IN VITRO PROPAGATION AND SURVEY OF *LEUCOJUM AESTIVUM* L. AND *SYRINGA JOSIKAEA* JACQ. FIL. EX RCHB ORIGINATING FROM NATURAL STANDS OF TRANSCARPATHIA

THESIS OF PhD DISSERTATION

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1. PRELIMINARIES AND AIMS

Transcarpathia thanks to its natural richness is one of the favoured areas of Ukraine. 826 plant and fungi species are listed in the Ukrainian Red Book; 268 of these can be found in Transcarpathia (GYIDUH 2010). This amounts to 32,4% of the protected species. However, the ever increasing human impact on the natural world urge for discovering and preserving the remained natural richness. As a biology teacher I have always perceive the importance to preserve natural richness. Experience proves that biology lessons in school do not give enough opportunity to raise children's awareness for preserving natural values. Regular conversations about nature conservation and environment protection in my immediate community have revealed that even children living in the countryside have lost their touch with nature to such an extent that they connect the idea of protection of natural richness with distant territories only. Therefore, I have decided to consciously create opportunities to help children to get insights into the natural world and learn more about the environment. To begin with study-groups for primary school-children, later on nature - study camps – which I have now been conducting for 15 years – were organised. The environmental education was started by introducing the Nagydobrony Wildlife Reserve — a significant national nature conservation area — located on the outskirts of my home village. During regular visits both with my students and campers aiming to observe changes in the wildlife I have paid attention to the plant species diversity of the Masonca wetland – part of the Nagydobrony Wildlife Reserve – especially to *Leucojum aestivum* and *Fritillaria meleagris,* two endangered species growing there. *Leucojum aestivum* is protected in Hungary and Ukraine, however because of its beauty it is an often collected geophyte species. Besides it is an important medicinal plant. Its populations are extremely vulnerable and the habitat sizes are reducing because of arid conditions and degradation. In 1999 the *University of Horticulture and Food Industry* in Hungary started affiliated horticultural training in the Transcarpathian Hungarian Teacher Training College. I got involved with the University and its lecturers, namely Jámborné dr. Benczúr Erzsébet and dr. Höhn Mária in 1999 when the Transcarpathian Hungarian Teacher

Training College recruited local tutors and tried to find places in Transcarpathia suitable for field-practice. The opportunities offered by the College as well as personal contacts with the University lecturers have encouraged me not only to continue my studies but with the professional guidance of Jámborné dr. Benczúr Erzsébet and dr. Höhn Mária to carry out organised and planned research. After numerous visits to the site and following phytocenological survey we have experienced that the Masonca grazed meadow it is at high risk. The process of afforestation as well as the continuously increasing aridity of the area has contributed greatly to a rapid reduction in the number of wetland plant species. Guided by this experience I have concluded that in addition to field study it would be important to preserve the genetic material of *Leucojum aestivum* population growing in the area of Masonca marshland. In addition, *in vitro* propagation technology of *Leucojum aestivum* would ensure the *ex situ* conservation possibility.

In the course of our research my doctoral supervisor drew my attention on another protected plant namely, *Syringa josikaea.* It is an endemic species with a highly disjunct area growing exclusively on a few localities in the Ukrainian (Erdős) Carpathians and the Transylvanian Western Mountains (Romania). It is the only palaeoendemic species of the Transcarpathian flora whose current stands in Transcarpathia have not been explored yet. The fact that *Syringa josikaea* has been included in the IUCN Red List – although with an information-lacking mark – justifies the need for a detailed registration of its natural stands. At the very beginning of the present research the review of the historical Hungarian as well as Soviet, later on Ukrainian specialized literature has revealed that there is no agreement on the number of existing habitats of *Syringa josikaea*. Therefore I aimed to update and specify those habitats in which this plant species still occur. and to do a detailed multiple uptake of phytoceonological data based on Braun-Blanquet method. *In vitro* propagation and preservation of the Hungarian lilac due to its good vegetative and generative abilities is not justified.

The aforementioned tasks have been summarised in my PhD thesis as follows:

- To carry out research of the *Syringa josikaea* habitats - a unique endemic species to Transcarpathia – and to characterise the habitats from floristic, phytocoenological and ecological points of view.
- To compare historical and modern Hungarian as well as Ukrainian literature on the subject, and to elaborate an accurate distribution map of *Syringa josikaea* stands. To enumerate the current geographic names by identifying their locations and evaluating the habitats by their nature protection status.
- Applying micropropogation technology of *Leucojum aestivum* on the genetic material of the Masonca wet-meadow habitat in Nagydobrony Wildlife Reserve. The process of micropropogation includes sterile medium development, optimization of the cultivation medium and rooting.
- Establishing cultivation material for *Leucojum aestivum* propagation for floricultural and pharmaceutical purposes by applying the elaborated micropropagation technology.

2. MATERIAL AND METHODS

2.1. *Syringa josikaea* Jacq. fil. ex Rchb.

2.1.1. *Syringa josikaea* habitats, site data collection

Between 2004 and 2012 I repeatedly localised and did floristic and phytoceonological study of *Syringa josikaea* (Hungarian lilac) habitats spending more than 35 days on field.

2.1.2. The used methods

To begin the research of the sites data from relevant academic literature and information of herbarium cards have been used. Besides foresters working in the area and local inhabitants have shared their knowledge on the subject. In addition to research data I have analysed data of the Department of Botany, Hungarian Natural History Museum, BUKA Herbarium in Bucharest, the University Herbarium of the Babes-Bolyai University Botanical Garden in Kolozsvár, Herbarium of the National University in Ungvár and Herbarium of the Institute of Botany, National Academy of Sciences of Ukraine. However, the identification of habitats enlisted in relevant literatures and those of the herbarium cards have caused many difficulties. In the past century *Syringa josikaea* habitats – because of historical border changes – were found on the territories of different countries and as a result each country used its own geographical names that are quite often unidentifiable. In numerous cases the locality-names have been changed moreover smaller settlements have become part of a different administrative unit. Thus, the linguistic and cartographic data processing demanded painstaking research.

On the territory of Ukraine I can confirm the existence of altogether 18 localities and their identification took place in the period of 2004-2012. Habitats have been marked with GPS reference points and a map of the existing Ukrainian populations was elaborated.

In the list of habitats I have included all the currently used geographical names with reference to Transcarpathia both in Hungarian and Ukrainian, on the basis of Google map data.

