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The impact of the Latvian plant physiologist Auseklis Veģis (1903 - 1973) in modern natural sciences

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Abstract

By showing that the dormant condition in plants is induced by external factors, Latvian plant physiologist Auseklis Veģis contributed to the formation of modern theory of plant dormancy. The present paper evaluates the impact of Veģis major publication, *Dormancy in higher plants*, published in the *Annual Review of Plant Physiology* in 1964, on modern natural sciences by means of citation analysis. This paper was cited 365 times within 40 years after its publication. In this respect, the Veģis paper ranks 86th among the most frequently cited papers of the 1082 review papers published in *Annual Review of Plant Physiology* from 1950 until 2001. It is interesting to note that the number of papers citing Veģis paper has been continuously increasing during the last six years. The increase is mostly contributed by publications in the field of seed dormancy and germination, dormancy of trees, and physical properties of disaccharide. The paper *Dormancy in higher plants* stands out among the most important and highly ranked plant physiology papers published so far.

Key words: citations, plant dormancy, plant physiology, seed germination.

Introduction

In 2003, along with several other scientific events, Latvian plant physiologists acknowledged the 100th anniversary of plant physiologist Auseklis Veģis. Born in Latvia in 1903 and starting his academic carrier at the University of Latvia in the 1930s, after 1945 Auseklis Veģis performed his investigations of plant dormancy in Sweden at the Institute of Physiological Botany, University of Uppsala.

His main contribution to plant physiology was a review paper published in 1964 in the *Annual Review of Plant Physiology* entitled *Dormancy in higher plants*. Now we can celebrate the 40th anniversary of the paper. The question addressed in the present paper is to assess the impact of this particular Veģis paper on plant physiology during these years.

Methods

Material for analysis of citations for plant physiology papers in *Annual Review in Plant Physiology* were found on Internet at the following address: http://www.garfield.library.upenn.edu/histcomp/annualreviews/annrevplantphys/. Data for citations of Auseklis Veģis papers were taken from the *Science Citation Index* (SCI) database (*Institute of*

Scientific Information, ISI). For other references, Internet searches were performed using freely available databases at http://www.scirus.com, http://www.altavista.com, http://www.google.com, http://www.hotbot.com, http://www.lycos.com.

Results and discussion

Regarding Auseklis Veģis major scientific achievements, it should be emphasized that the majority of investigators in the 1930s - 1950s believed that the dormant condition of plants originated autonomously during a particular season, and that it was hereditary characteristic. It was conceded, however, that external conditions might accelerate or delay the onset of dormancy, at least to a certain extent, but never prevent it. The crucial evidence against this point was given by Auseklis Veģis in the 1950s - 1960s, based on critical analysis of own experimental data as well as those of other investigators. He showed, that the dormant condition in plants is induced by external factors. In almost all plants that periodically become dormant, dormancy can be induced even in a season during normal active growth. This can be achieved only by changing external conditions, temperature, photoperiod, quality of light, nutritional conditions and water supply being most important. Environmental factors are important also for the termination of dormancy. As a result, a fundamental basis of the modern theory of plant dormancy was formed. The theory has also a great practical importance, as it predicts a possibility to control growth activity of plants by natural means.

By modern standards, the number of Veģis scientific publications is not especially high, and includes only 24. Nevertheless, the classic papers published by Ausekls Veģis still form the basis of modern textbooks in plant dormancy physiology. Most famous among them is a paper *Dormancy in higher plants* from the *Annual Review of Plant Physiology* published in 1964 (Vegis 1964). The ISI SCI database lists six papers published by Auseklis Veģis. Besides the mentioned paper, the remaining five account for 61 citations up to the year 2003. In contrast, the paper published in the *Annual Review of Plant Physiology* was cited 365 times within 40 years after its publication. The frequency of citation is better understood by analysis of citations for other papers from the same publication.

