle in the interphase, inhibiting the synthesis of nuclear proteins and DNA, changes in the duration of individual mitotic phases and others (Mohanty et al., 2004; Chauhan & Gupta, 2005; Şekeroğlu et al., 2013; Önen et al., 2018).

The data from the present study show that the solutions of "Actara WG" in their different concentrations affect not only the rate of mitotic division, but also the distribution of dividing cells in different phases. Statistically significant differences compared to controls were available for both prophase and telophase (for "Actara WG" SS and "Actara WG" 50) and for anaphase (for all the three concentrations tested). It is noteworthy that in the experimental samples the prophase index was lower and the anaphase and telophase indices were higher (Table 1), which demonstrates a delay in the rate of mitosis during prophase and its intensification in anaphase and telophase. Changes in the duration of mitotic phases under the influence of various pesticides have been previously reported by Prasad & Das (1977) and Hanif & Davies (1998). According to Liman et al. (2011), the accumulation of pesticides in the cell could be highly toxic and this could affect the duration of the individual phases of mitosis. The decreased rate of cell division in *Allium cep*a meristem cells reported in the present study after treatment with "Actara WG" probably provokes mitotic stress and in compensation for this - a subsequent accelerated course of certain phases (anaphase and telophase).

The genotoxic potential of various pesticides, including neonicotinoids, has been studied by a number of authors (Karabay & Oguz, 2005; Jemec et al., 2007; Kreutzweiser et al., 2007; Rodríguez et al., 2015, etc.). The data from the present study show the highest levels of genotoxicity with respect to "Actara WG" 50. The observed overall incidence of structural chromosomal changes in the root meristem of *Allium cepa* was statistically significantly higher at all experimental concentrations of the tested pesticide compared to the control (Table 2). The great variety of reported chromosomal aberrations (Figure 1) is due to various damages - fragmentations, adhesion of chromosomes without telomeres, loss or damage in the centromere regions, damage to the dividing spindle and others. In the current study, the presence of micronuclei was found for all tested samples, but the highest frequency of cells with micronuclei was found after treatment with "Actara WG" 50 (Table 2). Their presence (Figure 1) is further evidence of the genotoxic activity of the studied insecticide. Micronuclei are the result of dropped chromosome fragments or the loss of whole chromosomes with damaged centromeres (Fenech, 2000). The results of the present study are consistent with those reported by Karabay & Oguz (2005) and Rodríguez et al. (2015), who report increased genotoxic potential of other neonicotinoid insecticides tested by *Allium cepa*.

Conclusions

The neonicotinoid insecticide "Actara WG" has a negative effect on the rate of cell division in the root meristem of *Allium cepa*. The wide range of chromosomal structural changes found with significantly higher frequencies in the experimental samples of the neonicotinoid insecticide "Actara WG" compared to the control are evidence for its clear genotoxic effect.

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Physiological and Agro-biological Traits Evaluation of Several Local Grain Legumes under Climatic Condition of South-central Region of Bulgaria

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Abstract. The most important cool season grain legumes (dry pea, chickpea, broad bean, lentil, lupins, grass pea and common vetch) are widely grown in many parts of the world. The aim of the current study was to test some of them under the drought-prone area of Sadovo (south-central region of Bulgaria) and to evaluate their adaptability based on physiological and agro-biological traits. The investigation was carried out at the experimental field of IPGR including several local accessions of white lupin (*Lupinus albus* L.), chickpea (*Cicer arietinum* L.) and grass pea (*Lathyrus sativum* L.). The chlorophyll content index of the leaves, used as criteria for drought tolerance, was measured by portable CCM 200 plus- Chlorophyll Content Meter. The assessment of agro-biological traits was performed according to the International Descriptor for each crop. Under the drought stress conditions the highest value of the chlorophyll content index was observed in three lupin accessions (BGR 6341, BGR3080, BGR3085), in two from chickpea (BGR 40417 and B9E0149) and in four from grass pea (BGR 40415, BGR 4835, BGR4847, BGR4834). The highest yield potential was established in two lupin (BGR 6341 and BGR 3084), two chickpea accessions (BGR 23151 and B9E0149) and three grass pea (BGR4832 and BGR40415). The selected accessions were included in the list for further investigations concerning drought tolerance and maintenance of relatively high yield potential under drought stress conditions.

Key words: lupin, chickpea, grass pea, drought tolerance, agro-biological evaluation.

Introduction

Legumes are unique plants, with great value for agriculture and society because of that they contribute to many different functions and ecosystems services (Nemecek et al., 2008; De Faria et al., 2011). One approach that can contribute to mitigating climate change is including legume food, forage, and tree crops in farming systems (Jensen et al., 2012). Grain legumes crop have positive influence on global food and nutrition security. However, their production rate remains unsatisfying compared to their consumption rate due to biotic and abiotic stress factors (Ojiewo et al., 2018). Water deficit is one of the most important factors that not only affect plant growth and development but also limit productivity (Boyer, 1982; Choudhary & Suri, 2014). Under the stress of extreme drought, grass pea is the only productive crop and becomes the only food for the poor in some rural or marginal areas (Vaz Patto et al., 2006). Terminal drought still limits chickpea production and grain yield although, chickpea is considered as drought-tolerant, cool-season food legume. The seed yield can be reduced by 58−95% compared to irrigated crops due to terminal drought. Drought stress causing reduction in pod production and abortion (the chief factors affecting the overall grain yield) (Leport et al., 2006). The limiting factor in the development and yield of plants is strongly reduced water content in soil. It was observed the highest reduction of seed yield, as a result of drought occurring in the flowering period, in blue lupine, cultivated both in pure sowing and in mixture with barley (Podleśny & Podleśna, 2010). Synthesis of plastid pigments is of significant importance for the photosynthetic activity of plants. The photosynthetic pigments are one of internal factors which could limit the photosynthetic activity to a large extend. Their content in normal and stress environmental conditions has been widely studied and discussed (Mikiciuk et al., 2010; Wrobel et al., 2010; Aienl et al., 2011). It is proven that the reduction of the pigment concentration is an indicator of stress in cases as water and temperature stress, insufficiency or excess of mineral

elements, etc. (Hendry & Grime, 1993; Stoeva et al., 2010). A deficiency of water will additionally disturb effects such as water relations, membrane integrity, yield, development, osmotic adjustment, photosynthetic movement and pigment content (Benjamin & Nielsen, 2006; Kaur & Kumar, 2020). Notably, due to the severe pressure of global climate change and ever increasing demand for food production, implementation of high-throughput and cost-effective techniques is required, which would invariably support the traditional breeding schemes. Therefore, immediate attention needs to be placed towards large scale exploration and characterization of the available germplasm for abiotic stresses tolerance (Jha et al., 2014).

The aim of the current study was to assess the drought tolerance of several local accessions of white lupin (*Lupinus albus* L.), chickpea (*Cicer arietinum* L.) and grass pea (*Lathyrus sativum* L.) by their physiological and agro-biological traits under the drought-prone area of Sadovo.

Material and Methods

Two years (2019-2020) investigations were carried out on the experimental field of Institute of Plant Genetic Resources (IPGR) - Sadovo. The plants were grown by standard technology for field production on cinnamon-forest soils after a wheat precursor.

The subjects of the study were six white lupin accessions, six – chickpea and eleven local grass-pea. The seeds were obtained from the grain legums collections, stored in the IPGR gene-bank. Every one accession was sown in 5 to 10 rows (depending on number of seeds), at a depth of 4-6 cm and a distance between the rows of 30-50 cm.

The physiological assessment of the all studied accessions of lupin, chickpea and grass pea was carried out in the field. The plants were evaluated twice at two stages - beginning of flowering and end of flowering/beginning of ripening. Samples collection was performed during two periods of drought: end of April/May, 2019 and May/beginning of June, 2020. The temperature and precipitation sum during the periods of measurements are presented in Table 1. The precipitation during the first and second date of measurements in two years investigations was low (7.8 l/m^2 - 12.1 l/m^2; 12.6 l/m^2 - 14.1 l/m^2 , respectively).

Table 1. Temperature conditions and precipitation sum in May and June during the period 2019–2020.

Year	**2019**				**2020**			
Parameter	**Average temperature, (t°)**		**Precipitation (l/m^2)**		**Average temperature, (t°)**		**Precipitation (l/m^2)**	
Month	V	VI	V	VI	V	VI	V	VI
1-10 days	15.83	20.92	7.80	135.00	17.01	19.96	12.60	12.00
11-20 days	18.18	25.46	3.60	32.10	21.62	21.70	0.00	19.80
20-30 days	20.94	24. 99	5.90	12.10	15.54	23.25	27.50	14.10

The relative chlorophyll content, expressed by chlorophyll content index (CCI) in the leaves, was measured with Chlorophyll Content Meter (CCM 200 plus). The measurements were carried out on 20 leaves per accession. This apparatus is ideal for investigation of crop stress, leaf senescence, health determination. Furthermore, the affordability and ease of use make it an exceptional teaching tool for botany and plant science courses (Richardson et al., 2002; Arunyanark et al., 2008).

The structural elements of the yield were established by biometric analysis of 10 plants per accession. The traits evaluation was performed according to the descriptors for *Lathyrus* spp. (IPGRI, 2000), *Cicer arietinum* (UPOV, 2019) and *Lupinus* sp. (UPOV, 2004). The following quantitative characters were taken into consideration: plant height (cm), number of productive branches, height to the first pod (cm), number of pods per plant, number of grains per plant, number of grains per pods, mass of grains per plant (g) and mass of 100 grains (g). The length of vegetation period comprised the days from plant germination to 80% ripening of plants.

The analysis of variance and descriptive statistics (Mean, Error of mean, Minimum, Maximum, Range, Standard deviation and Coefficient of variation) were made with statistical program SPSS 19.0 for Windows. The means were compared by the three significance difference (LSD) at the 0.05, 0.01 and 0.001 probability level (p). The degree of variability of the studied traits, represented by a coefficient of variation (CV, %) was indicated according to the scheme of Mamaev (Shamov, 1998): up to 7% - very low; 7.1-12% - low; 12.1-20% - average; 20.1-40% - high; over 40% - very high.

Results and Discussion

Lupin (*Lupinus albus* L.)

Evaluation of the relative chlorophyll content, expressed by chlorophyll content index (CCI) in six white lupin accessions

The CCI (mean value of two years) during the first date of measurement in lupin accessions varied from 6.34 (BGR3086) to 14.01 (BGR3080) and they were significantly different (p=0.05), compared to the mean standard (Table 2). During the second date of measurement, CCI mean values in all analyzed accessions decreased considerably and high variation was not observed between the accessions – from 3.30 (B9E0202) to 5.88 (BGR6341). In some accessions, the CCI value dropped with 76.0% for BGR 3080 but only with 12.0% for BGR 3086 compared to the first date of measurement. The lupin accessions had high variation (29.3%) of the CCI values during the first measurement dates and average variation (18.9%) during the second date.

It is worth to pay attention on BGR 3080, BGR 3085 and BGR 6341 accessions characterized with high CCI during the first date of evaluation under the relatively low rate of precipitations.

Table 2. Mean value of Chlorophyll Content Index of six white lupin accessions in two years trial (2019–2020). Legend: n.s. no significance difference.

Accessions	Chlorophyll content index (first date)	Chlorophyll content index (second date)	Chlorophyll content index (mean)
B9E0202	8.14 n.s.	3.30 n.s.	5.72
BGR3084	9.08 n.s.	5.01 n.s.	7.05 n.s.
BGR3080	14.01*	5.04 n.s.	9.52 n.s.
BGR3085	10.82 n.s.	5.88 n.s.	8.35 n.s.
BGR3086	6.34*	5.57 n.s.	5.95 n.s.
BGR6341	13.37 n.s.	5.88 n.s.	9.62 n.s.
X-mean standard	10.29	5.11	7.70
Min	6.34	3.30	5.72
Max	14.01	5.88	9.62
CV (%)	29.3	18.9	22.4
p=0.05 *	3.35	2.96	2.32
p=0.01 **	4.47	3.94	3.08
p=0.001 ***	5.83	5.15	3.97

Descriptive statistics of quantitative traits in six white lupin accessions

High variation of almost all quantitative traits were observed in white lupin accessions except the vegetation period (CV-3.89%) and height to the first pod (CV-10.85%) (Table 3). Very high value of coefficient of variation (over 40.0%) were established between accessions in the following traits: mass of grains per plant, number of grains per plant, number of productive branches and number of pods per plant.

Several genotypes were selected based on their valuable traits. Two accessions (BGR 6341 and BGR 3084) had the biggest mass of grains per plant. They also had a big number of pods and grains per plant and big number of grains per pod.

The earliest accessions with shortest vegetation period were – BGR 3086, BGR 3080 and B9E0209. All genotypes of white lupin had vegetation period between 90 and 99 days.

Table 3. Evaluation of quantitative traits in six white lupin accessions in two years trial (2019-2020).