The maps were elaborated based on ESRI ArcGIS (Geographic Information System) programme (www.gis.com) and with the help of the ArcMap software. For the graphical projection of the maps the

widely used UTM (Universal Transverse Mercator) grid was chosen. The accurate location of the habitats was identified by WGS'84 (World Geodetic System 1984) system used by GPS satellites.

2.1.3. Sampling process, characterisation of the locations, sampling methods.

The Hungarian Lilac habitats are small in size and in most cases they are difficult to reach. After having identified the area a detailed floristic and phytoceonological study was carried out. However the phytoceonological recording could not be completed in each location either because of the small territory or the extremely low number of stands or due to the anthropogenic proximity.

Altogether 24 phytoceonological records have been made referring to 11 of the identified 18 habitats. The areas have been selected taking into consideration the abovementioned viewpoints and on the basis of field observations. The records were carried out in different periods of the year. I aimed at making at least one record on each site except for those that were too small in size. However, large distances and high ground water table during rainy periods have hindered or made the process of phytoceonological recording almost impossible. For the phytoceonological records the Braun–Blanquet (cit. in KÁRPÁTI – KÁRPÁTI 1968) method was applied. After repeated, detailed observations I have listed the plant species of the site. Besides photo documentation has been also done.

In accordance with the Braun-Blanquet scale or the Zürich-Montpellier school guidelines I have laid down the quadrants of 10m x 10m at the characteristic points of the habitats. A larger sample area was impossible to be estimated because of the habitat characteristics. In Ljuta where the habitat was situated along the banks of the stream in a narrow strip the quadrant area was 50m x 50m. Estimated A-D values ranged according to a 6 point scale (+, 1, 2, 3, 4, 5.).

The survey of 24 phytocoenological records has been summarised in a summarized coenological table.

Names of species follow Simon (2000) nomenclature. In case of species whose names have not been included into Flora Hungarica, the Flora Europaea scientific names were used. Ellenberg's indicator values were also applied. Based on the summarized coenological

tables I have defined the characteristic phytocoenological groups and their distribution, Simon's nature conservation categories (*TVK*) and Borhidi's social behaviour types.

Cluster analysis was performed based on presence-absence matrix of the species and Euclidean distance measure in PAST-programme (HAMMER 2001) was applied. Hungarian lilac habitats were analyzed based on the floral elements, life forms and Simon's nature conservation value categories of the local plant species according to TVK (SIMON 2002) and their distribution by Borhidi's social behaviour types (BORHIDI 1993). Accordingly, bioindicator research data of species was based on the distribution of relative humidity indicator values (WB), the distribution of the relative soil acidity values (RB), the distribution of the relative light indicator values (LB), the values of continentality (KB), the distribution of the relative nitrogen values (NB), and the distribution of the temperature indicator values (TB) (BORHIDI 1993).

These indices are described in detail for use in Ukraine. To illustrate the similarity of stands Sørrenson's Index of similarity was performed.

2.2. *Leucojum aestivum* L.

2.2.1. Micropropagation research of *Leucojum aestivum* L.

The research was carried out during the period 2006-2012 in the Micro-Reproductive Laboratory of the Department of Floriculture and Dendrology of the Corvinus University in Budapest. The plants used in the research were collected in the summer of 2006 from Masonca wet-meadow located on the territory of the Nagydobrony Wildlife Reserve. The natural reserve is situated in the north-eastern territory of the Szatmár–Bereg Plain on the outskirts of Nagydobrony. In terms of phytogeography the Bereg Plain belongs to the Észak Alföld (Northern Great Plain) floral district, the Samicum.

2.2.2. The method of micropropagation.

2.2.2.1. Physical conditions for *in vitro* propagation

The pH of the medium was adjusted to 5,6 1 N KOH prior to autoclaving. Then the medium was placed into 100 ml Erlenmeyer flasks and the autoclave pressure was set to 105 psig for 30 minutes. The flasks were incubated at 20-24^{o}C and shifted to 8/16 hours dark/

light conditions for 12 weeks. The phases of the experiment were documented by photos.

2.2.2.2. The propagation process

The collected bulbs were kept in refrigerator at 2-3°C in non-sterile environment for a week (first start, first experiment) and for 5 and 14 weeks respectively sterile in hormone-free culture in the second and third start–experiments. During the first experiment after cooling sterile bulbs were cut into small parts while still keeping part of their stem. The number of inoculums (explants) was 16. During the second and third experiments the upper part of the cooled bulbs (the upper part of the scale leaves) as well as the meanwhile sprouted green leaves were used as explants. The number of inoculums was 13-17.

The medium composition was E1, ½MS-alap 0,1 mgL^{-1} NES, 1 mgL^{-1} BA, 30 gL^{-1} sucrose.

2.2.2.3. The propagation

Four different hormones were used during the experiment, namely benzyladenine , kinetin , meta-topolin and paclobutrazol.

2.2.2.4. Propagation with benzyladenine and kinetin

Culture medium composition during the research with benzladenine and kinetin

Code of culture medium	Culture medium composition
E05	½MS-base 0,1 mgL^{-1} NES, 0,5 mgL^{-1} BA, 30 gL^{-1} sucrose
E1	½MS-base 0,1 mgL^{-1} NES, 1 mgL^{-1} BA, 30 gL^{-1} sucrose
C2	½MS-base 0,1 mgL^{-1} NES, 2 mgL^{-1} KIN, 30 gL^{-1} sucrose
C4	½MS-base 0,1 mgL^{-1} NES, 4 mgL^{-1} KIN, 30 gL^{-1} sucrose
E054	½MS-base 0,1 mgL^{-1} NES, 0,5 mgL^{-1} BA, 40 gL^{-1} sucrose
E14	½MS-base 0,1 mgL^{-1} NES, 1 mgL^{-1} BA, 40 gL^{-1} sucrose

2.2.2.5. Propagation with meta-topolin

Culture medium composition during the research with metatopolin

Code of culture medium	Culture medium composition
T1	½MS-base, 0,1 mgL^{-1} NES, 0,5 mgL^{-1} TOP, 30 gL^{-1} sucrose
T2	½MS-base, 0,1 mgL^{-1} NES, 0,5 mgL^{-1} TOP, 40 gL^{-1} sucrose
T3	½MS-base, 0,1 mgL^{-1} NES, 1 mgL^{-1} TOP, 30 gL^{-1} sucrose
T4	½MS-base, 0,1 mgL^{-1} NES, 1 mgL^{-1} TOP, 40 gL^{-1} sucrose

Data were analysed during September 15-28, 2009.