Within the particular volume the Veģis paper had second highest citation rate up to year 2001, with 308 citations. The highest citation rate (369) was for a paper of Cathey H.M. entitled *Physiology of growth retarding chemicals*. In comparison, the most ever cited paper from *Annual Review in Plant Physiology* was a paper by Hsiao T.C. entitled *Plant responses to water stress* published in 1973, which was cited 1300 times. The

Table 1. Relative distribution of papers (% of the total number) published in *Annual Review of Plant Physiology* within successive decades according to the number of citations up to the year 2001

Decades	No. of citations										
	0-49	50-99	100-199	200-299	300-399	400-499	500-599	600-699	700-799	800-899	>900
1950-1959	59.3	30.7	9.3	0.7	-	-	-	-	-	-	-
1960-1969	43.1	34.3	16.7	2.9	2.5	-	-	0.5	-	-	-
1970-1979	21.8	19.9	29.9	10.4	9.0	4.0	2.0	1.0	1.0	0.5	0.5

question is, how to evaluate the overall citation frequency of the particular Veģis paper? It is evident that, in terms of citation frequency, for a particular paper to qualify as a highly cited paper, certain criteria should be met. Direct comparison of the number of citations is a somehow misleading method because of an increase in the absolute number of citations for whole plant physiology over time, which reflects an increase in number of published papers. This trend is illustrated in Table 1 by comparing the change of relative distribution of papers according to citation numbers in successive decades. The highest rank among the top 10 % of papers in respect to the rate of citation in the period of 1950 - 1959 was achieved by a number of citations above 99, in 1960 - 1969 with a number of citations above 164 and in 1970 - 1979 with a number of citations above 383. Comparison with more recently published papers makes less sense because of a relatively shorter time allowed for citing of the particular paper. It is evident that the Veģis paper is among the top 2.5 % of highly cited papers for that particular decade. However, for the following decade, the paper with a particular frequency of citation (above 300) ranked only with a top 14 %. In spite of that, *Dormancy in higher plants* still holds as the 86th most frequently cited paper among all 1082 review papers published in *Annual Review in Plant Physiology* from 1950 until 2001 placing it among the top 8 %.

Is this high rank of the Veģis paper confirmed also when plant physiology papers from other publications are taken into account? The Veģis paper was mentioned among the 90 plant physiology articles published from 1949 until 1972 which were most cited during the period between 1961 and 1972 (Garfield 1975). With the number of citations as high as 51, the paper ranked number 85. Considering the total number of papers in plant physiology used for evaluation (obviously, several thousands yearly, the number itself was not given there) this value can be used to evaluate the Veģis paper as among the most important plant physiology papers of that period.

Another aspect concerning scientific publications is the longevity of their impact. This can be studied by analyzing a time course of citations of a particular paper. We conducted an analysis for *Dormancy in higher plants* for citations within the period from 1991

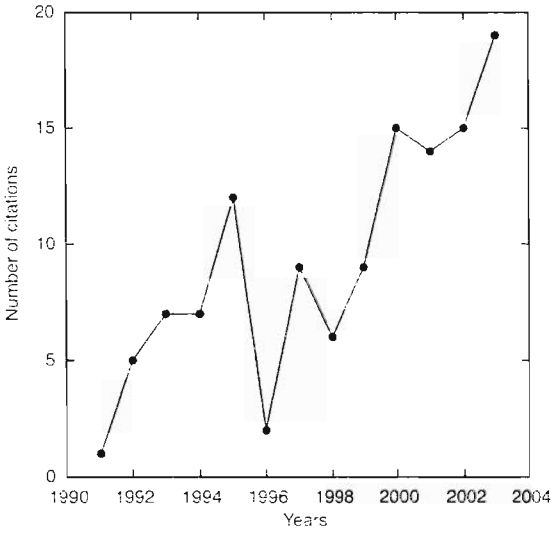

Fig. 1. Time course of number of citations of *Dormancy in higher plants* in scientific papers.

until 2003 inclusive using SCI database. With an average number of citations per year of 9.4, the present period is characterized by a relatively high frequency of citations in comparison with first nine years after the publication of the paper (in average 5.7 citations per year). A higher average yearly frequency of citation for the paper was for the period of 1973 - 1990 (10.7 citations per year). Data in Fig. 1 clearly shows that the number of papers citing Vegis paper has been continuously increasing within the last six years.

The complete list of journals where the papers were published citing Vegis paper in the period from 1991 until 2003 is given in Table 2 (according to ISI SCI). There is an impressive list of 67 journals from different branches of biology and even from material science, physical chemistry and other subdivisions of physics. In addition, when searching the Internet, we found five papers citing the paper of interest not included in the ISI SCI database (in order of publication):

– Friend A.D. 1995. PGEN: an integrated model of leaf photosynthesis, transpiration, and conductance. *Ecological Modelling* 77: 233–255;

– Alba F., Daz de la Guardia C. 1998. The effect of air temperature on the starting dates of *Ulmus*, *Platanus* and *Olea* pollen seasons in the SE Iberian penninsula. *Aerobiologia* 14: 000–000;