Characteristics	Range	Min	Max	Mean		Std. Deviation	Variance	CV (%)
				Statistic	Std. Error			
Plant height (cm)	37.10	30.33	67.43	48.13	5.03	12.33	152.07	25.62
Height to the first pod (cm)	8.40	24.17	32.57	29.10	1.29	3.15	9.95	10.85
Number of productive branches	4.26	0.17	4.43	1.82	0.66	1.61	2.60	88.72
Number of pods per plant	12.97	2.17	15.14	6.23	1.91	4.68	21.95	75.28
Number of grains per plant	59.26	5.17	64.43	20.73	9.14	22.39	501.49	108.01
Number of grains per pods	2.95	2.08	5.03	3.11	0.46	1.13	1.27	36.16
Mass of grains per plant (g)	21.32	0.88	22.20	6.50	3.30	8.08	65.34	124.38
Mass of 100 grains (g)	13.25	23.50	36.75	30.01	2.56	5.73	32.85	44.26
Vegetation period (days)	10.00	91.00	101.00	94.33	1.50	3.67	13.47	3.89

The combined analysis of variance revealed significant differences among genotypes for all studied traits indicating wide genetic variation among genotypes and this provides basis for selection among these genotypes. Our study is consistent with those of Abo-Hegazy et al. (2020). Abo-Hegazy et al. (2020) who also reported for significant differences among tested accessions by the most important quantitative traits.

Promising white lupin accessions were selected by carried out screening under drought stress conditions, based on their CCI and quantitative traits. BGR 6341, BGR3080 and BGR3085 possessed the highest value of the chlorophyll content index in the first date of measurement. The genotype BGR 3080 was distinguished with tall plants (63.86 cm) and large grains (40.80 g) while BGR 3085 had big number of pods and grains per plant (14.43 and 60.14, respectively) and high yield per plant (22.14 g). The genotype BGR 6341 had tall plants (67.43 cm), big number of productive branches (4.43), high values of number of pods per plant (15.14), number of grains per plant (64.43), number of grains per pod (5.03) and large grains (22.20 g). These accessions can be used as an initial gene sources in the breeding program.

The lowest reduction of the CCI between the first and second date of assessment in BGR 3086 accession could be considered as type of drought stress tolerance. This accession characterized also with a big number of grains per plant (52.86), tall plants (68.43 cm) and the shortest vegetation period (90 days), which is a reason to be selected as gene source. Similar results were reported by Juson et al. (2019), in a study of drought resistance in yellow lupin and pea cultivars.

Chickpea (*Cicer arietinum* L.)

Evaluation of the relative chlorophyll content, expressed by chlorophyll content index (CCI) in six chickpea accessions

The CCI (mean value of two years) during the first date of measurement in chickpea accessions varied from 2.72 (BGR23151) to 5.10 (BGR40417) and the first one was significantly different (p=0.05), compared to the standard (Table 4). Three genotypes (BGR23151, A9E0121 and A8E0412) had low CCI values with significance difference compared to standard during the first measurement date. During the second date of measurement, CCI mean values in all analyzed accessions decreased weakly and high variation was not observed between the accessions – from 2.80 (A8E0412) to 5.11 (B9E0149). Two accessions - BGR 23151 and A8E0412 had low mean values of CCI with significantly difference compared to standard. Two other accessions (BGR40417 and B9E0149) had the highest and stable values of CCI during the first and second date of measurements. The chickpea accessions had high variation of the CCI values during the first and second measurement dates (27.9% and 23.8% respectively).

Table 4. Chlorophyll Content Index of six chickpea accessions in two years trial (2019-2020). *Legend:* n.s. no significance difference.

Accessions	Chlorophyll content index (first date)	Chlorophyll content index (second date)	Chlorophyll content index (mean)
St. BGR 40417	5.10	4.46	4.78
BGR 23151	2.72*	3.08 n.s.	2.90 *
B9E0001	3.31 n.s.	4.19 n.s.	3.75 n.s.
B9E0149	5.34 n.s.	5.11 n.s.	5.22 n.s.
A8E0412	2.97*	2.80 n.s.	2.89 *
A9E0121	2.88*	5.05 n.s.	3.97 n.s.
X	3.72	4.11	3.92
Min	2.72	2.80	2.89
Max	5.34	5.11	5.22
CV (%)	27.9	23.8	24.4
p=0.05 *	1.88	2.10	1.54
p=0.01 **	2.50	3.14	2.04
p=0.001 ***	3.26	4.07	2.62

Descriptive statistics of quantitative traits in six chickpea accessions

High value of coefficient of variation was observed in number of pods and grains per plant (VC-41.83% and 43.31% respectively) in tested chickpea accessions (Table 5). There was a small difference among accessions in plant hight, number of grains per pod and vegetation period. These characters were slightly variable with coefficient of variation up to 12%.

Two accessions (BGR23151 and A9E0149) had big number of pods (58.17 and 59.25, respectively) and grains per plant (51.33 and 52.50, respectively). They also had tall plants-44.33 cm and 49.17 cm, respectively.

The vegetation period for all chickpea accessions varied between 83 and 107 days. The shortest vegetation period was established in following accessions: A8E0412 (83 days), A9E0121 (87 days) and A9E0149 (88 days).

Table 5. Qualitative traits observed on six chickpea accessions in two years trial (2019-2020).

Characteristics	Range	Min	Max	Mean		Std. Deviation	Variance	CV (%)
				Statistic	Std. Error			
Plant height (cm)	9.17	40.00	49.17	43.75	1.31	3.22	10.36	7.40
Height to the first pod (cm)	12.00	16.83	28.83	20.76	1.75	4.28	18.31	22.35
Number of prodoctive branches	1.92	1.08	3.00	2.15	0.27	0.65	0.43	27.55
Number of pods per plant	36.00	22.17	58.17	35.14	6.38	15.62	244.04	41.83
Number of grains per plant	39.08	20.17	59.25	35.67	6.66	16.31	265.91	43.31
Number of grains per pods	0.23	1.00	1.23	1.10	0.03	0.08	0.01	7.06
Mass of grains per plant (g)	13.68	9.55	23.23	13.98	2.21	5.41	29.25	36.39
Mass of 100 grains (g)	15.25	37.00	52.25	42.54	3.01	7.37	54.30	17.00
Vegetation period (days)	24.00	83.00	107.00	94.33	3.93	9.63	92.67	10.37

Mafakheri et al. (2010) and Maqbool (2017) reported significant variability in chickpea germplasm as a response to drought stress expressed as drought escape, drought avoidance and drought tolerance. These mechanisms prevent chickpea crop from harmful effects of drought. In our study promising chickpea accessions were selected under drought stress conditions, based on their CCI and quantitative traits. BGR 40417 and B9E0149 possessed the highest value of the chlorophyll content index during the first and second date of measurement. The same accessions possessed also some other valuable traits which could be interesting from breeding point of view. The genotype BGR40417 formed first pods high (28.83 cm) while accessions B9E0149 had tall plants (49.17 cm) and big number of pods and grains per plant (51.33 and 52.50, respectively). BGR 40417 and B9E0149 accessions will be used as initial gene sources in breeding program. The slight variation observed in our study in several quantitative traits is in contrast to Petrova & Angelova (2011) and Petrova & Stamatov (2013) who established wide genetic variation in agro-morphological traits of evaluated chickpea collection. This fact could be explained with the smaller number of tested accessions in our study.

Grass pea (*Lathyrus sativus* L.)

Evaluation of the relative chlorophyll content, expressed by chlorophyll content index (CCI) in eleven grass pea accessions

The CCI (mean value of two years) during the first date of measurement in grass pea accessions varied from 9.55 (BGR4833) to 16.78 (BGR40415) and the second one was significantly different (p=0.05), compared to the mean standard (Table 6). During the second date of measurement, CCI mean values in all analyzed accessions decreased considerably and not high variation was observed between the accessions – from 1.63 (BGR33111) to 2.83 (BGR4830). All accessions showed strong decrease in CCI values, an average to 80.00%, during the second measurement date. All studied genotypes showed no significance difference in CCI values during the second measurement date (Table 5). The grass pea accessions had average variation of the CCI values during the first and second measurement dates (16.10% and 19.40% respectively).

Descriptive statistics of quantitative traits in eleven grass pea accessions

The grass pea accessions had low variation of the most of studied traits – vegetation period (2.44%), number of grains per plant (9.02%), mass of 100 grains (9.53%) and plant height (11.89%) (Table 7). Big differences were observed in the mass of grains per plant, number of grians per plant and number of pods per plant. These characters were the most variable with high value of coefficient of variation. The vegetation period varied between 106 and 115 days. The earliest accession, with the shortest vegetation period, was BGR 4334 (106 days) and the latest one – BGR4833 (115 days). Those two genotypes had tall plants, with high first formed pods and large

grains. The accession BGR4832 had the highest number of pods and grains per plant and mass of grains per plants while genotype BGR 4847 had the lowest one.

Table 6. Chlorophyll Content Index of eleven grass pea accessions in two years trial (2019-2020). Legend: n.s. - no significance difference.

Accessions	Chlorophyll content index (first date)	Chlorophyll content index (second date)	Chlorophyll content index (mean)
BGR40415	16.78*	2.41 n.s.	9.59*
BGR4830	10.14 n.s.	2.83 n.s.	6.48 n.s.
BGR4831	11.55 n.s.	2.35 n.s.	6.95 n.s.
BGR4832	10.19 n.s.	1.99 n.s.	6.09 n.s.
BGR4833	9.55 n.s.	2.26 n.s.	5.91 n.s.
BGR4834	11.52 n.s.	2.81 n.s.	7.16 n.s.
BGR4835	12.53 n.s.	2.69 n.s.	7.61 n.s.
BGR4836	11.66 n.s.	3.09 n.s.	7.38 n.s.
BGR4847	13.43 n.s.	1.88 n.s.	7.66 n.s.
BGR33111	11.81 n.s.	1.63 n.s.	6.72 n.s.
BGR43334	12.48 n.s.	1.92 n.s.	7.20 n.s.
X-mean standard	11.87	2.35	7.16
Min	9.55	1.63	9.59
Max	16.78	3.09	5.16
CV (%)	16.1	19.4	13.8
P=0.5 *	4.19	0.89	1.96
P=0.01 **	5.53	1.17	2.58
P= 0.001 ***	7.11	1.51	3.31

Table 7. Qualitative traits observed on eleven grasspea accessions in two years trial (2019–2020).

Characteristics	Range	Min	Max	Mean		Std. Deviation	Variance	CV (%)
				Statistic	Std. Error			
Plant height (cm)	28.17	54.83	83.00	70.11	2.51	8.34	69.53	11.89
Height to the first pod (cm)	15.50	19.50	35.00	25.08	1.28	4.25	18.04	16.94
Number of prodoctive branches	1.67	3.00	4.67	3.65	0.14	0.46	0.21	12.66
Number of pods per plant	17.33	16.67	34.00	23.70	1.69	5.59	31.23	23.58
Number of grains per plant	33.58	42.17	75.75	55.31	3.64	12.09	146.11	21.85
Number of grains per pods	0.74	2.43	3.17	2.69	0.07	0.24	0.06	9.02
Mass of grains per plant (g)	6.15	7.68	13.83	10.25	0.62	2.05	4.21	20.01
Mass of 100 grains (g)	5.60	18.10	23.70	20.79	0.60	1.98	3.92	9.53
Vegetation period (days)	9.00	106.00	115.00	110.55	0.81	2.70	7.27	2.44

According Donskoy (2013) the highest seed productivity in grass pea is determined by the weight of the plant, the number of pods per plant and the weight of the pods per plant. The efforts of researchers should be directed in growing the grass pea mainly for grain (Polignano et al., 2005).

During the current study promising grass pea accessions were selected, based on their CCI and quantitative traits expressed under drought stress conditions. BGR 40415, BGR 4835, BGR4847 and BGR4834 possessed the highest value of the chlorophyll content index in the first date and mean for the two date of measurement. These accessions differentiated also with valuable

yield characters as: BGR40415 had big number of pods and grains per plant (29.75 and 75.75, respectively); BGR4835 – with tall plants (67.00 cm) and large grains (23.00 g); BGR4847 – with high first formed pod (35.00 cm) and big number of grains per pod (3.17) and BGR4834 – with plant height (64.83 cm) above the average standard and relatively short vegetation period (112 days). In similar study, Rybinski et al. (2008) reported average grain productivity between 7.20 g to 21.40 g in 106 grass pea lines. The selected accessions will be considered as starting materials for breeding.

Conclusions

Several local accessions of white lupin (*Lupinus albus* L.), chickpea (*Cicer arietinum* L.) and grass pea (*Lathyrus sativus* L.) were selected under the drought stress conditions of Sadovo region and based on their quantitative traits. Three white lupin accessions (BGR 6341, BGR3080, BGR3085) were selected as drought tolerant and with relatively high yield potential. Another lupin genotype (BGR 3086) had the lowest decrease of the CCI between the both dates of assessment, which could be considered as type of drought stress tolerance. Two promising chickpea accessions (BGR 40417 and B9E0149) were selected under the same environment as drought tolerant. The same accessions possessed also some other valuable traits which could be interesting from breeding point of view. Four grass pea accessions (BGR 40415, BGR 4835, BGR4847 and BGR4834) possessed drought tolerance and valuable yield characters. The selected accessions can be included in the list for further investigations concerning drought tolerance and maintenance of relatively high yield potential under drought stress conditions.