2.2.2.6. Propagation with paclobutrazol

Culture medium composition with benzyladenine and paclobutrazol

Code of culture medium	Culture medium composition
E0,5	½MS-base BA 0,5 mgL^{-1}, NES 0,1 mgL^{-1}
E1	½MS-base BA 1,0 mgL^{-1}, NES 0,1 mgL^{-1}
E2	½MS-base BA 2,0 mgL^{-1}, NES 0,1 mgL^{-1}
PB1	½MS-base BA 0,5 mgL^{-1}, NES 0,1 mgL^{-1}, PB 2,5 mgL^{-1}
PB2	½MS-base BA 1,0 mgL^{-1}, NES 0,1 mgL^{-1} PB 2,5 mgL^{-1}
PB3	½MS-base BA 0,5 mgL^{-1}, NES 0,1 mgL^{-1} PB 0,25 mgL^{-1}

The bulbs were placed on growth medium on 28th October, 2010. Research analysis was done during the 21-23 March, 2011.

2.2.2.7. Rooting

Culture medium composition used in rooting

Code of culture medium	Culture medium composition
E0	½MS-base 30 gL^{-1} sucrose
EG1	½MS-base , 0,1 mgL^{-1} NES, 30 gL^{-1} sucrose
EG2	½MS-base, 0,1 mgL^{-1} NES, 40 gL^{-1} sucrose
C2H	½MS-base, 0,1 mgL^{-1} NES, 2 mgL^{-1} KIN, 30 gL^{-1} sucrose
C4H	½MS-base, 0,1 mgL^{-1} NES, 4 mgL^{-1} KIN, 30 gL^{-1} sucrose

2.2.2.8. Data recording and method of analysis

During the data analysis 20-30 explants were considered in each culture medium except for the starting.

I have counted the newly grown bulbs; I measured their length and counted the number of roots. I also measured their length. (In the rooting experiment I have weighed the bulbs). The differentiation process of shoots (bulblets) was documented by photos. Electron and light microscopy images of the differentiation process of shoots (bulbets) were taken at the Central Laboratory of Corvinus University of Budapest.

Statistical analysis was carried out and graphs presented using the QtiPlot programme.

3. RESULTS

3.1. Research results of the *Syringa josikaea*

3.1.1. Localisation of the identified habitats

I have located and identified altogether 16 habitats in Transcarpathia and two in Lviv region in the period of 2004-2012. During the surveys of the 11 habitats 190 vascular plant species were recorded.

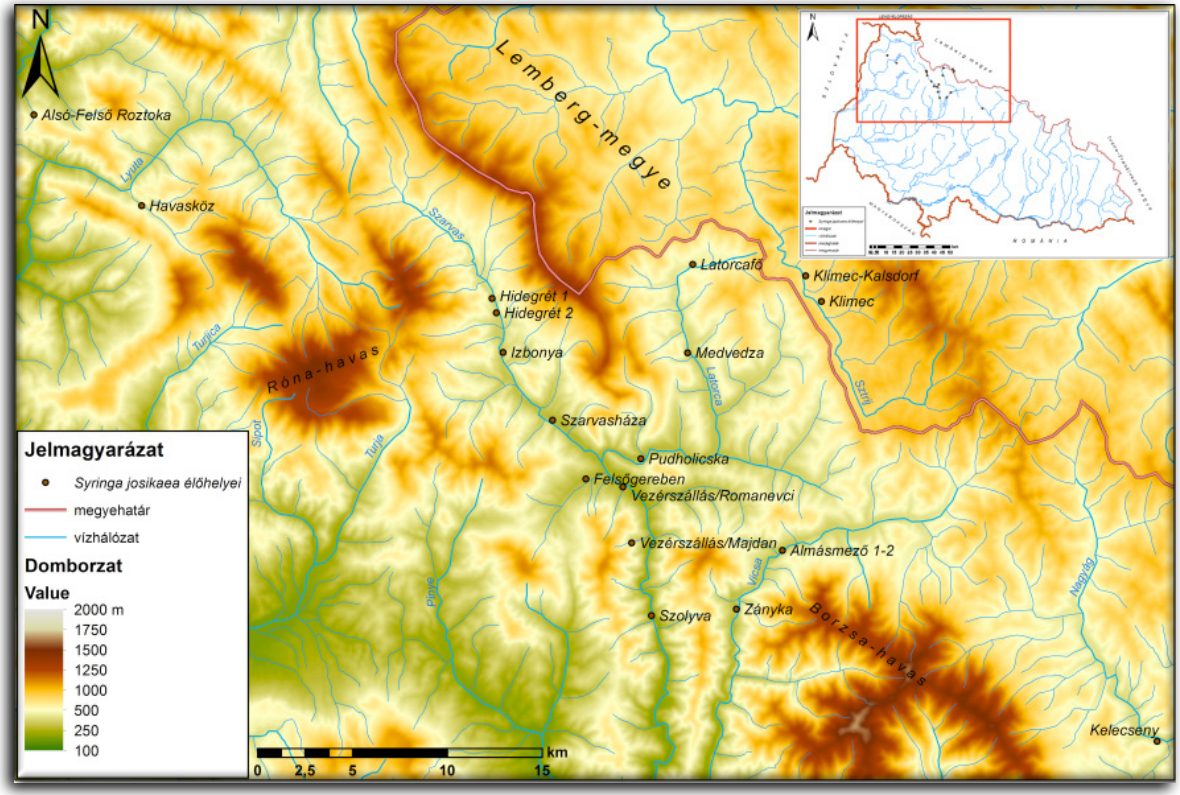
Based on 24 records from 11 habitats I was able to separate 9-13 characteristic coenological groups

13 groups were determined from Szarvasháza and Hidegrét 1. The least number, 9 were experienced in Izbonya, Almásmező and Medvefalva. The majority of the species in most of the habitats belonged to the *Fagetalia, Fagion, Querco-Fagetea* group (with the exception of the Izbonya stand with *Alnetea* being dominant). The floristic element composition presented relatively narrow spectrum. The studied territories were dominated by Eurasian species with 34.0%, followed by the European species, 23.3%. Circumboreal species' rate was significant 19% that is due to the cool and humid atmosphere of the alder woods and mountain fens. The ratio of Central European elements was 12%. In all studied localities the abundance of native species, characteristic for wet habitats, was high. There is a lack of adventive species whereas the number of cosmopolitan species is characteristic for wetlands in their natural environment.