– Chytry M., Tichy L. 1998. Phenological mapping in a topographically complex landscape by combining field survey with an irradiation model. *Applied Vegetation Science* 1: 225–232;

– Schütz W. 2000. Ecology of seed dormancy and germination in sedges (*Carex*). *Perspectives in Plant Ecology, Evolution and Systematics* 3: 67–89;

– Jato V., Mendez J., Rodriguez-Rajo J., Seijo C. 2002. The relationship between the flowering phenophase and airborne pollen of *Betula* in galicia (N.W. spain). *Aerobiologia*

Table 2. Citation of *Dormancy in higher plants* in different journals within the period of 1991 - 2003. Data are from *Science Citation Index* database (*Institute of Scientific Information*)

	Journal	Year of citation
1	Acta Agriculturae Scandinavica sect. B	1995
2	Acta Botanica Neerlandica	1995
3	Acta Oecologica	1993; 1999; 1999; 2001
4	American Midland Naturalist	2000; 2003
5	American Naturalist	1993
6	American Potato Journal	1995
7	Annals of Applied Biology	1998
8	Annals of Botany	1994; 2002; 2003
9	Annals of the New York Academy of Sciences	1999
10	Applied Physics A - Materials	2002
11	Aquatic Botany	1997; 2000
12	Australian Journal of Ecology	1992
13	Australian Journal of Experimental Agriculture	2001
14	Biological Reviews of the Cambridge Philosophical Society	1992
15	Botanical Review	1994
16	Bulletin of the Torrey Botanical Club	1995
17	Canadian Journal of Botany	1992; 1994; 1995; 1996; 2000; 2002; 2003
18	Canadian Journal of Plant Science	1997; 2001

(continued)

	Journal	Year of citation
19	Climatic Change	1998; 2001
20	Comptes Rendus de l Academie des Sciences ser. III	1993
21	Ecological Research	1997
22	Euphytica	2002; 2003
23	Field Crops Research	2000
24	Forest Science	1992
25	Genetics	2003
26	Global Biogeochemical Cycles	1997
27	Global Change Biology	2000
28	Grana	2000
29	Heredity	1998
30	HortScience	1997; 2003; 2003; 2003; 2003
31	International Journal of Biometeorology	2001; 2003
32	International Journal of Plant Sciences	1992
33	Journal of Applied Ecology	1994
34	Journal of Chemical Physics	1999; 1999
35	Journal of Ecology	1991; 1995
36	Journal of Experimental Botany	2000
37	Journal of Molecular Structure	1999
38	Journal of Physical Chemistry B	1997; 1998; 1999; 2000; 2001; 2001; 2002; 2003
39	Journal of Physics - Condensed Matter	1999
40	Journal of Range Management	1995
41	Journal of the American Society for Horticultural Science	1998
42	Journal of the Japanese Society for Horticultural Science	1997
43	Journal of the Torrey Botanical Society	2000; 2001
44	Journal of Theoretical Biology	2000
45	Molecular Crystals and Liquid Crystals	2002
46	New Phytologist	2001
47	NMR Physica B	2000
48	Oecologia	1997; 2000
49	Philosophical Magazine B	2002
50	Physica Scripta	2001
51	Physica A	2002
52	Physica B	2001
53	Physiologia Plantarum	1993; 1993; 1993; 1997; 2001
54	Plant Biosystems	2003
55	Plant, Cell and Environment	1993; 1994; 1995; 2002
56	Plant Cell Tissue and Organ Culture	2000; 2003
57	Plant Ecology	2003
58	Plant Growth Regulation	2003
59	Plant Journal	2001
60	Scandinavian Journal of Forest Research	1998; 1999; 2001; 2003
61	Seed Science and Technology	2002
62	Seed Science Research	2000; 2000; 2001; 2002; 2003
63	Tree Physiology	1995; 1995; 2003
64	Vegetatio	1995
65	Weed Research	1995; 1996; 2002; 2003
66	Weed Science	1994; 2002
67	Wetlands	2002; 2003
	Total No. of citations	***122***

18: 55–64.

However, these papers were not included in a further analysis because even then we can not be sure that all the papers citing the particular Veģis paper are listed.