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Functional and Structural Feature of Photosynthetic Apparatus of Some Halophytic and Glycophytic Representatives from Genus Lactuca (Asteraceae)

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Abstract. In the present study, chloroplast ultrastructure, PSII functionality and lipid and fatty acids pattern of isolated chloroplasts have been used in order to characterize structural and functional peculiarities of photosynthetic apparatus in some halophytic and glycophytic *Lactuca* species. The comparative studies of *Lactuca tatarica* (L.) C.A. Mey, *L. serriola* L. and *L. quercina* L. have shown distinctive features of thylakoid membrane system, chlorophyll thermoluminescence emission and kinetic parameters of PSII oxygen-evolving reactions. The analysis of lipid classes and fatty acids composition of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG) and phosphatidyldiacylglycerol (PG) show the existence of qualitative and quantitative differences that can contribute in this regard. The results show specific characteristics of photosynthetic membranes in halophytic and glycophytic *Lactuca* species, reflecting different adaptive strategies of the studied species to environmental conditions in their natural habitats.

Key words: chloroplasts, fatty acids, lipid composition, *Lactuca quercina, Lactuca serriola*, *Lactuca tatarica*, oxygen evolution, photosynthetic activity, thermoluminescence.

Introduction

In natural habitats, the environmental factors (temperature, water, light intensity, salinization, etc.) have a complex influence on the plants. Fluctuations in environmental factors as well as various unfavorable situations induce physiological adaptation by influencing the activity of primary metabolic reactions such as photosynthesis, which is a major physiological process that determines plant growth and productivity (Lichtenthaler, 1998). The properties of photosynthetic apparatus may contribute to a great extent to plant habitat separation and to adaptation to environment factors governing mechanisms for effective light energy utilization. The physiological properties of chloroplast membrane such as permeability, selectivity, etc., varied in dependence of environment conditions, resulting in changes in the physical orientation of membrane lipids and functional activity of thylakoids. The response of the cell in such cases is a series of quantitative and qualitative alterations in the lipid composition in order to restore the initial orientation and the physical properties of the membrane.

The genus *Lactuca* L. (Asteraceae) comprises about 100 wild species occurring in Europe, Asia, Africa and North America. Since ancient times, some *Lactuca* species have been well known as dietary and medicinal plants. Plants of the genus *Lactuca* have been shown to produce sesquiterpene lactones (SL) (Wang et al., 2010) as their characteristic secondary metabolites. Sesquiterpene lactones may play a highly significant role in human health, as pharmaceutical agents, due to their potential for the treatment of cardiovascular disease and cancer (Chadwick et

al., 2013). Wild *Lactuca* species e.g., *L. virosa* L. and *L. saligna* L. are being used in breeding programs for the introduction of virus resistance into commercial lettuce (Tamaki et al., 1995)

In Bulgaria, seven *Lactuca* species can be found: *Lactuca tatarica* (L.) C.A. Mey, *L. serriola* L., *L. quercina* L., *L. viminea* (L.) J.Presl & C.Presl, *L. saligna* L., *L. perennis* L., *L. aurea* (Vis. & Pančić) Stebbin (Stoyanova et al., 2015). In the present study were selected three species, a halophyte *L. tatarica* and two glycophytic species *Lactuca serriola* and *L. quercina*.

The aim of this study was to investigate the structural-functional peculiarities of chloroplast membranes influenced by the specific environmental conditions in different natural habitats of some halophytic and glycophytic *Lactuca* species.

Materials and Methods

Plant material and habitats. Sampling of leaves from selected *Lactuca* species was made in flowering stage from different natural habitats during the period July - August 2019. The glycophyte *Lactuca serriola* is a drought-resistant species mainly found in solar habitats, urban places, along railways, landfills, etc. (D'Andrea et al., 2009). The plants were collected around the city of Sofia from agricultural field near Lozen village (42.6017° N, 23.4827° E, altitude 650 m). The glycophyte *Lactuca quercina* inhabits mainly shadow and a semi-shade oak, beech forests and shrubland communities. The plants were collected in Rila Mountain in beech forest on the territory of Rila Monastery Nature Park (42.1334° N, 23.3401° E, altitude 1200 m). In nature, halophyte *Lactuca tatarica* grows in meadows, in steppes and semi-deserts, as well as in salty soils, for example, along sea coasts. The samples were collected from the Bulgarian Black sea coast near the village of Shabla (43.5379° N, 28.5352° E, altitude 47 m).

Isolation of broken chloroplasts (thylakoids). Averaged samples of leaves of 3-4 plants collected were homogenised in 50 mM Na-tricine (pH 7.8), containing 3 mM Na-ascorbate, 10 mM NaCl, 5 mM MgCl2, 0.4 M sucrose and 5% PEG-6000. The resulting slurries were passed through 8 layers of cheesecloth and the broken chloroplasts (thylakoids) were collected by centrifugation at 1000 x g for 10 min. The pellets were washed twice in 10 mM Na-tricine (pH 7.8) containing 0.4 M sucrose, 10 mM NaCl and 5 mM MgCl and then resuspended to concentration of 1 mg Chl/ml in 50 mM Na-Mes (pH 6.5) instead of Tricine buffer and stored on ice for 1 h in the dark before measurements. The pigment content was determined spectrophotometrically (Lichtenthaler, 1987).

Oxygen-evolving reactions. Oxygen-evolving reactions were measured using polarographic oxygen rate electrode (Joliot-type) and thylakoid membranes (100 µl sample volume, 300 µgChl/ml) without any artificial electron acceptors, as described in (Zeinalov, 2002). The initial oxygen burst was recorded after irradiation with continuous white light (450 µmol photons m^{-2} s^{-1}). Deconvolution of the oxygen burst decay was performed by fitting of the function with two exponential components: $A_1e(tk_1) + A_2e(tk_2)$, where A_1 and A_2, and k_1 and k_2 were the rate constants of the fast and slow components of the oxygen burst decay, respectively.

Thermoluminescence. Thermoluminescence (TL) measurements were carried out in darkness using computerized equipment, described in detail in (Zeinalov & Maslenkova, 1996 a). The samples were kept in the dark for 2 h before measurements. Samples of isolated thylakoid membranes ware illuminated at 2-5°C to generate charge pairs within the PSII reaction centres and then rapidly cooled down in liquid nitrogen to trap those charge-separated states. Subsequent warming of the samples reveals thermoluminescence emission with characteristic peaks (Sane & Rutherford, 1986). Decomposition an analysis of TL glow curves was carried out using Origin Pro 8.

TEM analysis. For TEM analysis small segments (1-2 mm^2) from the middle part of fully expanded leaves were taken and fixed in 3% (m/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 12 h at 4°C. The leaf segments were post fixed in 1% (m/v) $KMnO_4$ in the same

buffer for 2 h at room temperature. After dehydration by increasing concentrations of ethyl alcohol (from 25 to 100%), the samples were embedded in Durcupan (Fluka, Buchs, Switzerland) and cross-sectioned with Reichert-Jung (Wien, Austria) ultramicrotome. Observation and documentation were performed by JEOL 1200 EX (Tokyo, Japan) electron microscope.

Lipid extraction. The lipids were extracted with chloroform/methanol/water as described by Bligh & Dyer (1959). The fresh aerial part of plant (25-30 g) was homogenized with 20 ml methanol and refluxed for 5 minutes in order to inactivate the lipases. An equal volume of chloroform was added and after 24h the mixture was filtered, and an equal amount of water was added. The lower layer (total lipophilic extract) was evaporated under vacuum and kept at –30°C. The amounts of lipophilic components were determined gravimetrically.

Lipid and fatty acid analysis. For lipid classes and fatty acids analyses part of the total lipophilic extract (50 mg) was applied on 20×20 cm silica gel G (Merck) plate (layer thickness 0.5 mm) and then the plate was developed with chloroform-methanol-acetone-acetic acid (70:14:24:0.4 v/v/v/v) as a mobile phase. The spots of the main lipid classes were visualized under UV-light, scrapped off with the silica gel layer and transferred in small vials with Teflon screw caps. Five ml of 15% acetyl chloride in absolute methanol were added and the vials were heated for 4 hours at 55°C (Christie, 1989). After cooling, the samples were diluted with water and the obtained fatty acids methyl esters (FAME) were extracted twice with hexane (2×5 ml). The FAME in combined hexane extracts were purified by preparative thin–layer chromatography (TLC) on 20×20 cm silica gel G (Merck) plates (layer thickness 0.5 mm) developed with hexane–acetone (95:5 v/v). The spots of the FAME were visualized under UV light, scrapped off with the silica-gel layer and eluted with diethyl ether. The amount of each sample was determined gravimetrically.

Fatty acid analysis was performed using Gas Chromatograph with Flame Ionization Detector Agilent 7890B, equipped with Agilent7693 Autosampler with 10 μl syringe and with capillary column SGE BPX70 (60 m x 0.25 mm x 0.25 μm). Nitrogen was the carrier gas at flow rate of 1.2 ml/min. The column temperature was programmed from 80°C (hold for 1 min) to 130°C by step of 8°C/min and then to 250°C by step of 5°C/min. The injector and detector temperatures were 245°C and 255°C, respectively; split 15:1. Instrument control, data acquisition and data processing were performed by GC software Clarity v.8.0.0.125. The fatty acids were identified by comparison of the retention times with that of reference mixture F.A.M.E. Mix C8-C24 (Sigma-Aldrich). The relative amounts of the fatty acids were determined from peak areas of the respective methyl esters.

The fluidity of the membrane lipids was expressed by the level of unsaturation, calculated as double bond index (DBI = 18:1 x 1 + 18:2 x 2 + 18:3 x 3).

Results and Discussion

Due to their immobile lifestyle, plant organisms are able to survive only by their ability to build rapid and highly adaptive responses to ever-changing environments. Under field conditions the situation is frequently much more complicated, since various interferences between numerous factors co-occur. As a means of overcoming abiotic and biotic limitations, plants have different adaptive and protective strategies.

As model plants to study structural-functional peculiarities of chloroplast membranes influenced by the specific environmental conditions some glycophytic and halophytic *Lactuca* species that inhabits different areas are chosen. *L. serriola* is a glycophytic drought-resistant species mainly found in solar habitats whether glycophytic *L. quercina* generally inhabits humid areas preferably in shadow or in partial shade, and is considered drought intolerant. *L. tatarica* is an extreme halophyte plant from Bulgarian Black sea coast.

The influence of environmental factors is manifested in alterations in structure of thylakoid membranes and the photochemical efficiency of photosystems, especially photosystem II (PSII) as main stress sensitive site in plants. The possibility of fast and reliable monitoring of the effectiveness of the operation of PSII oxygen-evolving enzyme complex of thylakoid membranes is the first prerequisite to solve the site and mechanisms of injury and adaptation to specific environmental conditions in different natural habitats.

TL glow curve parameters were used to access the functional features of PSII. TL proved to be a very sensitive and reliable biophysical method for investigation of the functioning of both PSII donor and acceptor side components (Sane, 2004). TL signals have been assigned to result from the thermal-activated recombination of the trapped electrons and stabilized positive equivalents on the reduced quinone acceptors (QA or QB) and on the S_2 (or S_3) oxidation state of the water-splitting complex, respectively. The illumination of dark-adapted chloroplast suspensions isolated from fully hydrated *Lactuca* leaves by continuous white light revealed a glow curve with a B band temperature maximum positioned at around 28.5–31 ◦C in *L. tatarica* (Table 1). The respective maximum in *L. seriola* and *L. quercina* chloroplast membranes appeared at lower temperature of 25-26°C and 23-24°C, respectively. The overall intensities of TL B-band were maximal in *L. seriola* samples and reaching minimal value in *L. tatarica* chloroplast membranes.

It is generally accepted that the amplitude of TL B-band is proportional to the number of centers having $S_{2(3)}$ Q_B^- charge pairs after flash illumination, while the maximal emission temperature of this band is a measure for redox span between the separate charges (Sane & Rutherford, 1986). It is reasonable to suggest that the dynamics in the relative number and stability of PSII reaction centers of the investigated *Lactuca* species can reflect some specific adaptive characteristics of the photosynthetic system of halophytic plants and glycophytic species with different drought tolerance to the environmental conditions in their natural habitats.

Table 1. Changes in the kinetic parameters of thermoluminescence (TL) and oxygen-evolving reactions of isolated *Lactuca* thylakoids. T_{max} (ºC) and B-band (%) are the emission temperature and the amplitude of the main TL B-band, recorded after one turn-over flash; A is the oxygen burst under continuous irradiation; A_1 and A_2, represent amplitudes of fast and slow components of initial oxygen burst.

Species	**T_{max} (°C)**	**B-band (%)**	**A (%)**	**A_1/A_2**
Lactuca seriola	25-26	100.0	100.0	2.46
Lactuca tatarica	28.5-31	70.7	72.3	2.22
Lactuca quercina	23-24	87.8	86.5	2.84

Another reliable approach used to study the properties of PSII complex in *Lactuca* thylakoids was to compare the kinetics of oxygen-evolving reactions (Table 1). The amplitude (A) of the initial oxygen burst and the area under the curve (which is a measure of the oxygen volume evolved) are proportional to all functionally active oxygen-evolving centers (i.e., both PSIIα in the grana and PSIIβ in the stroma domains). The decay kinetics after the oxygen burst are fitted with two exponential decay functions and the ratio A_1/A_2 of the obtained amplitudes for the fast (A_1) and the slow (A_2) components corresponds to the ratio of functionally active PSIIα to PSIIβ centers. The results suggest some decrease in the proportion of functionally active PSIIα centers in thylakoids in halophytic and drought tolerant *Lactuca* species which could be attributed to the reduced grana formation and dominant operation of the cooperative mechanism of oxygen evolution in stroma situated PSIIβ centers. (Maslenkova et al., 1993). It is supposed that the cooperative mechanism is realized by diffusion of oxygen precursors mainly within PSIIβ centers and is

characterized by a time constant lower than that of the non-cooperative Kok's mechanism, realized by PSIIα centers (Zeinalov & Maslenkova, 1996 b). The cycling of the rest operating PSIIα was typical for higher plants, thus suggesting no peculiarities in this parameter of investigated *Lactuca* species (data not shown).