The majority of the species present along the studied sites are characteristic for wet and mireland habitats. Accordingly, the rating of the 10 species occurring in the highest number is the following:

1.	*Caltha palustris*	37,3134%
2.	*Salix cinerea*	24,0941%
3.	*Syringa josikaea*	23,2072%
4.	*Cardamine amara*	23,2072%
5.	*Chaerophyllum hirsutum subsp. glabrum*	16,7157%
6.	*Nasturtium officinale*	16,7157%
7.	*Filipendula ulmaria*	15,4403%
8.	*Oxalis acetosella*	15,3846%
9.	*Alnus incana*	15,0060%
10.	*Asarum europaeum*	14,7522%

The stands were analysed based on the floristic composition and the rate of the phytocoenological groups. The results have been summarised and are presented in the table below.



Identified habitats of the Hungarian lilac in Transcarpathia

Geographic coordinates, habitat, characteristics, and height above sea level of the Hungarian lilac stands

	Ung river valley	Brief characteristics of the habitat	φ	λ	Height above sea level
1	Lower-Upper Roztoka (Kosztrinszka Roztoka) Borszucsinó- slope Kostrynska Roztoka	A small willow fen immigrating into beech forest	N 48°55.228’	E 22°36.747’	550 m
2	Havasköz (Ljuta Bisztricska) Lyuta	Ravines	N 48°52.578’	E 22°41.310’	430-570 m
	Latorca river valley				
3	Szarvasháza (Zsdenyijevo) Memorial local natural area Zdenievo	Montane grey alder bog	N 48°46.199’	E 22°58.782’	440 m
4	Izbonya Zbun (Zbine) Zbine	Small willow fen	N 48°48.166’	E 22°56.729’	450 m
5	Hidegrét 1 (Paskivci) Paskivci	Small willow fen	N 48°49.706’	E 22°56.314’	490 m
6	Hidegrét 2 (Paskivci) Paskivci	Small willow fen adjacent to peatmoss willow fen	N 48°49.295’	E 22°56.482’	470 m
7	Vezérszállás/Majdan (Pidpolozja/Majdan) Pidpolozzya/Majdan Majdan – Memorial local natural area	Grey alder bog, alderwood rich in spring geophytes	N 48°42.653’	E 23°02.047’	340 m
8	Vezérszállás/Romanevci (Pidpolozja/ Romanevci) Pidpolozzya /Romanevci	Alder bog and wood immigrating into deciduous forest	N 48°44.253’	E 23°01.734’	350m
9	Felsőgereben (Verhnya Hrabovnyica) Verhnya Hrabivnytsya		N 48°44.505’	E 23°00.148’	380 m
10	Szolyva (Szvaljava) Svaljava	Gummy and grey alder bog	N 48°40.572’	E 23°02.808'	320 m
11	Medvedza (Medvezsa) Tyshiv	Low-moor, tall herb species Bogs, Tall herb species	N 48°48.014’	E 23°04.671’	475 m
12	Latorcafő (Latyirka) Latirka		N 48°50.522’	E 23°05.001’	570 m
13	Almásmező 1 (Jablonyevo) Jablonyevo Almásmező 2 (Jablonyevo) Jablonyevo	Low- moor ponds with grey alder	N 48°42.320’	E 23°08.514’	440 m

Geographic coordinates, habitat, characteristics, and height above sea level of the Hungarian lilac stands

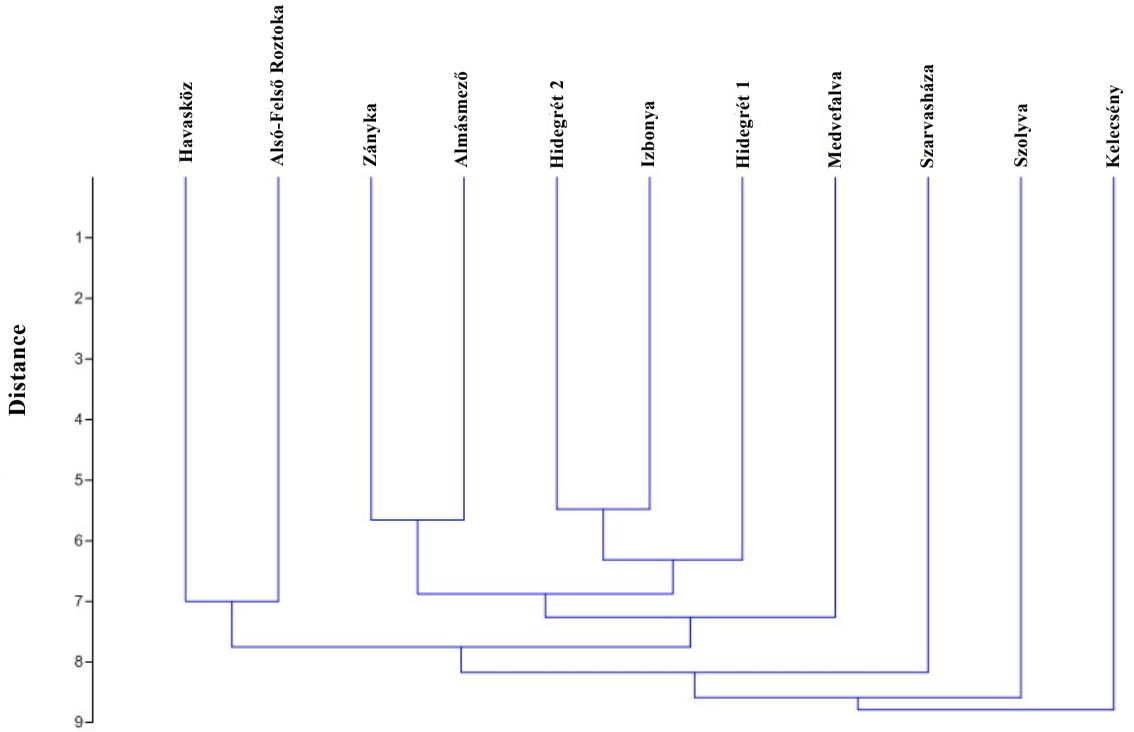
14	Zányka (Zanyka) Zanyka	Montane alder woods scattered with willow fens	N 48°40.690’	E 23°06.470’	420m
15	Pudholicska (Jalove) Jalove	Alder bogs with willows	N 48°45.037’	E 23°02.538’	390 m
	Nagyág (Rika) river valley				
16	Kelecseny (Kelecsenyi) Kelecenyi	Both spots are alderwoods rich in tall herb species	N 48°36.582’	E 23°24.349’	500 m
	Sztrij river valley				
17	Klimec (Klimec) Klimets	Alder wood bog of grey alder	N 48°49.370’	E 23°10.495’	740-760 m
18	Klimec-Kalsdorf (Klimec) Klimets	Alder wood bog of grey alder	N 48°50.110'	E 23°09.852'	741 m

3.1.2. Cluster analysis based on the species composition of stands

On the UPGMA dendrogram based on Euclidean distance calculation and the presence-absence matrix of the species occurring in the habitat sites several groups. were differentiated.