These 122 papers citing Veģis from 1991 - 2003 can be divided in the following general categories: (i) seed dormancy and germination (53 papers); (ii) dormancy of trees (20); (iii) properties of disaccharide in aqueous solution (18); (iv) climate change effects on forest trees (11); (v) dormancy in plant tissue culture (6); (vi) dormancy and flowering of herbaceous perennials, incl. grasses (6); (vii) flowering of trees (3); (viii) general aspects of dormancy and plant seasonality (2); (ix) effect of chilling stress on herbaceous perennials (2); (x) genetics of adaptive traits of trees (1). The time course analysis of publications from the three major categories (Fig. 2) revealed an increase of the overall rate of citations within the last years (Fig. 1) that was due mostly to papers from the present categories, namely, seed dormancy and germination, dormancy of trees, and properties of disaccharide.

Several recent papers (published in 2000 - 2003) representing the main general categories of the publications were analyzed further to reveal the context of citation of Veģis paper.

The majority of papers (52, about 43 % of the whole list) was devoted to different aspects of seed dormancy and germination. Seed germination ecology was one of the most widely used contexts among them. Objects investigated included weeds, endangered plant species, critical wetland plants, and samples stored in seed banks. The paper by Alvarado

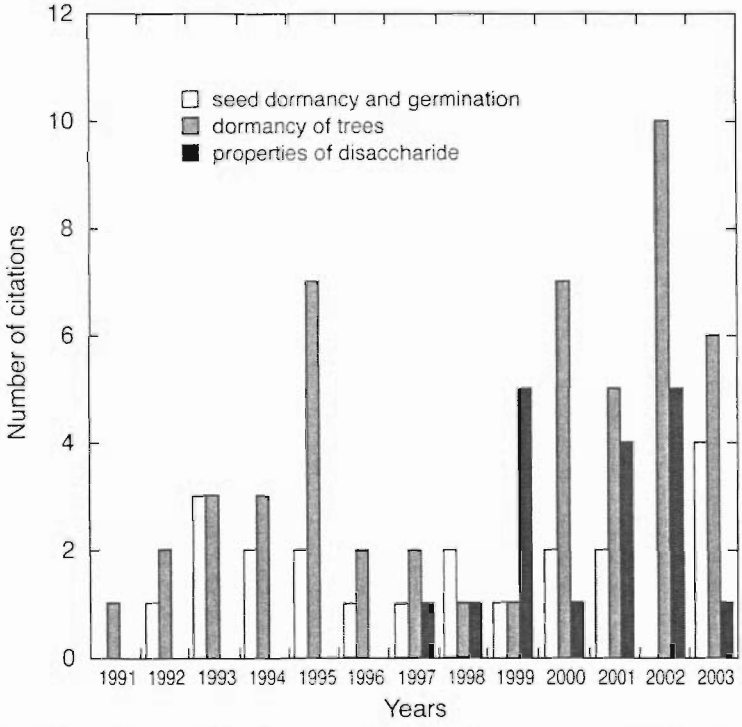

Fig. 2. Distribution of papers citing *Dormancy in higher plants* among major thematic groups.

and Bradford (2002) used Veģis results on a temperature range sensitive to the dormancy status of seeds to develop a hydrothermal time model that can describe seed germination timing and percentages at various temperatures. Further, the paper gave experimental evidence for that model. Data on shifts in the normal distribution in a threshold water potential that just prevents germination of the seed populations in response to dormancy-regulating factors were used by the authors to explain the common observation mentioned by Veģis that the temperature limits for germination widen as dormancy is released and narrow as dormancy is imposed.

The second largest group of papers citing *Dormancy in higher plants* was that of papers studying different aspects of tree dormancy. The paper from this group by Rinne et al. (2001) studied the mechanism by which cessation of morphogenic activity in shoot apex of overwintering perennials is regulated at the end of the growing season when the apex transforms into a bud which is dormant and freezing-tolerant. Veģis paper was cited in respect to an initial stage of the process, which depends on measuring the length of the photoperiod by means of the phytochrome system.

It was somewhat odd to find a relatively large group (18) of papers citing the Veģis paper in respect to properties of disaccharides in aqueous solution. On closer examination of one of them (Prabhu et al. 2002), it appeared that the particular group of papers focused on the physico-chemical mechanisms of disaccharide-associated protective effect against dehydration and freezing. The general hypothesis tested there was that disaccharides, in particular, trehalose, obstruct the crystallization process by reducing the amount of freezable water, namely destroying the network of water compatible with that of ice. This hypothesis was examined experimentally by means of ultrasonic techniques, Raman scattering, NMR etc. Veģis work was cited in these papers as providing an evidence for a biological phenomenon of trehalose biosynthesis in plants under stress conditions.