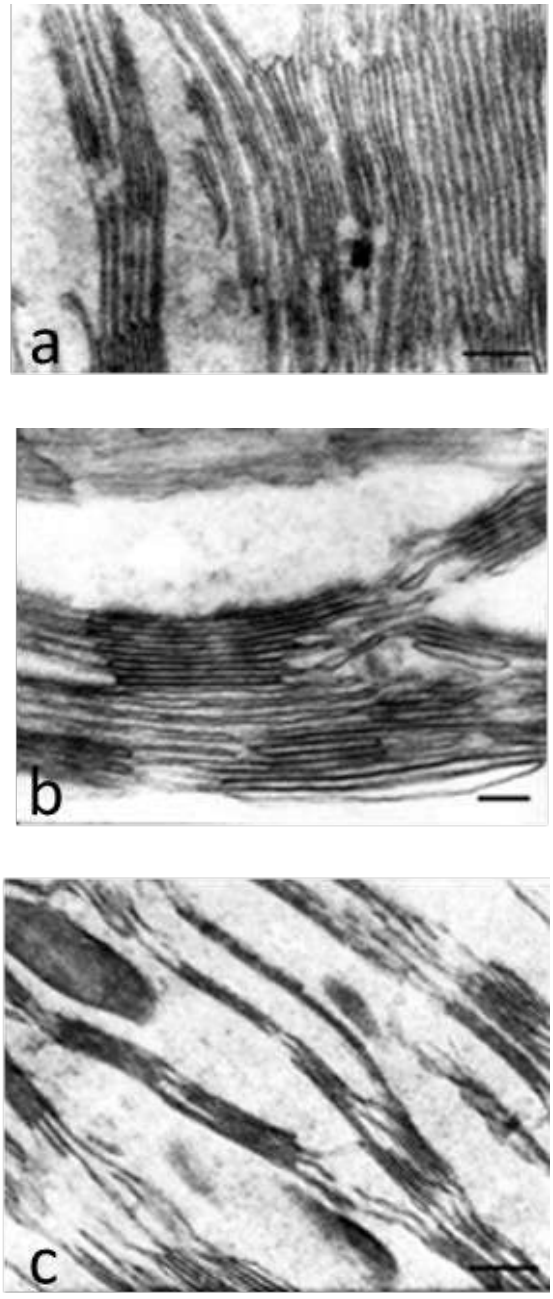

Fig. 1. Chloroplast ultrastructure in different *Lactuca* species:
a - *L. seriola,* **b** – *L. quercina,* **c –** *L. tatarica* (scale bar 200 nm).

The microscopic investigations obtained by TEM-analysis revealed that the structure of the chloroplasts in *L. seriola* were characterized by an elliptical shape and well-developed inner membrane system (Fig. 1-a). The grana represented different height. The number of thylakoids in them varies from 8-10 to 35, connected by evenly spaced stromal thylakoids of different lengths.

Chloroplasts in *L. quercina* are characterized by a well-developed inner membrane system composed of broad grana areas, most of them of low (5-8 thylakoids) and medium hight with about 15 thylakoids (Fig. 1-b). Most stromal thylakoids are short and fragmented between some faces.

Chloroplasts in halophyte *L. tatarica* species are characterized by a visibly smaller volume of the inner membrane system (Fig. 1-c). The grana are low (2 to 5-6 thylakoids) evenly distributed in the stroma and associated with long stromal thylakoids.

It is well known that lipids play an essential role in maintaining the integrity and functional activity of chloroplast membrane (Lichtenthaler, 1987). Differences in lipid and fatty acid composition may be species-specific or due to adaptation to varied environmental conditions. The lipid restructuring in the membranes as well as changes in the unsaturation of fatty acids play an important role in the acclimation of the photosynthetic machinery to changes in various forms of environmental stress (Allakhverdiev et al., 2010). In this respect the elucidation of the specific difference in the lipid classes and fatty acid composition of *Lactuca* membranes could bring important information in the interpretation of the obtained results of their functional activity (Table 2).

Table 2. Lipid classes in thylakoid membranes isolated from *Lactuca* leaves. The values obtained are means ± s.e. from three parallel measurements.

Species	Lipid classes (% of total)				
	MGDG	DGDG	SQDG	PG	DGDG/MGDG
L. tatarica	36.67±0.3	27.77±0.4	17.77±0.3	17.77±0.2	0.76
L. seriola	35.84±0.1	26.41±0.1	22.64±0.1	15.09±0.7	0.74
L. quercina	40.91±0.1	30.68±0.5	12.50±0.5	15.91±0.1	0.75

Photosynthetic membranes of plants are characterized by a high content of glycolipids dominated by galactolipids MGDG and DGDG. The fractions of MGDG and DGDG were higher in drought intolerant *L. quercina*, but the DGDG/MGDG ratio is nearly equal in all samples (Table 2). The fractions of the minor lipid constituents of thylakoid membrane show a gradual decrease of the amount of SQDG from *L. seriola* (22.64%) to *L. tatarica* (17.77%) and *L. quercina* (12.5%) while the difference in the amount of PG between investigated species was smaller.

Six common fatty acids were detected in the lipid classes of the respective thylakoid preparations, including palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) and the species variations in their relative content are presented in Table 3. The most abundant fatty acid in all the lipid classes was linolenic acid followed by palmitic and linoleic acids. The high level of unsaturation (Table 3), calculated as double bond index (DBI = 18:1 x 1 + 18:2 x 2 + 18:3 x 3) was most obvious for MGDG reaching slightly lower values in *L. quercina*. The content of polyunsaturated fatty acids in the lipid matrix is one of the major factors determining membrane fluidity. The high level of lipids unsaturation provides the mobility of lateral separated pigment-protein complexes and electron carriers in the electron transport chain thus affecting the effectiveness of photosynthetic machinery. It has been proven that polyunsaturated fatty acids in thylakoid lipids play an important role in the stability of oxygen evolving machinery and increases in unsaturated fatty acids in membrane lipids protects PSII against photoinhibition (Sui & Han, 2014).

Table 3. Species variations in relative fatty acids content of lipid classes. The values obtained are means ± s.e. from three parallel measurements; double bond index (DBI = 18:1 x 1 + 18:2 x 2 + 18:3 x 3); n.d. – not detected.

Lipid classes	Species	16:0	16:1	18:0	18:1	18:2	18:3	DBI
MGDG	*L. tatarica*	6.0±0.1	n.d.	1.6±0.2	0.5±0.7	2.6±0.2	86.5±0.1	265.2
	L. seriola	4.1±0.3	0.2±0.1	0.8±0.1	0.5±0.4	0.9±0.1	85.5±0.3	258.8
	L. quercina	10.5±0.3	n.d.	2.1±0.1	0.9±0.1	3.7±0.2	71.1±0.2	221.6
DGDG	*L. tatarica*	44.7±0.2	0.54±0.1	5.7±0.3	1.5±0.1	2.1±0.1	21.1±0.5	47.9
	L. seriola	23.9±0.5	1.0±0.2	2.8±0.1	0.8±0.1	1.5±0.1	24.7±0.3	77.8
	L. quercina	30.6±1.1	n.d.	5.6±0.1	1.5±0.4	3.2±0.1	45.0±0.3	97.9
SQDG	*L. tatarica*	40.2±1.3	8.9±0.2	5.2±0.2	2.2±0.3	8.6±1.8	25.6±0.1	96.2
	L. seriola	13.3±0.6	0.6±0.1	3.5±0.1	8.5±1.0	9.0±0.8	26.3±0.7	105.4
	L. quercina	33.0±0.1	7.0±0.3	5.1±0.1	2.0±0.1	9.3±0.2	33.2±1.1	120.2
PG	*L. tatarica*	32.1±1.1	10.5±0.5	5.9±0.1	2.4±0.1	11.2±0.1	25.8±0.3	102.2
	L. seriola	19.1±0.2	5.6±0.1	5.7±0.2	4.1±0.1	4.2±0.1	27.3±0.1	94.4
	L. quercina	32.9±0.4	10.2±0.2	3.5±0.2	1.8±0.2	11.8±0.1	24.8±0.1	99.8

Conclusion

During our studies of halophytic *L. tatarica* and glycophytic species *L. seriola* and *L. quercina*, using a highly sensitive TL and polarographic techniques were demonstrated high capability of chloroplast membranes to maintain efficient PSII function. In the same time, we observed some peculiarities of PSII redox reactions and mechanisms of oxygen evolution that can reflect specific adaptive strategies of the photosynthetic system, under environmental conditions in their natural habitat. Salt and drought tolerant species demonstrated some stabilization of PSII charge pairs, evidenced by higher temperature maximum of the main TL B peak and a decrease in the proportion of functionally active PSIIα centers which could be attributed to the reduced grana formation and dominant operation of the cooperative mechanism of oxygen evolution in stroma situated PSIIβ centers. The analysis of lipid classes and their fatty acids composition show the existence of qualitative and quantitative differences that can contribute in this regard. The results of our experiments show specific characteristics of photosynthetic membranes in halophytic and glycophytic *Lactuca* species, reflecting different adaptive strategies to environmental conditions in their natural habitats.

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Evaluation of Cytotoxic and Genotoxic Effects of Commonly Used Food Additives on the Root Meristem Cells of Allium cepa

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Abstract. The effects of the food preservative sodium benzoate, food sweetener aspartame and food colorant carmoizine have been studied on root tips of *Allium cepa* L. Roots of *A. cepa* were treated with a series of concentrations of food additives for 72 h. The results indicate that these food additives reduced values of mitotic index in meristem cells of *A. cepa* compared with the control samples. All concentrations of these chemicals showed an inhibitory effect on cell division. The frequencies of chromosomal aberrations were generally increased with increasing of substances's concentrations. All studied food additives induced c-mitosis, lagging and vagrant chromosomes and fragments and micronucleuses. Sodium benzoate and aspartame were the reason for the formation of anaphase bridges and diagonal anaphases. Only in the root tips were treated with aspartame were established pulverized chromosomes and multipolar metaphases and anaphases. The research of the cyto- and genotoxic effects of food additives used in food products are relevant, given their wide application and their unclear effect on human health.

Key words: carmoisine, sodium benzoate, aspartame, root meristem cells, *Allium cepa*.

Introduction

Since the middle of the 20th century and still today, the use of food additives is becoming more widespread. Manufacturers use them in various food products, medicines and cosmetics in order to improve their consistency, taste, achieve the desired organoleptic properties, increase their use value and longer shelf life (Leathwood et al., 2007, Carocho et al., 2014). Unfortunately, however, these additives can have adverse health effects (Jarskog, 2006; Kitano et al., 2002).

The use of food additives is regulated in Codex Alimentarius and is justified only when it does not pose a risk to the health of consumers, does not mislead the consumer and gives an advantage to the product concerned, which cannot be achieved in any other economic and technological way (Codex Alimentarius, FAO, 2011).

The legislation of most countries adheres to a list of permitted and prohibited additives and their maximum permitted amounts. As a result of numerous studies on the negative effects of synthetic colours on the human body, their number in the permitted lists is constantly decreasing. It has been shown that regular consumption of azo colours in food can lead to allergic reactions, anemia, binding to blood hemoglobin, kidney and liver diseases, hyperactivity and hyperexcitability in children, asthma, especially if they are used in larger quantities (Amichova et al., 2015; Basu & Kumar, 2015; Oplatowska-Stachowiak & Elliot, 2017).

Toxic, mutagenic and carcinogenic effects of azo colours, including carmoisine, have been found in studies by Zhang & Ma (2013a). Carmoisine is a widely used colouring agent whose application is responsible for altering biochemical markers in murine vital organs, modifying the secondary structure of serum proteins (human serum albumin and bovine serum albumin), and increasing the conformational changes in the DNA of bovine cell lines (Amin et al., 2010; Arvin et al., 2013; Datta et al., 2013).

Carmoisine can cause side effects in asthmatics and people allergic to aspirin, (Martin, 2007). This colorant is banned in the United States (Mahfoz et al., 2010). A study by Macioszek and

Kononowicz (2004) showed that some colouring agents and food additives can cause genotoxic effects in human lymphocyte cells and in the root meristem of *Vicia faba in vivo*. The effect of this synthetic colouring agent on mitotic activity, induction of abnormalities in the course of mitosis and changes in DNA structure was also studied using the *Allium cepa* test system (Mahfoz et al., 2010). The results obtained show that carmoisine causes a decrease in the mitotic index, changes in mitotic phase indices and induces a wide range of chromosomal abnormalities in dividing cells. These changes are accompanied by variation in the content of nucleic acids. Electrophoretic analysis has shown that the colorant has the ability to change the size of proteins (Mahfoz et al., 2010).

Antimicrobial additives (benzoic acid and benzoates, including sodium benzoate) are of limited use and act by destroying the membrane and inhibiting metabolic reactions in the microbial cell (Brul and Coote 1999).