As it is obvious from the dendrogram below Kelecseny is the most outstanding habitat. This is not only because of the geographical distance (Nagyág valley) but it is also due to the greater extent of disturbance. Many disturbance indicator species are present in this habitat. The high diversity of species present in Szarvasháza and Szolyva makes these habitats to group separately from the others situated along the Latorca valley. Within the large group of habitats along the river Latorca, and Ung latter with two habitats form a separate group, whereas the population of Izbonya, Hidegrét and Medvefalva localized along the upper river Latorca form a single group.

Similar results were obtained based on Sørrenson’s similarity index calculation. Sites within the same river basin show higher similarity levels. Szarvasháza and the closely located Hidegrét 1, Hidegrét 2 and Izbonya have higher similarity values. The stands of Roztoka and Havasköz located in the Ung river valley diverge from the others. Kelecseny has intermittent position being distinctly separated from the 5 stands however, the similarity index is not high (varies between 0,51 and 0, 63)..



UPGMA dendrogram of the studied Syringa josikaea habitats based on the presence-absence species' matrix

3.2. Micropropagation results for *Leucojum aestivum* L.

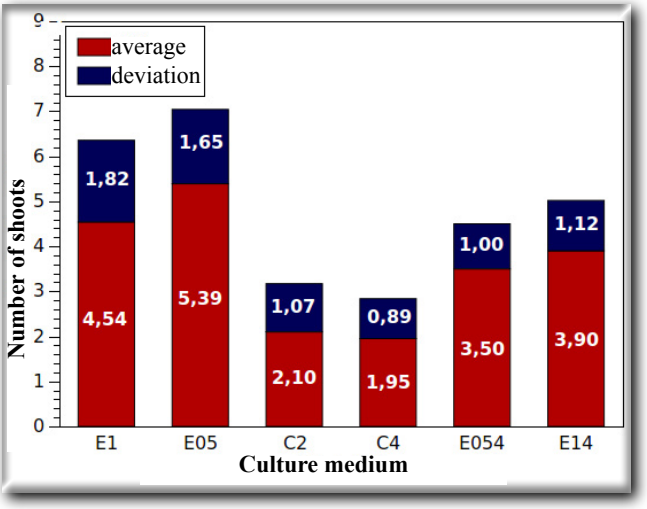
3.2.1. Starting: The five-week long cooling period proved to be the best out of all the cooling periods.

Researched characteristics	First starting from bulblets	Second starting		Third starting		
		From bulblets	From bulb leaves	From bulblets	From bulb scale leaves	From bulb leaves
Sterility %	81.3	92.3		100		
Inoculum with shoots %	69.2	100	100	62.4	68	85
Number of shoots	12.44	11.77	7.2	6.1	2.88	7.41
Length of shoots (mm)	2.7	3.7	1.5	1.75	1.91	1.2
Number of roots	1.8	1.2	27.5	1.71	1	1
Length of roots (mm)	27.5	53.4	20	47	17.5	12.3
Root formation %	38.5	38.5	20	23.1	1.2	4.3

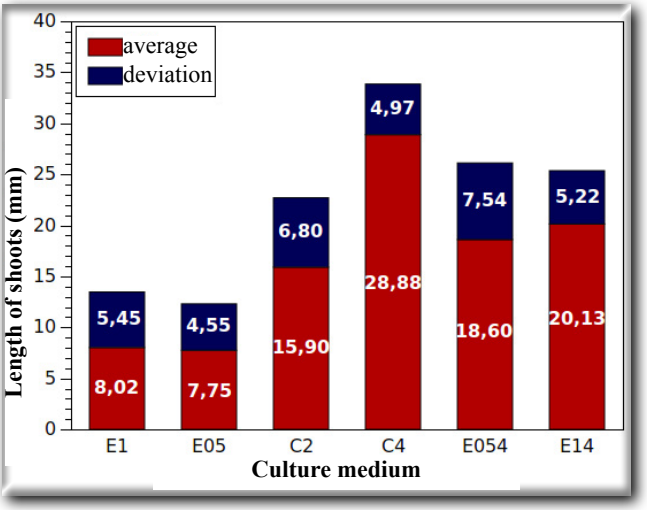
3.2.2. Results of propagation with various growth regulators

3.2.2.1. Propagation with benzyladenine and kinetin

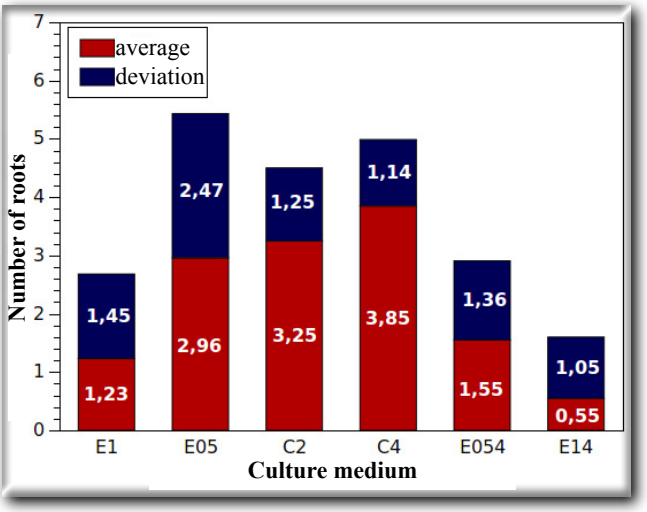
After cooling, I put the sterile bulbs cut into pieces into the starting culture medium which also served the propagation process. Propagation with kinetin did not prove to be efficient even with increased concentration compared to BA. However, with benzyladenine (0.5 and 1 mgL^{-1} BA + 0,1 mgL^{-1} NES) I could achieve 4.5 and 5.3 propagation rate in average, respectively. I have added 30 g L^{-1} sucrose to the culture media. The increased (40 g L^{-1}) sugar amount caused the decrease of the propagation rate, but the unwanted root formation increased.