A substantial group of papers (11) dealt with various aspects of climate change on forest ecosystems. One of them (Price et al. 2001) analyzed recruitment algorithms in forest gap models with particular regard to their suitability for simulating forest ecosystem responses to a changing climate. In these models of forest regeneration, special importance is attributed to seed production and germination success, which are especially climate-sensitive processes. Veģis work was cited there as one of the papers giving evidence for a need of vernalization for successful germination.

Dormancy is a focus of extensive studies also in plant tissue culture. The dormancy-like condition called 'slow growth' is a method for long lasting plant preservation in tissue culture (Pruski et al. 2000). On the other hand, during propagation of bulbous plants by means of tissue culture, high temperature-induced dormancy is an undesirable phenomenon (Langens-Gerrits et al. 2003). The two above papers on the subject cite Veģis paper on dormancy. Tissue culture can be used as a means for investigation of the physiological basis of dormancy, as described by the paper of Kalengamaliro et al. (2003). In alfalfa (*Medicago sativa* L.) two contrasting types of cultivars exist in respect to dormancy. The dormancy in alfalfa is not a true physiological dormancy according to Veģis because alfalfa plants can be forced out of dormancy by favorable conditions. Kalengamaliro and co-workers prepared cell cultures from alfalfa cultivars that genetically differed in dormancy to study physiological basis of the phenomenon. High growth rates of cells derived from rapid growing, non-dormant alfalfa cultivars were associated with rapid sugar uptake and higher cell respiration rates when compared to cells derived from

dormant alfalfa cultivars.

Theoretical considerations of Veģis about differences in true dormancy and relative dormancy have been widely used also in studies of dormancy in other perennial herbaceous plants, including summer dormancy in grasses. The experiments described by Ofir and Kigel (2003) examined naturally occurring variation in the onset of summer dormancy in populations of the geophytic perennial grass *Poa bulbosum* collected along an aridity gradient. In controlled conditions, plant age at the onset of dormancy positively correlated with mean annual precipitation at the site of origin of the population.

Several papers used ideas about dormancy in a broader sense to analyze seasonal phenomena in plant life. In a paper entitled *Aspects of seasonality*, Battey (2000) looked at flowering seasonality in perennials compared with annuals from both molecular and whole plant physiology perspectives. At first it was mentioned that Veģis ideas concerning the proposed mechanisms of bud dormancy, namely, that metabolic limitations (oxygen reduction) were among the internal control factors during dormancy, were exceeded by these in favor of hormonal control. However, the author emphasized that the theoretical considerations of Veģis about dormancy placing a restriction on the temperature range over which buds will grow formed 'a foundation for an analysis of the way in which the environment allows bud growth in the spring'.

We can conclude that the *Dormancy in higher plants* stands out among the most important and highly ranked plant physiology papers published so far. Bearing in mind the extreme longevity of the impact of the paper, one can ask what was so special in the paper of Veģis to hold the attention of so many scientists for so long period of time? One possible explanation is that Veģis was the first to suggest common mechanisms for seed and bud dormancy. From the present point of view, given the fact that these parts of plants utilize the same environmental signals, mainly light and temperature, as a cues for adaptation in a form of dormancy, this seems to be a trivial consideration. However, within 40 years after publication of *Dormancy in higher plants* no unifying general theory about the phenomenon has emerged. At least until that time, we will be waiting for further tribute to Veģis ideas in the form of citing his work.

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Latviešu augu fiziologa Ausekļa Veģa (1903 - 1973) ietekme uz modernajām dabaszinātnēm

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Kopsavilkums

Pierādot, ka miera stāvokli augos inducē ārējie faktori, latviešu augu fiziologs Auseklis Veģis piedalījās modernās augu miera perioda teorijas izveidē. Dotais raksts novērtē Veģa svarīgākās publikācijas (*Dormancy in higher plants*, *Annual Review of Plant Physiology*, 1964) ietekmi uz mūsdienu dabaszinātnēm, analizējot tās citējumus. Veģa raksts citēts 365 reizes 40 gadu laikā pēc tā publicēšanas un tāpēc tas ieņem 86 vietu visvairāk citēto rakstu vidū no 1082 apskata rakstiem, kas publicēti izdevumā *Annual Review of Plant Physiology* laikā no 1950. līdz 2001. gadam. Ir interesanti atzīmēt, ka to rakstu daudzums, kuros citēts Veģa raksts, ir pieaudzis pēdējo sešu gadu laikā. Šis pieaugums pārsvarā saistīts ar publikācijām tādās nozarēs kā sēklu miera periods, miera periods kokaugiem, kā arī disaharīdu fizikālās īpašības. Secināts, ka *Dormancy in higher plants* ir viena no pasaulē nozīmīgākajām publikācijām augu fizoloģijā.