The preservatives benzoic acid and sodium benzoate have been tested in vitro and are considered non-toxic, but some authors have found their clastogenic, mutagenic and cytotoxic effects using the *Drosphila* SMART test, the *Allium* test and human lymphocyte cell lines (Nair, 2001; Yilmaz et al. 2008, 2009; Zengin et al. 2011). Sodium benzoate has also been reported to intercalate with DNA in the nucleus of bovine thymus cells at concentrations up to 4.5x10-5 mol/L (Zhang and Ma 2013b).

A synergistic effect of sodium benzoate and food colouring agents, expressed in hyperactivity in children and students was found in a study by Beezhold et al. (2014), which confirms the need for further studies.

Flavoring agents are additives used to change the taste of food and are divided into 3 subgroups: sweeteners, natural and synthetic flavours and flavour enhancers.

Aspartame is a widespread low-calorie sweetener. Extensive studies have been conducted on aspartame, and while some ensure its safety (Jeffrey and Williams, 2000; Mukhopadhyay et al., 2000), others have drawn alarming conclusions (Ashok et al., 2013; EFSA 2013; Rycerz & Jaworsaka-Adamu, 2013). Other studies have shown that long-term consumption of aspartame can lead to hepatocellular damage and changes in liver antioxidant status and behaviour in rats (Abhilash et al., 2011; Ashok et al., 2013).

The cytotoxic and genotoxic effect of aspartame concentrations of 100, 300 and 400 mg/l was established by De la Cruz et al., (2013) in the root meristem cells of onion bulbs. The authors found a decrease in the mitotic index depending on the concentration of the sweetener. The progressive increase in nuclear bud formation as a function of the increasing concentration of aspartame proves the possible genotoxicity of this sweetener. Nuclear buds are probably the result of the elimination of excess genetic material obtained from the processes of polyploidization and hyperamplification of DNA, which subsequently become micronuclei (Fernandes et al., 2007; Prieto et al., 2008).

There are certain advantages associated with the use of plant test systems in procedures for cytogenetic analysis and screening for toxic and mutagenic effects of various substances in the environment (Grand, 1999; Iganci et al., 2006; Leme & Marin-Morales, 2008; Samoilov et al., 2019). Compared to other tests that use animals or cell cultures, the *Allium* - test turns out to be less complicated and expensive and more sensitive, and the *Allium cepa* species is indicated as an effective system for assessing cytotoxicity (Leme & Marin-Morales, 2008; Gomez et al., 2013; Nunes et al., 2011), which facilitates the analysis to detect changes in the intensity of cell division (Kuras et al., 2006; Tabrez et al., 2011) and damage to the structure of DNA (Carita & Marin-Morales, 2008; Herrero et al., 2012).

Studies of the cyto- and genotoxic effects of colouring agents, preservatives and sweeteners used in food products are relevant, given their wide application and their unclear effect on human

health. For our country, such studies have not been conducted, which determines the interest in developing a combined approach for cytogenetic characterization of their impact in vivo. The purpose of this study is the assessment of cytotoxic and genotoxic effects of the commonly used food additives carmoisine (E122), sodium benzoate (E211) and aspartame (E951) on the root meristem cells of *Allium cepa*.

Material and Methods

For the purposes of the study, untreated bulbs of Allium cepa, Asenovgradska kaba variety with a diameter of 2.5-3 cm were used, provided by the Maritsa Vegetable Crops Research Institute in the city of Plovdiv. The food additives were purchased from Trimart Ltd. and accompanied by an analytical certificate for use in the food industry. The solutions of the colorant carmoisine, the preservative sodium benzoate and the sweetener aspartame (E951) with different concentrations presented in Table 1.

Table 1. Concentrations of the tested food additives (by Prajitha & Thoppil, 2016; Yilmaz et al., 2008; De la Cruz et al., 2013).

Carmoisine (E122)	Sodium benzoate (E211)	Aspartame (E951)
0.005% (0.05 g/l)	50 mg/l	400 mg/l
0.01% (0.1 g/l)	100 mg/l	600 mg/l
0.1% (1 g/l)	200 mg/l	800 mg/l
1% (10 g/l)	500 mg/l	1000 mg/l

Five bulbs per each concentration were placed in tubes with a volume of 50 ml containing food additive solutions, away from direct sunlight for 72 hours. In the control sample, distilled water was used. After the treatment period, the sprouted roots were separated from the bulbs with the help of a scalpel, fixed with a Clarke's fixative and stored in 70% ethanol at 4˚C.

Acetocarmine-stained root apical meristem was used to prepare the temporary microscopic slides. From each experimental sample and from the control one, 5 preparations were prepared, which were observed under a light microscope at a magnification of x400 and pictures were taken with a digital camera for microphotography. At least 1000 cells for each preparation were analyzed.

The effect of different concentrations of substances on the intensity of cell division was determined by calculating the mitotic index according to the following formula:

$$\text{IM}\,(\%) = \frac{N'}{N} \times 100,$$

Where: IM is the mitotic index calculated in%,
N′ is the number of dividing cells, and
N is the total number of cells analyzed.

The genotoxic effect of food additives was analyzed using an anaphase method and a micronucleus mutagenicity test. The frequency of chromosomal aberrations in the meristem cells of *Allium cepa* was calculated as a percentage of the number of dividing cells and relative to the total number of analyzed cells according to the formulas:

$$CAI^{N'}(\%) = \frac{CA}{N'} \times 100,$$

Where: CAI $^{N'}$ is the frequency of chromosomal aberrations relative to the number of dividing cells, calculated in %,
CA is the number of cells with chromosomal aberrations, and

N' is the number of dividing cells.

$CAI^N(\%) = \frac{CA}{N} \times 100$, Where: CAI ^{N} is the frequency of chromosomal aberrations relative to the total number of cells, calculated in %,
CA is the number of cells with chromosomal aberrations, and
N is the number of all cells analyzed.

Different types of aberrations have been reported at different stages of cell division - lagging and 'vagrant' chromosomes, acentric fragments, chromosome bridges, micronuclei, C-mitosis and asynchronous mitosis.

The spectrum of chromosomal aberrations was analyzed as an indicator of the specificity in the mutagenic action of food additives.

Comparisons between treated and control samples were subjected to statistical analysis using Student's t-test to assess the significant differences between them.

Results

The effect of carmoisine (E122), sodium benzoate (E211) and aspartame (E951) on the intensity of cell division, expressed by the value of the mitotic index in the various experimental sample and in the control sample is presented in Table 2.

Table 2. Values of mitotic index and phase indexes (%) (X±SD) in the control and experimental samples treated with different concentrations of carmoisine (E 122), sodium benzoate (E 211) and aspartame (E951). Legend: *p<0.05, **p<0.01, ***p<0.001.

Food additives		Mitotic index %	Phase indexes %			
			Prophase index	Metaphase index	Anaphase index	Telophase index
E 122	0.05 g/l	48.65±11.34	92.96±3.27	2.81±1.3	1.5±0.79	2.72±1.5
	0.1 g/l	48.56±5.41*	89.34±4.17	4.22±1.96	3.26±1.31	3.17±1.35
	1 g/l	50.85±4.37	89.57±1.86	4.02±0.5	3.59±0.82	2.81±0.93
	10 g/l	31.12±11.1***	90.24±4.16	5.21±2.88	2.06±1.04	2.49±1.51
E 211	50 mg/l	34.83±10.68***	86.73±12.59	6.04±4.48	3.08±2.35	4.16±5.4
	100 mg/l	33.79±10.3***	99.79±0.35	0.21±0.35	0	0
	200 mg/l	35.69±4.72***	100±0	0	0	0
	500 mg/l	27.95±7.26***	100±0	0	0	0
E 951	400 mg/l	52.88±3.59	92.26±2.74	3.26±1.8	2.7±0.89	1.43±0.68
	600 mg/l	44.8±5.88***	91.18±3.57	4.23±1.54	2.91±1.78	1.67±0.79
	800 mg/l	45.28±4.12**	95.76±1.23	1.78±0.66	1.91±0.5	0.55±0.4
	1000 mg/l	33.95±3.33***	100±0	0	0	0
Control		51.58±1.5	93.42±2.91	2.51±1.16	1.9±0.96	2.17±0.92

The data presented in Table 2 show that in all experimental samples (except the lowest concentration of sweetener) the values of the mitotic index are lower than the value in the control sample. The preservative sodium benzoate has the strongest effect on reducing the intensity of cell division. Significant differences in mitotic index values were found at all preservative concentrations studied and at the highest dissolved carmoisine concentration. At a concentration of

aspartame higher than 600 mg/l, the intensity of cell division is significantly reduced in the experimental samples.

The genotoxicity study was performed taking into account structural aberrations of chromosomes and other mitotic abnormality, most often associated with changes in DNA helix and changes in the structure of the dividing spindle, which are visualized as different changes in chromosome behaviour during different stages of mitosis. The analysis of the type and frequency with which the disorders are observed allows characterization and interpretation of the mutagenic effects of the chemical compounds.

The total number and frequency of chromosomal aberrations among dividing cells and among all analyzed cells in the experimental and control samples are presented in Table 3.

Table 3. Total number and frequency of chromosomal aberrations among dividing cells and among all analyzed cells in the experimental and control samples (X±SD) of carmoisine (E 122), sodium benzoate (E 211) and aspartame (E951).

Food additives (concentration)		**Total number of chromosomal aberrations**	**Frequency of chromosomal aberrations among:**	
			Dividing cells	**All analyzed cells**
E 122	0.05 g/l	8±2*	1.66±0.72***	0.76±0.2***
	0.1 g/l	12.8±2.59***	2.58±0.4***	1.25±0.25***
	1 g/l	12.2±3.96***	2.3±0.81***	1.16±0.36***
	10 g/l	12.2±4.38***	4.09±1.55***	1.18±0.42***
E 211	50 mg/l	10.8±5.02**	3.77±3.66**	1±0.46***
	100 mg/l	4.4±9.84	1±2.24	0.43±0.97
	200 mg/l	5.4±3.51	1.5±0.94**	0.52±0.35*
	500 mg/l	-	-	-
E 951	400 mg/l	9.6±4.56*	1.65±0.9***	0.85±0.38***
	600 mg/l	12±4***	2.67±1.07***	1.16±0.39***
	800 mg/l	12.2±1.3***	2.53±0.43***	1.13±0.1***
	1000 mg/l	-	-	-
Control		5.6±2.88	0.53±0.29	0.27±0.24

The total number of chromosomal aberrations in all colorant concentrations was significantly higher than the number of aberrations in the distilled water samples. The lowest concentration of carmoisine showed an average of 8 aberrations per microscopic slide, and in the three higher concentrations the number of cells with different aberrations in the chromosomes or in the structure of the dividing spindle is on average from 12.2 to 12.8.

An interesting dependence was found in the samples with different concentrations of the preservative E211. At the lowest concentration, an average of 10.8 aberrations per microscopic slide were detected. In the next two concentrations (100 mg/l and 200 mg/l) the number of aberrations was 4.4 and 5.4. These values are lower than the value in the control sample, although the differences are insignificant. This can be explained by the low value of the mitotic index and index of metaphase, and by the lack of dividing cells in anaphase and telophase (Table 2). The registered aberrations in these slides are micronuclei and those associated with disorders in the formation of the dividing spindle (C-mitosis).

At the highest concentration of the preservative, dividing cells are found only in prophase. This is also observed in the highest concentration of the sweetener. The dates in Table 2 show that sodium benzoate in concentrations above 200 mg/l and aspartame in concentrations above 1000

mg/l block cell division in prophase. In the lower concentrations of aspartame, an average number of chromosomal aberrations from 9.6 to 12.2 per slide were registered.

More precise indicators of the genotoxic effect of food additives in the cells of the growing root meristem of onion are the percentages of aberrations to the number of dividing cells and the total number of analyzed cells.

At all concentrations of food additives studied, the frequency of chromosomal aberrations was significantly higher than in the control sample. The presence of dividing cells only in prophase, without chromosomal aberrations, is the reason why in the highest concentrations of preservative (E211) and sweetener (E951) no values were recorded for these two indicators.

The specificity in the mutagenic action of the analyzed substances is expressed in the spectrum of chromosomal aberrations that provoke.

All three studied additives induce C-mitosis, lagging and vagrant chromosomes and fragments and micronuclei. Sodium benzoate and aspartame cause the formation of anaphase bridges and diagonal anaphases. Only in the cells of the roots treated with aspartame, pulverized chromosomes and multipolar metaphases and anaphases are found.

Discussion

The mitotic index is the indicator that correlates directly with the intensity of cell division. According to Fiskesjo (1985), the study of cell proliferation activity, expressed by the mitotic index, can detect the presence of cytotoxic effects from various environmental factors (Fiskesjo, 1985). In all experimental samples the values of this indicator are lower than its value in the control sample. An exception is found only in samples with a concentration of aspartame 400 mg/l, but the differences between this value and the one registered in the control sample are insignificant.

At a concentration of aspartame higher than 600 mg/l, the intensity of cell division is significantly reduced in the experimental samples. The preservative sodium benzoate reduces cell proliferation the most. Significant differences in mitotic index values were found at all studied preservative concentrations, as well as at the highest dissolved carmoisine concentration.