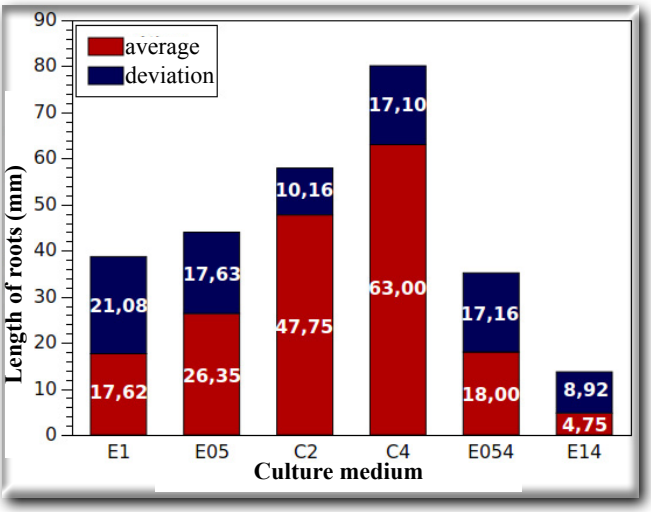
The influence of various culture media on the number of shoots after a twelve-week culture period



The influence of various culture media on the length of shoots after a twelve-week culture period



The influence of various culture media on the number of roots after a twelve-week culture period



The influence of various culture media on the length of roots after a twelve-week culture period

Having compared the six culture media, it can be stated that kinetin is not worth applying in the multiple shoot formation period because of the low number of shoots and the numerous roots. The culture medium containing 1 mgL^{-1} BA can be considered as being optimal because there I have found the least roots and the lowest root formation percentage with a relatively high number of shoots.

3.2.2.2. Propagation with meta-topolin and paclobutrazol

To investigate the further process of propagation I have applied meta-topolin (TOP) as cytokinin instead of BA in different concentrations. Besides I applied "small" and "big" bulbs from previous experiments as secondary explants in the investigation. The significantly best culture medium for propagation of small bulbs proved to be the one containing 1 mgL^{-1} TOP + 1 mgL^{-1} NES + 30 gL^{-1} sucrose. In this medium the propagation rate was 3.4, and the unwanted root formation was minimal. To propagate large bulbs I have considered the above-mentioned supplementary combination the best (5.2 average bulblet number), however, I have reached more bulblets with an increased amount of sucrose (40 g L^{-1} 7.2 pieces), but the roots were larger and the increasing of the amount of the sucrose with 10 g L^{-1} increases the cost of cultivation to a great extent. The advantage of the first medium is the absence of the unwanted root formation. Further improvement was achieved on T3 culture medium (0.1 mgL^{-1} NES + 1 mgL^{-1} TOP and 30 gL^{-1} sucrose) applying BA combined with paclobutrazol (PB). The medium that proved to be the best consisted of the following: 0.5 mg L^{-1} BA + 0.25 mg L^{-1} PB + 0.1 mg L^{-1} NES. In this medium the propagation rate was 7.8 which is significantly the best compared to the other media. This is the best result achieved during the experiments.

3.2.2.3. The results of root formation

The bulblets, – though they rooted in hormone-free medium as well – during the rooting experiments rooted best in the medium containing 0.1 mg L^{-1} NES and 40 g L^{-1} sucrose in all aspects. In this medium the number of roots was the biggest (4.52 pieces), root length was the shortest (23.24 mm). I obtained the biggest bulblets (2.04 g) in this culture medium as well. The latter two values proved to be better than the results achieved in other media. Therefore, I can confirm that I have successfully elaborated the *in vitro* propagation technology for *Leucojum aestivum.*

3.3. New scientific results

1. Up-to-date distribution of *Syringa josikaea* was compiled on the territory of Transcarpathia by processing historical data and by performing field studies. I have located altogether eighteen existing habitats.
2. I have described and characterised habitats of Transcarpathian *Syringa josikaea* from floristic, phytosociological and ecologic points of view.
3. I have elaborated the micropropagation technology of the endangered *Leucojum aestivum*.

Within this:

- I have elaborated the preliminary sterile cooling method, and thus the starting processes can be safety begun.
- I have defined the composition of the optimal culture medium needed for multiple shoot formation, and I was the first to apply successfully meta-topolin and paclobutrazol in the propagation phase.
- In addition, I have identified the composition of the culture medium appropriate for root formation.

4. CONCLUSIONS

4.1. Characteristic features of the *Syringa josikaea* Jacq. fil. ex Rchb. habitat

I could identify, locate and study altogether 18 Hungarian lilac habitats during my investigations, out of which 16 can be found in Transcarpathia in the valleys of the rivers Ung (Uzh), Latorca (Latorytsia) and Nagyág (Rika) 2 habitats are situated in Lemberg (Lviv) region in the valley of the river Stryi.

Even after several occasions of survey, I have not found certain stands, namely: the stands between Kis-Pásztély and Nagy-Pásztély, in Oroszmocsár (Ruszkij Mocsar), the area close to the Verecke pass and the Szőlősgyula area. The habitat called the Rónafüred (Lumsori) stand by the Ukrainian academic literature cannot be identified unambiguously, either.

Syringa josikaea stands are present most frequently along mires of the rivers and streams in the beech forests and fir-beech forest zones. It appears in the shrub layer of the alder woods. The lowest appearance is 350 m above sea level at Vezérszállás in the valley of the river Latorca. The highest altitude is between 740–760 m at Klimec, in the valley of the river Stryi. *Alnus glutinosa,* and *A. incana* are dominant species in these mountain alder thicket forest habitats; however, there are smaller stands dominated by shrub willows, for example Stand No4 at Izbonya (Zbine).

Fagetalia, Querco-Fagetea and *Abieti-Piceion* species can immigrate into the edges of these mires but the herb layer more often is characterised by tall herb species. The result of the cluster analysis on the basis of the presence-absence matrix of the species reveals that the stands are mainly grouped according to their geographical position, thus species composition of the stands in the same river valley is found to be quite similar. Kelecsény habitat along the Nagyág (Rika) bank is characterised by the most distinct species composition which is the only known population in this river valley. In its species composition, alder species dominate, though several weed plants are also present that show anthropogenic influence (e.g. *Arctium lappa, Elymus repens, Galeopsis ladanum, Anthriscus sylvestris, Festuca arun-*

dinacea). This stand is probably strongly disturbed by the closeness of the motorway and its extensive use. Habitat protection, or separating the stand from the motorway and the drivers stop at the edge of the road could guarantee the survival of the stand and further invasion of weeds.