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Ovarian cancer in Latvia is highly attributable to recurrent mutations in the *BRCA1* gene

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Abstract

Most hereditary ovarian cancers are associated with germline mutations in the *BRCA1* and *BRCA2* genes. The aim of this study was to estimate the role of *BRCA1* mutations in ovarian cancer in Latvia and to determine the mutation profile and frequency of founder mutations in ovarian cancer patients. The analysis of the entire *BRCA1* gene was carried out in 34 ovarian cancer patients by SSCP/HD analysis and automatic sequencing of the variants detected. The screening for founder mutations was performed in 75 ovarian cancer patients recruited for the Project "Genome database of Latvian population" and in 86 consecutive ovarian cancer patients treated at the Latvian Oncology Centre. Six of seven pathogenic mutation carriers identified by the analysis of the entire *BRCA1* gene were carriers of founder mutations. A high proportion of *BRCA1* mutation carriers was revealed also by the screening for recurrent mutations. Altogether 44 mutation carriers in 195 ovarian cancer patients were identified in our study. The high frequency (24.6 %) of two founder mutations in Latvian ovarian cancer patients allow us to suggest that testing for these mutations should be offered to all women with ovarian cancer diagnosed before the age of 65 years.

Key words: *BRCA1* gene, founder mutations, genetic testing, Latvian population, ovarian cancer.

Introduction

Despite significant advances in therapy over the past 30 years, ovarian cancer remains the most deadly gynaecological cancer with over 100,000 deaths worldwide. Most women with ovarian cancer still develop recurrent disease and die within five years. Minimal symptoms in the early stages of the disease and the concealed position of the ovaries in the abdomen mean that only 30 % of patients are diagnosed with early-stage disease. Treating patients at an early stage of the disease remains the most important factor in successful treatment.

It has been noted that ovarian cancer tends to cluster in some families and significant impact of genetic factors in ovarian cancer development have been detected. The discovery of the breast/ovarian cancer susceptibility genes *BRCA1* (Miki et al. 1994) and *BRCA2* (Wooster et al. 1995) facilitated detection of individuals predisposed to cancer. These tumour supressor genes are involved in many important cellular processes, including DNA damage recognition, DNA repair, chromatin remodelling and control of transcription. Inactivating germline mutations in *BRCA1* and *BRCA2* genes account

for highly penetrant cancer predisposition, mostly limited to carcinomas of breast and ovaries. Inherited mutations in the genes predisposing to cancer are considered now as most important risk factors. The characteristic features of hereditary cancer cases are earlier onset of disease and recurrence of cancer with high probability.

The mutation prevalence and spectrum in the *BRCA1* gene have been assessed in different populations (Risch et al. 2001; Sarantaus et al. 2001; Meindl 2002; Menkiszak et al. 2003) and the screening for identification of individuals with elevated risk of cancer is widely used in clinics of all developed countries. The nature of germline mutations depends on the ethnicity of population. Recurrent mutations has been identified in the Ashkenazi Jewish population, and another recurrent mutation was identified in the Icelandic population (Liede, Narod 2002). Similarly, founder effects have been observed in Eastern European populations (Gayther et al. 1997; Csokay et al. 1999; Gorski et al. 2000; Oszurek et al. 2001). A high prevalence of recurrent mutations facilitates the identification of the risk individuals. Early diagnosis of cancer is associated strongly with better survival of patients. Screening procedures has been shown to reduce breast cancer mortality by approximately 35 % (Vainio, Bianchini 2002), and even more in ovarian cancer patients.

The lifetime risk of ovarian carcinoma has been estimated to be ~60 % in *BRCA1* mutation carriers (Easton et al. 1995) and ~30 % in *BRCA2* mutation carriers (Ford et al. 1998).

Early molecular changes has been analysed in ovaries removed prophylactically from *BRCA1* mutation carriers who are at significant risk for ovarian cancer development, and a higher rate of potentially premalignant lesions was revealed compared to controls (Werness et al. 1999). However, contrary results have been published as well (Stratton et al. 1999). Phenotypic differences have been detected in ovarian surface epithelium in women from families with a cancer history and in women with no cancer in their family, and more data are needed to understand better the difference between hereditary and sporadic cancers to tailor better the therapeutic and prophylactic procedures to individual patients.