De la Cruz et al. (2013) reported a cytotoxic effect of aspartame concentrations of 100, 300 and 400 mg/l on the root meristem of onions and a decrease in the mitotic index depending on the concentration of the sweetener, which is confirmed as a trend also in the present study (Table 2) (De la Cruz et al., 2013).

When treating onion seeds for 24 and 48 hours with 0.25%, 0.5%, 0.75% and 1% solutions of carmoisine, Khan et al. (2020) found that increasing the concentration of the colouring agent decreases the mitotic index. In the study we have conducted we found a similar relationship - the highest concentration of carmoisine of 10 g/l (which corresponds to the highest in the study of Khan et al., 2020 of 1%), is the reason for the lowest value of mitotic index.

A lower value of the mitotic index in the experimental samples compared to the control one demonstrates suppression of cell proliferation and hence the growth and development of organisms affected by the test compounds (Hoshina, 2002; Mahfoz et al., 2010). Samoilov et al. (2019) report a synergistic effect of the colorant carmoisine and the sweetener sucrose, expressed in slowing down the growth of onion roots. Inhibition of cell division may be associated with prolongation of cell differentiation time (Fusconi et al., 2006), decreased protein synthesis or other effects on the apical meristem (Webster & MacLeod, 1996).

The reported structural aberrations of chromosomes and other mitotic abnormalities are evidence of the mutagenic effect of food additives on the DNA molecule and the structures that make up the dividing spindle. They are visualized as different changes in the behaviour of chromosomes during the different stages of mitosis. The analysis of their type and frequency allows to characterize the specifics in the action of the chemical compounds on the chromatin and the cell division apparatus.

The percentage of chromosomal aberrations in relation to dividing cells and in relation to the total number of analyzed cells are the lowest in the control sample, respectively 0.53 ±0.29 and 0.27±0.24. Although low in number and frequency, aberrations of the C-mitosis type and vagrant chromosomes were reported in the control samples (Fig. 1 and 2).

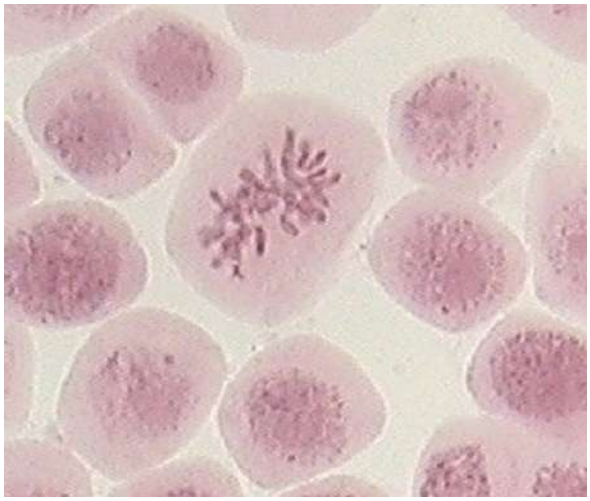

Fig. 1. C-mitosis in control sample of *A. cepa*, magnification x 400.

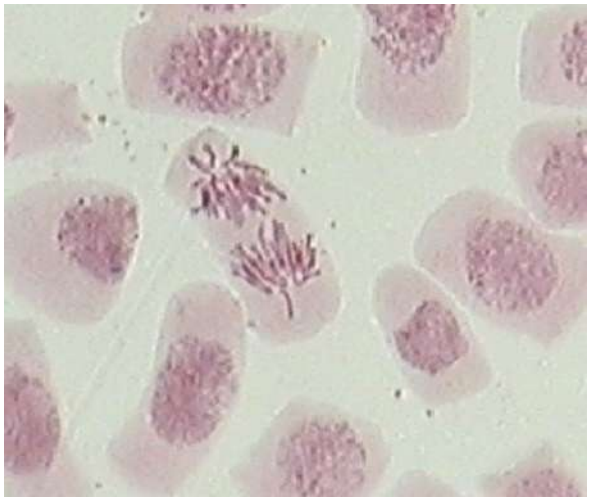

Fig. 2. „Vagrant" chromosome in control sample of *A. cepa*, magnification x 400.

They are due to the automutagenic effect, which is explained by individual characteristics or by peculiarities in the cultivation of the plants. In their study, Azhar et al., 2019 found no chromosomal aberrations in *A. sativum* control cells, but in the *A. cepa* control, micronuclei were found in 0.11% of the cells in interphase.

In all carmoisine solutions, the frequency of chromosomal aberrations was significantly higher than in the control sample (Table 3). Aberrations of the C-mitosis type, lagging and vagrant chromosomes, fragments and cells with micronuclei were found (Fig. 3 and 4).

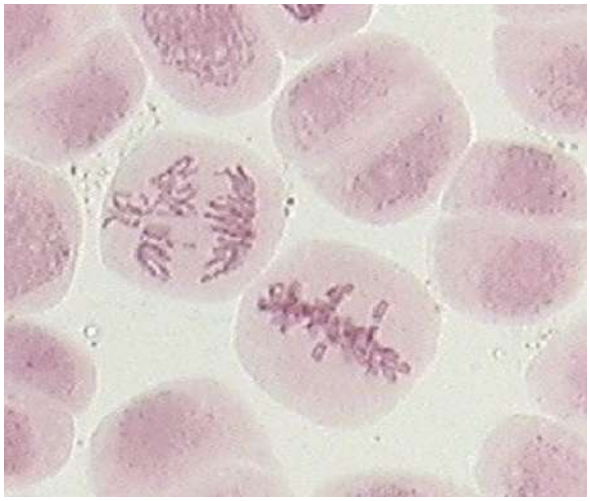

Fig. 3. Lagging chromosomes, vagrant chromosomes and fragments in sample of *A. cepa* treated with carmoisine, magnification x 400.

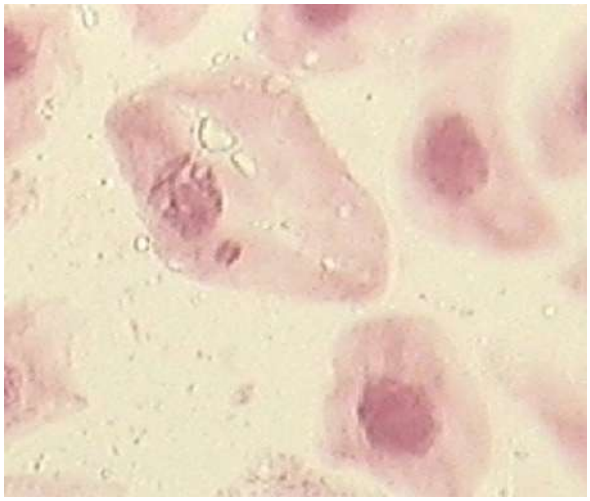

Fig. 4. Micronucleus in sample of *A. cepa* treated with carmoisine, magnification x 400.

In the three lower concentrations of the preservative E211, the frequency of chromosomal aberrations was found to be from 1 to 3.77% in relation to dividing cells and from 0.43 to 1% in relation to the total number of analyzed cells. No dependence of the frequency of aberrations on the concentration of sodium benzoate was established. The frequencies are highest in solutions with a concentration of 50 mg/l, and in the next two concentrations, these percentages decrease.

This unusual trend can be explained by the lower values of the mitotic index and the index of metaphase (sodium benzoate at concentration of 100 mg/l) and the established lack of cells in metaphase, anaphase and telophase (sodium benzoate at concentrations of 200 mg/l and 500 mg/l). At the two highest concentrations of the preservative, dividing cells are detected only in prophase. The registered aberrations are associated with disorders in the formation of the dividing spindle (Fig. 5 and 6) and the micronuclei formation. At the highest concentration of sweetener, the mitotic index is also the lowest and all dividing cells are in prophase. This indicates that sodium benzoate at concentrations above 200 mg/l and aspartame at concentrations above 1000 mg/l block cell division in prophase.

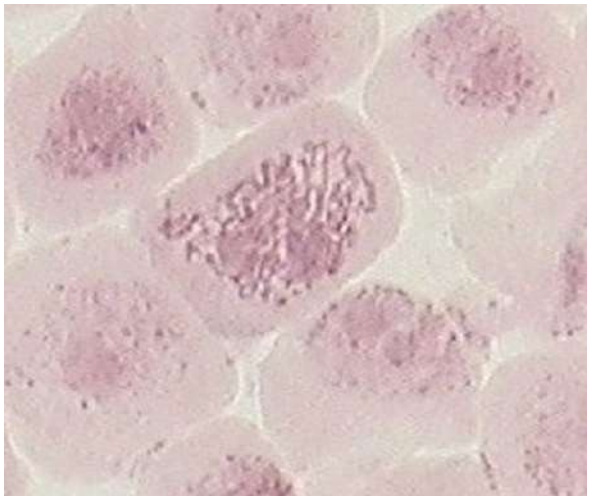

Fig. 5. Disturbed spiralization in cells of *A. cepa*, treated with sodium benzoate, magnification x 400.

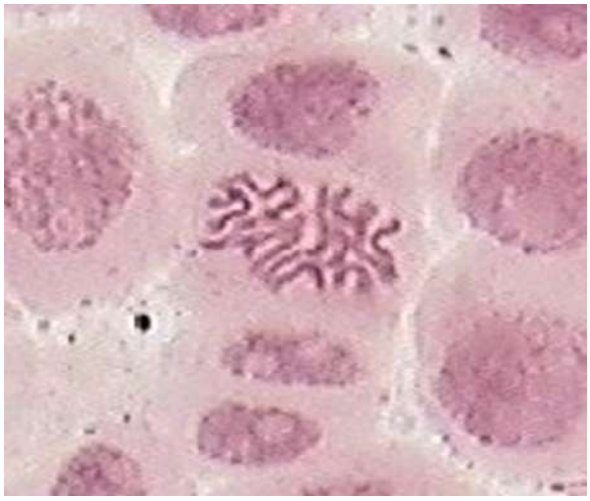

Fig. 6. C-mitosis in cells of *A. cepa*, treated with sodium benzoate, magnification x 400.

Zabka et al. (2012) found that treatment with certain chemicals disrupts the proper course of the cell cycle and activates the mechanisms of control and retention of cell division known as 'reference points'. When cells are exposed to adverse conditions, for example under the influence of a toxic agent, this could disrupt division and lead to abnormal mitosis (Rybaczek et al. 2007).

In addition to the chromosomal aberrations: C-mitosis, lagging and vagrant chromosomes, fragments and cells with micronuclei, found in the samples treated with the colouring agent carmoisine, diagonal anaphases and anaphase bridges were found in the meristem cells of onions under the influence of sodium benzoate (Fig. 7 and 8).

The spectrum of structural mutations in chromosomes and mitotic disorders provoked by the various tested concentrations of aspartame is the widest. The studied sweetener additionally causes deviations of the type: multipolar metaphases and anaphases and pulverized chromosomes (Fig. 9-12).

C-mitosis occurs as a result of improper organization of the microtubules that make up the filaments or the dividing spindle itself (Fiskesjo, 1988; Haliem 1990). The most likely cause of anaphase bridges is adhesion as a result of chromatid separation errors (Gaulden, 1987), caused by

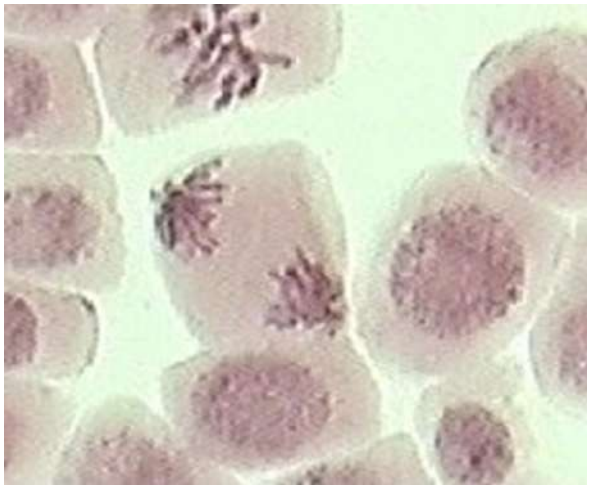

Fig. 7. Diagonal anaphase in cells of *A. cepa*, treated with sodium benzoate, magnification x 400.

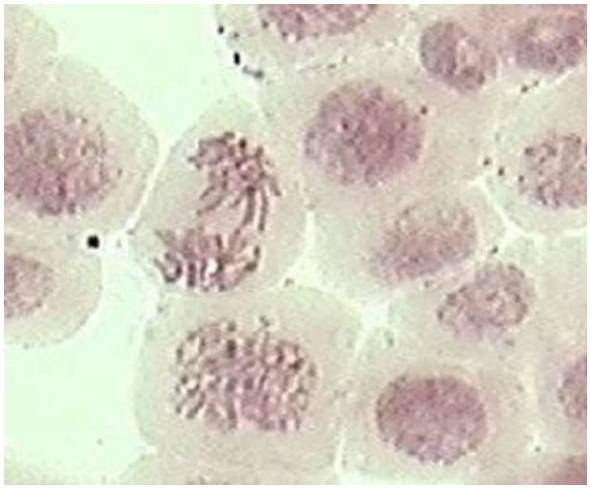

Fig. 8. Anaphase bridge in cells of *A. cepa*, treated with sodium benzoate, magnification x 400.