Based on the species composition and dominance it can be stated that Transcarpathian Hungarian lilac stands do not show characteristic features of a relictary habitat. The territories are characterized by alder and willow communities characteristic for mountain river and stream banks. These communities having in their composition tall herb species however are commonly spread in the area of the Eastern Carpathians.

Though the Natura 2000 territories are not singled out in Ukraine, based on the EUNIS, EUR 27 *Syringa josikaea* habitats belong to the 91E0 category, –alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior (Alno-Padion, Alnion incanae, Salicion)*.

Similar habitat types are mentioned in the habitat directive of Romania. These habitats are categorised into Group 2.4.3. in the summary work 'Habitats of Romania' (DONITA et al.2005): marsh and alluvial forests and shrub forests. Within this group the Romanian lilac stands are mentioned as a separate group of habitat (R4413 south-eastern Carpathian shrub forests with *Syringa josikaea*). However, it is crucial to note that most of the Transylvanian and Transcarpathian *Syringa josikaea* habitats differ greatly from each other. The Romanian stands grow on the rocky bottom of fast-flowing montane stream valleys. Here mire communities with stagnant water spread out are not typical. Though the Havasköz (Ljuta) stand in the basin of the river Ung (Uzh) is similar to the Romanian habitats, most of the Transcarpathian ones differ from them greatly.

Syringa josikaea is a protected species of Ukraine. Though it is also included in the National Red List, most habitats are not under protection. Only the habitats mentioned below are protected:

Lower-Upper Roztoka (Kosztrinszka Roztoka) and Borszucsinó are natural memorials of local importance, which is part of the Javornyk Mountain natural memorial place of national importance. The Javornyk Mountain, and thus the stand, too, is situated on the territory of the Uzhansky National Park.

Nature conservation value of the Havasköz (Ljuta) stand is not known, as it is not listed by the environmental protection authorities. However, it is found on the territory of the Uzhansky National Park, therefore it is also protected.

The Szarvasháza (Zsdenyijevo) stand is a Hungarian Syringa Botany Reserve of local importance.

The Vezérszállás (Pidpolozja) stand is a Hungarian Syringa Botany Natural Memorial of local importance and so it is the Klimec stand.

Most of the habitats are situated close to inhabited areas, therefore they are highly endangered. We can assume that the size of habitats by the roads became smaller due to changeable use of lands. In the case of the Szarvasháza (Zsdenyijevo), Izbonya (Zbine), Hidegrét (Paskivci), Kelecsény (Kelecseny) stands it can be assumed, while in the case of the Vezérszállás (Pidpolozja) and Pudholicska (Jalove) stands it can be unambiguously proved that the area of the Syringa habitats decreased. A part of the Vezérszállás stand was destroyed during the process of building the motorway. The Pudholicska stand was degraded by laying down gas pipes. Forestry must also be mentioned as an endangering factor of the populations. Worth to be mentioned that for forest rangers the tree of the lilac is economically valueless and its protection rather hinders their work. Therefore, even if they know about still existing habitats, they try to conceal it from the conservationist. Further danger for the habitats near inhabited areas is meant by trampling due to pasturing (Szarvasháza (Zsdenyijevo), Zányka , Almásmező (Jablonyevo).

S. josikaea is known both as an ornamental plant and as a medicinal plant, therefore local people collect it with pleasure not only when blooming. The common experience is that in the gardens of villages close to the natural stands uncultivated specimens of the Syringa can be found (Klimec, Sóhát (Csornoholova), Havasköz (Ljuta), Felsőgereben (Verhnya Hrabivnyica), Vezérszállás (Pidpolozja), Zányka, Ivaskivci). It is evident that the lilac can renew itself well by vegetative propagation; therefore digging out the sprouts does not cause the destroying of the plants. However, the specimens planted close to each other enlarge the real size of the population. In the case of Klimec, the number of the garden shrubs might exceed the number

of the specimens existing in the wild, and it connects the two subpopulations living along the stream Stryi.

Different species of *S. vulgaris* having flowers that are more impressive than those of *S. josikaea* are kept everywhere, therefore collecting *S. josikaea* as an ornamental plant will possibly not become considerable. The specimens living in the gardens increase the genetic connectivity and reproduction ability of small populations, so their existence is also acceptable from the point of view of environmental protection.

Besides the anthropogenic influence mentioned above, the fact that the populations are endangered is caused by their small size because of which their fertility is low, and in the small stocks the influence of an occasional natural catastrophe (tree falling, landslide, forest fire, flood) or of a later human impact can be dangerous. The drying of territories is also a threatening factor, which can only be partly considered due to an anthropogenic influence (Vezérszállás).

Based on my experience, the habitats in the best conditions are the ones situated farther from inhabited areas, for instance, Havasköz (Ljuta), Szolyva, Borszucsinó, or the territories proclaimed protected, e.g. Szarvasháza. The Szolyva stand is especially rich in species and it is also characterized by many protected species. I found specimens with the highest number of inflorescences and the most beautiful flowers in Szarvasháza.

4.2. Experiences of micropropagation experiments with *Leucojum aestivum* L.

Starting

Out of the three starts I obtained the best result after the five-week long cooling period by applying the small bulb parts. One can be considered as a new method the keeping of the sterile bulbs cooled until dissection and putting them into the starting culture media, respectively. Concerning the length of cooling, the optimal cooling period was of similar length as that applied with the Narcissus poeticus (JÁMBORNÉ BENCZÚR et al. 1989). Difference is that the cooled Narcissus bulbs were not sterile. The culture medium used by me was similar to what STANILOVA et al. (1994) described, but I did not apply kinetin for starting.

Propagation with benzyladenine and kinetin

Comparing my results with the data from the academic literature I can state that they do not coincide exactly with the results of any author dealing with the *in vitro* propagation technology for *Leucojum aestivum*, though in case of regeneration from callus there is no sense of data comparison. I obtained similar results to those of STANILOVA et al. (1994) in case of the cultivation started from bulbs.