The diagnosis of hereditary breast/ovarian cancer syndrome indicates that the development of ovarian cancer several years after breast cancer may represent the common case and that the knowledge about the genetic basis of disease may be very useful for appropriate management of cancer patients.

Nearly 300 women are diagnosed with ovarian cancer in Latvia annually and a high mortality of women with ovarian cancer is associated with late diagnosis of disease (approximately 70 % of them are diagnosed at stages III - IV).

The aim of our study was to estimate the hereditary fraction of ovarian cancer in the Latvian population, to characterise the mutation spectrum of *BRCA1* gene in ovarian cancer patients and to assess the possibilities and criteria for identification of women with elevated risk of ovarian cancer.

Materials and methods

Permission for the study of hereditary ovarian cancer was obtained from the Central Ethics Committee of Latvia.

Ovarian cancer patients were recruited at the Latvian Oncology Center (LOC). The

criteria for including patients in mutation analysis of the entire *BRCA1* gene were the following:

(i) agreement of the patient to participate in *BRCA1* gene testing,

(ii) diagnosis of ovarian cancer before the age of 55 years,

(iii) breast and/or ovarian cancer in the family and the diagnosis of ovarian cancer before the age of 61 years.

Three recurrent mutations were screened in ovarian cancer patients recruited for the National project "Genome database of Latvian population". Information concerning the personal and family history of cancer was obtained from questionnaires filled by the patients. Screening for 4154delA and 5382insC mutations in consecutive ovarian cancer patients treated at LOC was carried out also.

The DNA was isolated from blood by standard phenol-chloroform procedure and from blood of consecutive ovarian cancer patients by a non-enzymatic salting out procedure.

Oligonucleotide PCR primers for exons 2 - 10 and 12 - 24 were used according to Friedman et al. (1994) and according to sequences available in the BIC (Breast cancer information core) database (http://research.nhgri.nih.gov/projects/bic) for exon 11.

Amplification reactions for all coding exons including flanking intronic sequences of the BRCA1 gene (40 different fragments – 220 - 350 bp) were performed using the reagent kit (Fermentas, Lithuania) and the PTC 100 (MJ Research Inc., USA) thermocycler.

Amplified DNA fragments were analysed for mutations by SSCP (single strand conformation polymorphism) and/or HD (heteroduplex) analysis. Gels were visualised by silver staining.

The analysis of recurrent mutations was carried out by two or three loadings of samples on each gel with appropriate intervals. A positive control was included in each loading.

The DNA was reamplified if variant bands were detected, and the PCR product was directly sequenced using the ABI 3100 PRISM Genetic Analyser (Applied Biosystems, USA). The sequencing reactions were performed using an Amersham Exo (exonuclease I) and SAP (shrimp alkaline phosphatase) pre-sequencing kit and a BigDye Terminator Cycle Sequencing Kit.

Results and discussion

The analysis of the entire *BRCA1* gene was carried out in 34 ovarian cancer patients to characterise the mutation profile in ovarian cancer patients in the Latvian population.

Seven carriers of four different deleterious mutations (5382insC, 4154delA, 300T>G and 962del4) were detected by the analysis of the entire *BRCA1* gene (Table 1). The 962del4 mutation was reported earlier in Austria and Germany (Meindl et al. 2002), but it was not detected in our previous studies of breast cancer patients (Csokay et al. 1999; Tihomirova et al., unpublished data).

Missense mutations of uncertain clinical significance were detected as well: 162A>G (novel, not registered in the BIC database), 1186A>G and 4158A>G. Several common polymorphisms were observed in this study: 3232A>G, 3634A>G, 3667A>G, 4227T>C, 5272+66G>A and others (in exons 8, 9, 12, 17).

Seventy-five ovarian cancer patients were screened for three recurrent mutations prevalent amongst breast cancer patients in Latvia and detected also by the analysis of the entire *BRCA1* gene in ovarian cancer patients. One 300T>G mutation carrier, six

Table 1. *BRCA1* gene mutation analysis in ovarian cancer patients from Latvia (number of carriers detected). *, pathogenic mutations: 5382insC, 4154delA, 300T>G, 962del4

	No.of patients	Age range (average age)	No. of pathogenic mutation carriers*
Analysis of the entire gene	34	20 - 61 (43.5)	7
Screening for three mutations (5382insC, 415delA, 300T>G)	75	24 - 71 (50.3)	18
Screening for two mutations (5382insC, 4154delA)	86	34 - 81 (60.7)	19

4154delA and eleven 5382insC mutation carriers were identified (Table 1).