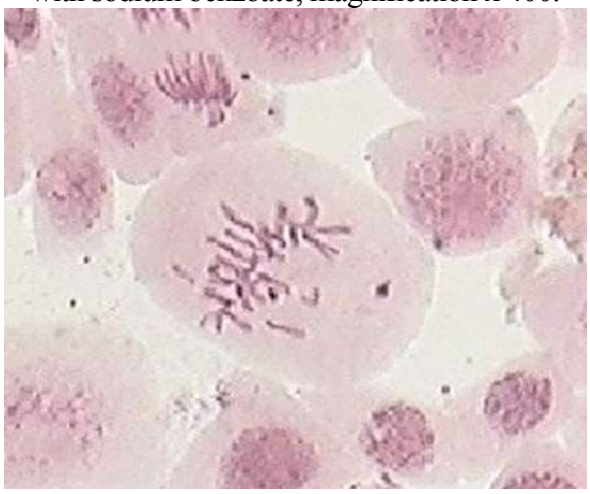

Fig. 9. Lagging chromosomes and fragments in metaphase in cells of *A. cepa* treated with aspartame, magnification x 400.

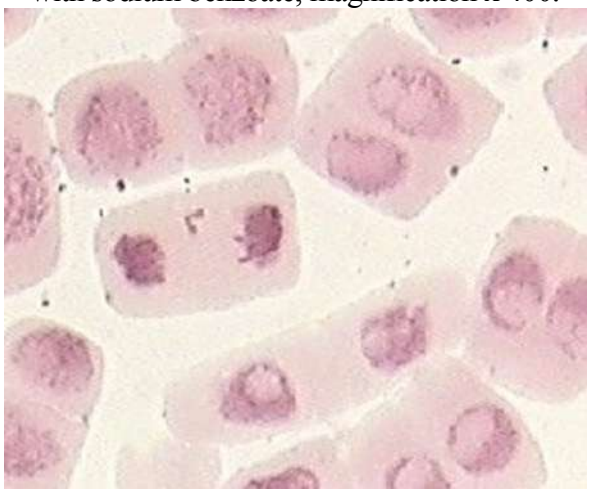

Fig. 10. Acentric fragment in telophase in in cells of *A. cepa* treated with aspartame, magnification x 400.

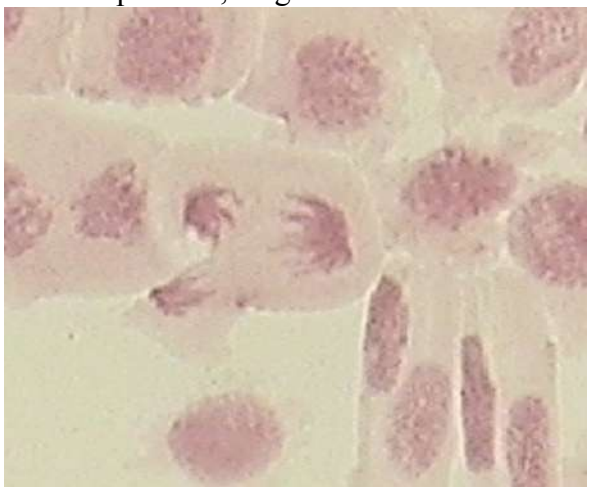

Fig. 11. Multipolar anaphase in cells of *A. cepa*, treated with aspartame, magnification x 400.

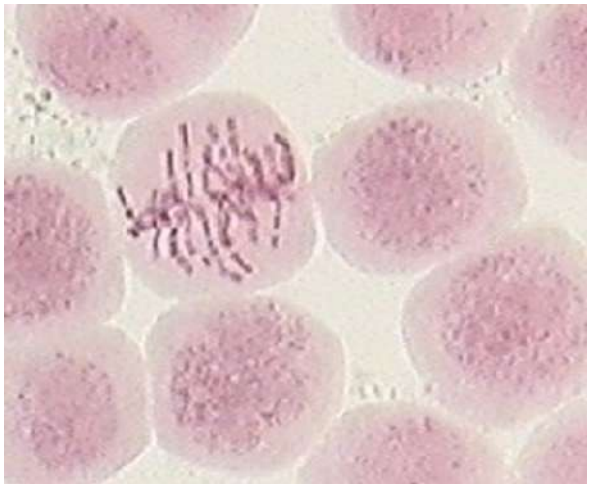

Fig. 12. Pulverized chromosomes in cells of *A. cepa*, treated with aspartame, magnification x 400.

The mutagenic effect of carmoisine has been found in human, murine and bovine cell lines (Zhang & Ma, 2013a Amin et al., 2010; Arvin et al., 2013; Datta et al., 2013).

A study by Macioszek & Kononowicz (2004) showed that some colorants and food additives can cause genotoxic effects in human lymphocyte cells and in the root meristem of *Vicia faba in vivo*. Using the *Allium cepa* – test system, Mahfoz et al. (2010) showed that carmoisine induces a wide range of chromosomal abnormalities in dividing cells. Tripathy & Rao (2015) found decondensation of chromosome arms in *Allium cepa*, treated with the colorant.

Sodium benzoate has a clastogenic and mutagenic effect, demonstrated by applying the Drosophila SMART test, *Allium* – test and in human lymphocyte cell lines (Nair, 2001; Yilmaz et al. 2008; 2009; Zengin et al. 2011). At concentrations up to 4.5x10-5 mol/L, sodium benzoate is intercalated with DNA in the nucleus of thymus cells in cattle (Zhang & Ma 2013b).

In the cells of the root meristem of onion De la Cruz et al. (2013) found a genotoxic effect of aspartame in concentrations of 100, 300 and 400 mg/l. The genotoxicity of increasing aspartame concentrations was observed as polyploidization and hyperamplification in Oreochromis niloticus and *Allium cepa* cells. The elimination of this additional DNA is the probable cause of the formation of nuclear buds, which subsequently turn into micronuclei (Fernandes et al., 2007; Prieto et al., 2008).

Conclusions

As a result of the study conducted and analysis of the results obtained, it can be concluded that carmoisine, sodium benzoate and aspartame used in food production have a cytotoxic effect on the cells of the root meristem of *Allium cepa*. It has been expressed in the suppression of cell division and the reduction of the mitotic index. The studied food additives have a specific genotoxic effect. It has been established by anaphase method and micronucleus mutagenicity test that all three substances cause chromosomal aberrations of the C-mitosis type, 'vagrant' and lagging chromosomes, fragments and micronuclei. Sodium benzoate and aspartame provoke the formation of chromosomal bridges and diagonal anaphases, and only the sweetener aspartame causes the formation of pulverized chromosomes and multipolar metaphases and anaphases.

Acknowledgements

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Overcoming Phytoplasma Infection in Paulownia tomentosa by Meristem In vitro Culture

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Abstract. Paulownia witches' broom (PaWB) is caused by phytoplasma of Aster Yellows group and may result in severe damage to the growth and premature death of the infected tree. *In vitro* cultures of meristems are important tools for solving problems with plant diseases. In the present study, meristem cultures from phytoplasma infected *Paulownia tomentosa* have been created in an attempt to obtain healthy plants. The faster procedure with longer explants generated 95% regeneration but no healthy plants. The combination of smaller explants and thermotherapy resulted into the generation of plantlets with a healthy phenotype. The PCR analyses showed the absence of phytoplasma only in one of the produced plants. Despite the low efficiency, expressed in one phytoplasma-free regenerant of a total of 480 explants, these results can serve to continue the work on optimizing the used strategy.

Key words: meristem plant tissue culture, thermotherapy, Paulownia witches' broom (PaWB).

Introduction

Paulownia tomentosa (royal paulownia, empress, or princess tree) is a deciduous and fast-growing tree of the family Paulowniaceae. It has several desirable characteristics and nowadays is grown in many countries including China, U.S.A., west and south Europe (Newman et al., 1998; Ipekci & Gozukirmizi, 2004). It is not only an ornamental tree but also multipurpose species with potential medicinal uses, a source of renewable energy and also used for reforestation and reclamation of mine sites (Rao et al., 1996; Ozaslan et al., 2005; Barton et al., 2007). Paulownia trees have been cultivated for their commercial importance but they are susceptible to Paulownia witches' broom (PaWB), which is one of the most devastating diseases caused by phytoplasma of the Aster Yellow group "*Candidatus Phytoplasma asteri*" (Doi et al., 1967). Aster yellows (AY) group (16SrI) phytoplasmas are associated with over 100 economically important diseases worldwide and represent the most diverse and widespread phytoplasma group. Phytoplasmas are cell wall-less plant pathogenic bacteria that belong to the class Mollicutes and have unique biology among bacteria. These obligate intracellular parasites need plants and insects for survival in nature and they can efficiently reproduce in both hosts (Hogenhout, 2009). The geographical distribution and impact of phytoplasma diseases depend on the host range of the phytoplasma as well as on the presence and the feeding behaviour of the insect vector. Characteristic symptoms associated with phytoplasma infection include witches' brooms (proliferation of auxiliary or axillary shoots), short internodes, yellowing or reddening of leaves, phyllody (the development of floral parts into leafy structures), stunting and decline, virescence (the development of green flowers and the loss of normal flower pigments), sterile flowers and necrosis (Khadhair et al., 2001; Namba, 2002; Bayliss et al., 2005; Fan et al., 2015). Plants infected by members of the 16SrI subgroups include general

stunting (little leaves, small flowers, shortening of internodes), leaf curl or rolling, small and faintly coloured flowers, and some symptoms that are typical of the AY syndrome (Lee et al., 2004).

Studies have been carried out to understand the interaction of paulownia with phytoplasma but the molecular mechanism of PaWB occurrence remains elusive (Liu et al., 2013; Cao, 2014).

In vitro culture techniques represent successful strategies for the production of pathogens-free plants (Panattoni et al., 2013; Smith et al., 2017). Meristem tip culture is extensively used for eradication of plant pathogens, which involves the removal and *in vitro* placement of dome-shaped meristematic tissue on the culture media for the development of the whole plant. This technique has been used alone or in combination with other treatments for virus elimination from plants. Meristem tip culture of 0.3 mm has been useful to eliminate the virus in sweet potato and cassava. However, meristem tips of larger size (1-2.5 mm) have also given satisfactory results in virus elimination when heat treatments are provided (Nehra & Kartha, 1994; Varveri et al., 2015).

The aim of the present study was to regenerate healthy plants from PaWB-infected *Paulownia tomentosa* using the meristem cultures technique. Plant regeneration has been achieved from excised tissues with variation in their sizes and combined with thermotherapy and callus tissue.

Material and Methods

Plant materials. PaWB-infected plants were cultivated on MS media (Murashige & Skoog, 1962) containing 30 g/L sucrose and 7 g/L plant agar. The pH of the media was adjusted to 5.8 before autoclaving. The culturing conditions were 16-h photoperiod with cool-white fluorescent tubular lamps at 25±2 °C.

Meristem plant tissue culture. Under a laminar flow hood, meristem tips were removed by sterile dissection under microscope from apical shoots and axillary buds of infected plants. The explant comprises the apical dome and a limited number of the youngest leaf primordia and has the potential for excluding pathogenic organisms that have been present in the donor plants. The explant size plays a major role in meristem culture and we tested variants. The first pattern for meristem culture was with tips 1-2.5 mm in length, which made the experiment faster and easier, compared to the second variant where the meristem tips were with smaller size (0.5 mm). In the third variant, we used explants 0.5 mm in length but donor plants were preliminarily cultivated for one week at 40 °C (thermotherapy). Tips from the described three experimental patterns were cultured in Petri dishes, each with 20 mL of initiation MS media containing 0.2 mg/L NAA (α-naphthalene acetic acid) and 0.5 mg/L GA_3 (gibberellic acid) (20 tips per Petri in two repeats). Each experiment was performed in triplicate. The samples were cultured at 25±2 °C in the dark for two weeks. Thereafter, they were transferred at a light with 16/8 h (light/dark) photoperiod for another week. Twenty-one days after the beginning of initiation, produced shoots were excised and transferred to elongation MS media containing 1 mg/L BAP, 0.1 mg/L NAA, and 1 mg/L GA_3. Four weeks after the beginning of elongation, growing seedlings were observed for morphological changes.

In the fourth pattern, a combination of callus and meristem tissue culture was tested, where explants (1-2.5 mm in length) were introduced to MS media with 5 mg/L BAP (6-benzylaminopurine) and 0.5 mg/L GA_3. Two weeks explants were in the dark at 25±2 °C, thereafter Petri dishes were moved to cool-white fluorescent tubular lamps light (16/8 photoperiod) for two more weeks. The formed callus was transferred to MS media with 1 mg/L BAP and 0.1 mg/L NAA for shoot induction. Every four weeks callus tissue was transferred to fresh media. Excised shoots were grown on MS media without plant growth regulators.

The newly formed shoots with healthy phenotype were tested for PaWB detection through PCR analysis.

DNA extraction. Total DNAs were extracted from analysed shoots with innuPREP Plant DNA kit (Analytik Jena AG, Jena, Germany) following manufacturer's instructions. The quality and concentration of isolated DNA were estimated on 1% agarose gel and spectrophotometric analysis.