Propagation with meta-topolin

As in conclusion, it can be stated that in case of the cultivation started from small bulbs the high concentration of sucrose influenced unfavourably the formation of shoot bulbs because I got the most shoot bulbs in the optimal T3 medium containing 30 gL^{-1} sucrose. The T4 medium with 40 gL^{-1} sucrose content proved to have a hindering effect on shoot differentiation. The shoot number was low in the T1 medium because here the meta-topolin concentration was small, only 0.5 mgL^{-1}.

In case of the cultivation started from small bulbs during the statistical analysis of root formation I found that more roots were formed in medium T4 (average 2.60 pc) and in medium T2 (average 2.00 pc). The longest roots grew in medium T2 (average 64.10 mm), and the shortest roots were in the small bulbs in medium T3 (average 4.05 mm). In the other media I obtained various measures that were significantly different from the results obtained from the medium with the shortest root length and that of the longest root length. The elevated sucrose content also increased the number of roots, which is unwanted in the multiple shoot formation process. So culture medium T3 proved to be optimal for cultivation started from small bulbs.

In case of the cultivation started from large bulbs one can state that the number of shoots was the biggest in media T2s (7.20) and T4 (6.70). However, shoot length was the longest in medium T1 (13.50 mm). In media T3 and T4 the length values were approximately the same. The number of shoots (1.70 pc) the length of shoots (22.40 mm) were 'the best' in medium T4, which was, however, disadvantageous from the point of view of the multiple shoot formation process. There was no root growing in medium T3. I measured different values in the other media that were significantly different from the results obtained from the medium with the shortest root length and that of the

longest root length. The elevated sucrose content did not influence the root number notably, though the average root length was increased considerably. Besides, it increased the average size of the bulblets, although significant difference could not be pointed out because of the high standard deviation of the measured values. Comparing the size of bulbs used as explants it can be defined that the starting matter of bigger size produced significantly better results in all types of the culture media.

Finally, comparing the culture media it is possible to conclude that medium T3 (0.1 mgL^{-1} NES + 1 mgL^{-1} TOP and 30 gL^{-1} sucrose) is optimal in case of both small and big *Leucojum aestivum* bulbs. In case when the cultivation started from small bulbs I found the least number of roots and the smallest root formation ratio, while in case when the cultivation started from large bulbs I did not find any root formation. The shoot number was relatively big in this case and the shoot bulbs were well developed.

Propagation with paclobutrazol

The aim of my experiments conducted with paclobutrazol was to find the most optimal composition of culture medium among the various media for multiple shoot formation of *Leucojum*. Ideal would be the case of development of shorter roots, and less roots alongside with the numerous differentiations of bulblets because these might be disadvantageous in further *in vitro* multiple shoot formation processes. Taking this into account, one can declare that the three culture media containing paclobutrazol showed the best results. Among these PB 3 was significantly outstanding. In this medium, the paclobutrazol concentration was 0.25 mgL^{-1}, the benzyladenine concentration was 0.50 mgL^{-1}, so an average of 7.80 shoot bulbs was differentiated. Paclobutrazol concentration in PB 1 and PB 2 media was 2.50 mgL^{-1} meaning that the smaller concentration is more effective, the bigger concentrations have a hindering effect. With regard to the length of shoots, though shorter shoots were formed in PB 3 medium, this can be accounted for the high number of shoot bulbs which was accompanied by shorter shoots in other plants as well. This is proved and it can be well seen from the experiment conducted with meta-topolin. Having a look at the number and length of roots, the culture media containing paclobutrazol also produced higher averages. This is not

surprising because this hormone was originally applied to help root induction. This kind of effect can also be singled out here; however, because of the favourable number of shoot bulbs the application of paclobutrazol is justified.

Comparison of the impact of growth regulators based on three propagation experiments

During my experiments I investigated the impact of BA, KIN, TOP and PB. The first three, are cytokinins; this hormone group mainly contributes to differentiation and thus it is the essential compound of shoot induction. One can find a lot of data in the academic literature about the impact of BA and KIN, on the other hand this cannot be stated about the relatively new TOP.

I have defined that kinetin produced only two shoots on average, even in case of a higher concentration (4 mgL^{-1}), therefore its use is not effective in the propagation phase.

BA traditionally used for propagation of bulbous plants was successful. By using 1 mgL^{-1} concentration I could achieve a propagation rate between 4.10–4.50 that can be considered appropriate. With applying TOP this propagation rate could further be increased. The 1 mgL^{-1} TOP concentration resulted in 5.20 propagation rate with bigger shoots. Moreover, I experienced a propagation rate even higher than this one with 0.5 mgL^{-1} TOP using more sucrose than usual, (30 gL^{-1}) –40 gL^{-1}. However, this is not economic. The combination of 30 gL^{-1} sucrose and 1 mgL^{-1} TOP can be regarded as optimal in this case.

The paclobutrazol rarely used during propagation of bulbous plants cannot be considered as cytokinin, the academic literature mentions its positive effect mainly for root differentiation. However, in certain cases it influenced shoot formation positively. This is believed to be due to the synergist effect triggered by cytokinin.

Because there was hardly any data on the suggested concentration in the literature, I used an amount of 2.5 mgL^{-1}. But as it turned out from the experiment, its tenth (0.25 mgL^{-1}) proved to be even better combined with BA. The culture medium producing the best shoot number contained 0.5 mgL^{-1} BA and 0.25 mgL^{-1} PB, with this combination the average shoot number (multiplication rate) was 7.80, the highest during the propagation experiments. Of course, in the case of every culture medium the cultures also contained 0.1 mgL^{-1} NES. Fi-

nally, it can be concluded that it is optimal for propagation to combine BA with low concentration of PB.

Rooting

The rooting process of *Leucojum aestivum* did not cause difficulties because it can be rooted in hormone-free medium, too. However, the use of NES in 0.1 mgL^{-1} concentration increased the root number significantly from 2.80 to 3.70. This effect could be improved by adding 40 gL^{-1} sucrose instead of 30 gL^{-1}. This way I got 4.50 roots per bulbs. This medium also decreased the length of roots significantly, which is especially advantageous from the point of view of acclimatization during planting. It is interesting to note about the experiment that the bulblets also rooted well in kinetin media and multiplied less. Others authors also experienced such impact of kinetin on rooting (JÁMBORNÉ BENCZÚR, 1992; JÁMBORNÉ BENCZÚR, 2005). Based on the observed and obtained data I suggest the culture medium containing 0.1 mgL^{-1} NES combined with 40 gL^{-1} sucrose for rooting.

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