PaWB phytoplasma detection. In direct PCR assays, amplification of phytoplasma 16S rDNA was performed by using R16F2n (GAAACGACTGCTAAGACTGG) and R16R2 (TGACGGGCGGTGTGTACAAACCCCG) universal phytoplasma primers to amplify DNA fragments of 1.24 kb. Each reaction was performed and PCR products were analysed as previously described by Lee et al. (1993). DNA products from PCR assays were separated in 1% agarose gels and compared with a standard molecular marker (2-log DNA Ladder 0.1-10kb, New England Biolabs, Ipswich, Massachusetts, USA).

Results

Pattern 1. Meristem tissue culture from explants 1-2.5 mm in length.

This experimental pattern presented total of 114 plantlets arisen from 120 explants, used in the tree repeats, or av. 38 plants per 40 excised tips (Table 1), and none of the newly formed shoots had healthy phenotype (Fig. 1A).

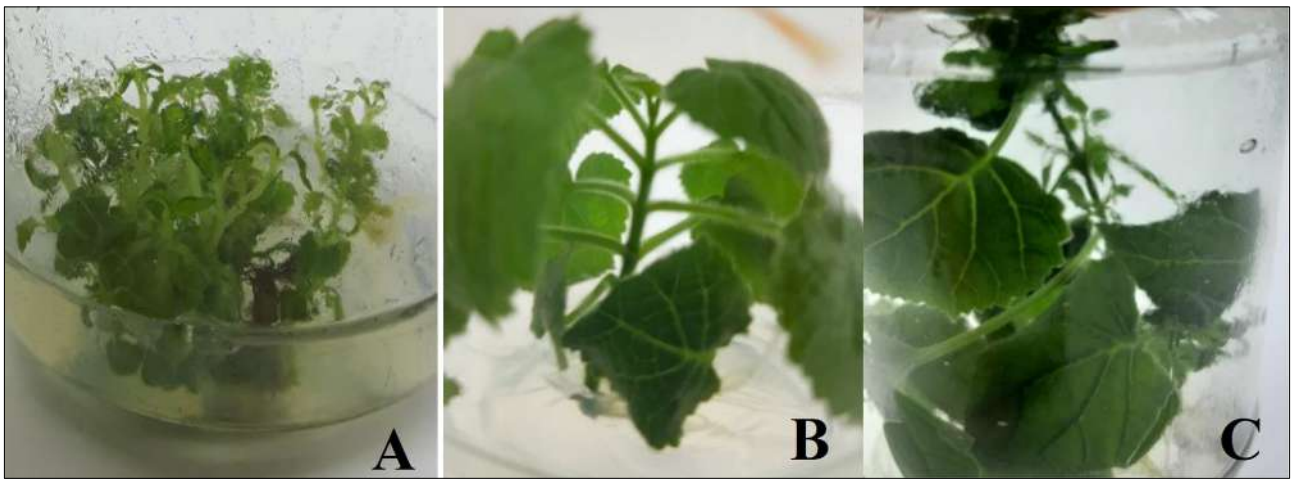

Fig. 1. Changes of morphology in *Paulownia tomentosa* plantlets: (A) with PaWB infection; (B) after thermotherapy and phytoplasma eradication; (C) after indirect regeneration from callus tissue that resulted in bigger leaves and enlarged internodes like healthy phenotype but with proliferation of axillary shoots that is witches' broom symptom.

Pattern 2. Meristem tissue culture from explants 0.5 mm in length.

The excised smaller explants have revealed lower regeneration efficiency (Fig. 2), with only 54 regenerants from a total of 120 used meristem tips (Table 1). In this variant, despite the smaller sizes of the selected tissues, all newly produced plantlets had no changes in their morphology, repeating PaWB symptoms – small light green to yellow leaves and short internodes with sprouting axillary buds.

Pattern 3. Thermotherapy and meristem tips – 0.5 mm in length.

When we combined smaller explants with thermotherapy, less than 40% of excised meristem tips regenerated to plantlets, presenting a decrease in regeneration efficiency, compared to the first and second experimental patterns (Fig. 2). On the other hand, changes in phenotype appeared and

three of the grown shoots regained a healthy morphology – bigger and depth green leaves, enlarged internodes, without developing axillary buds (Fig. 1B).

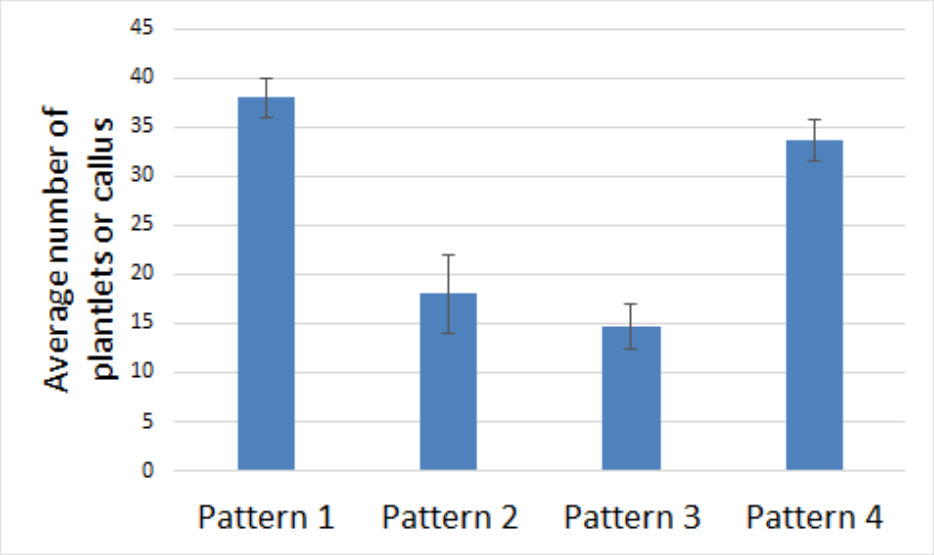

Fig. 2. Average number of plantlets (callus for pattern 4) in the applied experimental designs.

In the followed PCR analysis with universal R16F2/R2 primer pair, from the first newly formed plant with a healthy phenotype (3.1) produced through thermotherapy and 0.5 mm meristem-tips, there was no amplified phytoplasma 16S rDNA. However, 1.2 kb long products were amplified from 3.2 and 3.3 (Fig. 3). The results showed that tree promising samples were produced out of a total of 120 explants in this experimental pattern but only one was free from phytoplasma, with 0.83% efficiency in overcoming witches' broom infection.

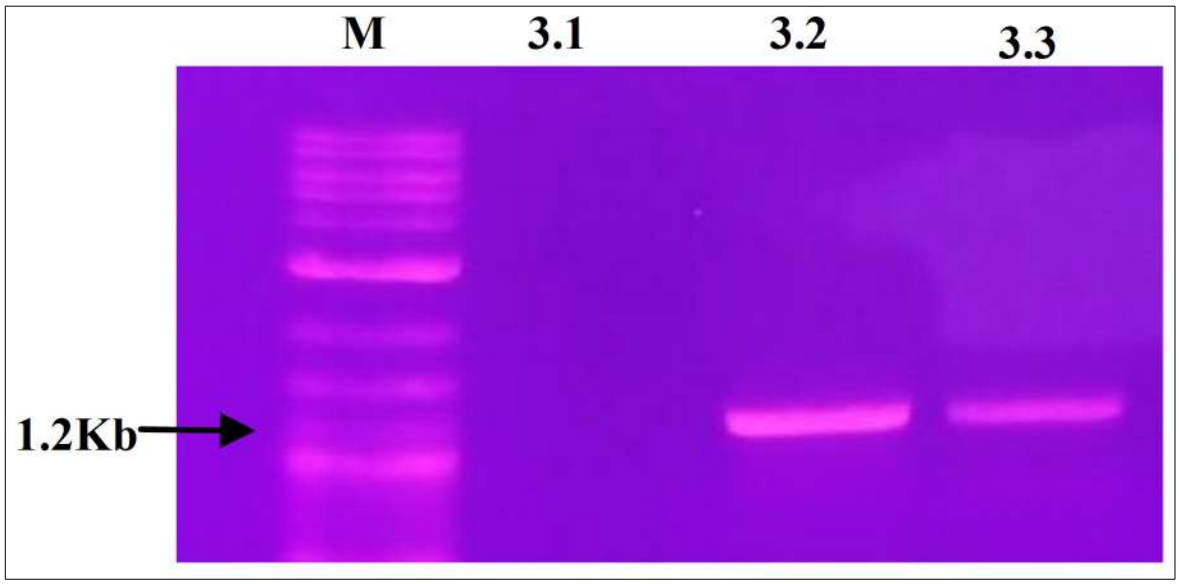

Fig. 3. Detection of phytoplasma 16SrDNA by PCR. **M:** DNA Marker; **1**: (3.1) the first regenerant with healthy phenotype in experimental pattern 3; **2:** (3.2) the second regenerant with healthy phenotype; **3:** (3.3) the third regenerant with healthy phenotype.

Pattern 4. Combination of callus and meristem culture with explants 1-2.5 mm in length. Most of the excised tips, for this experimental design, developed to callus tissue (Table 1). The induced calli were placed on a fresh medium, where they produced more than one shoots. Many of the excised shoots were asymptomatic (35.83%) but the healing effect was not permanent. In the prolonged cultivation, PaWB phenotype reappeared (Fig. 1C). This pattern was repeated three times with 214 shoots generated in total. After three months cultivation, one of the regenerated plants kept a healthy phenotype. To investigate the presence of pathogens' DNA, the plant that kept the changed morphology was tested with PCR. Through the analysis 1.2 kb fragments were amplified, revealing lack of success in overcoming phytoplasma infection (Fig. 4).

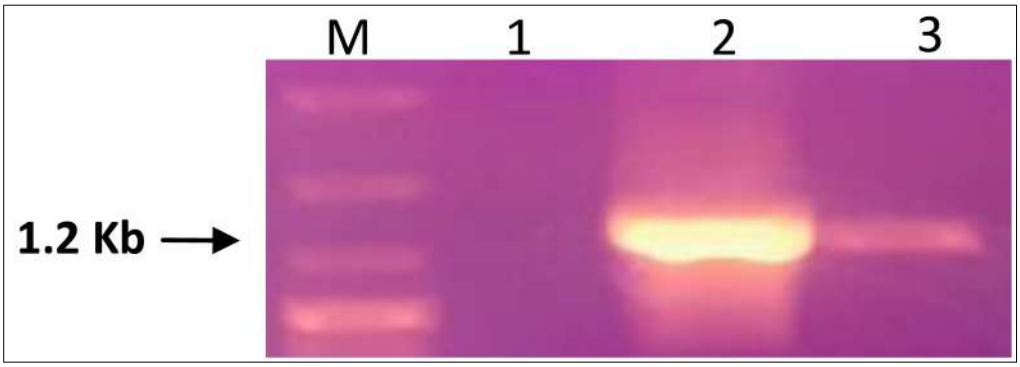

Fig. 4. Detection of phytoplasma 16SrDNA. **M:** DNA Marker; **1:** - Control (distilled water); **2:** +Control (PaWB infected plant); **3:** Regenerant with healthy phenotype.

Table 1. Efficiency of patterns. The regeneration efficiency in the fourth pattern was calculated about frequency of callus induction. Each experiment was repeated tree times.

Experimental pattern	Number of explants per experiment	Average number of plantlets or callus*	Regeneration efficiency	Total number of plants with changed phenotype	Total number of plants free from phytoplasma
Pattern 1	40	38±2	95%	0	0
Pattern 2	40	18±4	45%	0	0
Pattern 3	40	14.7±2.309	36.7%	3	1
*Pattern 4	40	33.7±2.081	84.17%	43	0

Discussion

In order to select pathogens free cells, the smallest possible meristem has to be taken but small explants could not survive because of desiccation, while large meristems do. After conducting four different strategies for meristem cultures, it has been observed the disappearance of PaWB symptoms in 46 produced regenerants of a total of 480 used explants. Mostly, the combination of meristem tissue with indirect regeneration through callus tissue reduced phytoplasma' symptoms (35.8%), compared to the variant with thermotherapy (2.5%), and lack of changes in the other two patterns. The healing effect turned to be permanent for one of the changed plantlets, and it was the product of a combination of smaller explants and thermotherapy.

As it is already known, the size of shoot tips is critical for pathogen eradication but it is also positively related to survival and shoot regeneration (Wang et al., 2018). In our studies, shoot regeneration levels were much higher (95%) in meristem culture from larger explants (1-2.5 mm) than that (45%) in meristem culture from tips 0.5 mm in length. When heat-treatment has been applied the regenerative ability of excised 0.5 mm tips reduced to 36.7%. Heat therapy is the oldest method used for the elimination of viruses, viroids and phytoplasmas from vegetatively propagated plants and has been used since the end of the nineteenth century (Varveri et al., 2015). High temperatures induce stress to plants and such stress is intensified as their durations increase (Wahid et al., 2007). Negative effects of prolonged thermotherapy durations on *in vitro* shoots and tips excised from the treated shoots were found in woody plants such as apricot, peach and cherry (Gella & Errea, 1998). In this study, PaWB-infected *in vitro* plants were heat-treated for one week at 40 ˚C and then used for meristem-tip cultures, which produced tree symptomless shoots and one of them was proved to be free from phytoplasma. Despite the low efficiency of the tested strategies, the provided data can serve to continue the study on the eradication of phytoplasma infection in paulownia plants. It has been suggested that the indirectly regenerated plants with changed morphology could be appropriate donor plats for further experiments with meristem tips for pathogen eradication.

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