A high proportion of mutation carriers (19 of 86) was detected by the screening for two recurrent mutations in the group of consecutive ovarian cancer patients (Table 1), in spite of the fact that such criteria as early onset of the disease or positive family history of cancer were not applied in this patient group.

The analysis of family history data given by patients included in this study allows us to characterise the most prevalent cancer sites in families of *BRCA1* mutation carriers identified (Table 2).

The *BRCA1* mutation spectrum in Latvian ovarian cancer patients differs insignificantly from that in breast cancer patients. Three pathogenic founder mutations (5382insC, 4154delA and 300T>G) detected in our studies of breast cancer patients were found in ovarian cancer patients approximately in the same proportions and women with founder mutations make up 86 % of mutation carriers identified.

Apart from protein-truncating recurrent mutations, the analysis of the entire *BRCA1* gene resulted in detection of the unique *BRCA1* frameshift mutation 962del4 and the unique missense mutation 162A>G in ovarian cancer patients of Latvian ethnicity. Two known missense mutations (1186A>G and 4158A>G) with unclear significance and several common polymorphisms were identified as well. 962del4, 4154delA and 5382insC are pathogenic protein-truncating mutations.

The 300T>G missense mutation leads to substitution of cisteine to glycine in an important RING-finger domain of breal protein and its strong pathogenic nature was proved unambiguously. In contrast to other Eastern European countries (Hungary, Poland) and Germany (Neuhausen 2000) the frequency of this mutation in Latvia is significantly lower. This fact was confirmed by the additional screening in another 100 breast/ovarian cancer patients with early onset of disease and a family history of cancer in most of them (unpublished).

The frequencies of pathogenic mutation carriers were similar in all patient groups tested: 20.6 % of mutation carriers were identified by the analysis of the entire *BRCA1* gene in 34 patients, 24.0 % mutation carriers by screening for three mutations in patients who donated blood for the Genome Database of the Latvian population, and 22.1 % by screening for two mutations in consecutive ovarian cancer patients (Table 1). Mutation analysis of the entire *BRCA1* gene is laborious because of the large size of the gene and the diversity of mutations detected. The results of our study indicate that the screening for

Table 2. Cancer sites reported in families of *BRCA1* mutation carriers

Cancer site	Number of cases
Breast	12
Ovary	10
Colon, rectum	9
Uterus	6
Lung	6
Kidney	2
Prostate	2
Stomach	2
Melanoma	1
Liver	1
Pancreas	1
Cervix	1
Neuroblastoma	1
Other	5

only two mutations (5382insC and 4154delA) allows easy identification of the majority of women with high risk of breast and ovarian cancer.

Studies of consecutive ovarian cancer patients unselected for age or family history provide useful information regarding the contribution of germline *BRCA1/2* mutations to ovarian cancer occurrence in a population. These studies have been performed in many populations with founder *BRCA1* or/and *BRCA2* mutations.

The proportion of the two founder mutations in unselected ovarian cancer patients in our population (22.1 %) is lower than the frequency of founder mutations in the Israel Ashkenazi Jewish population (29.0 %; Modan et al. 2001), but higher than in Polish (13.5 %; Menkiszak et al. 2003), Finnish (8.6 %; Sarantaus et al. 2001), Hungarian (11.0 %; Van Der Looji et al. 2000) and French Canadian (8.1 %; Tonin et al. 1999) populations.

The proportion of ovarian cancer cases diagnosed in different age groups is shown in Fig. 1A and the proportion of *BRCA1* mutation carriers identified in the same age groups are shown in the Fig. 1B. These figures show that the major proportion of mutation carriers among ovarian cancer patients can be identified by the screening of women diagnosed before 65 years.

The data concerning cancer history in the family reported by *BRCA1* mutation carriers (Table 2) show that, apart from breast and ovarian cancer in the family (the main criterion used for genetic testing of hereditary cancer), other cancer sites were reported as well. Colorectal, uterine and lung cancers are reported more frequently. Thus, the presence of other cancers in the family history is very important information for genetic counselling and important criterion to offer *BRCA1* testing in these families.

It has been suggested that ovarian cancer risk depends on the localisation of mutation in the *BRCA1* gene (Gayther et al. 1995). Mutations in the 3'-end of the *BRCA1* gene are more strongly associated with breast cancer and mutations in the middle of the gene

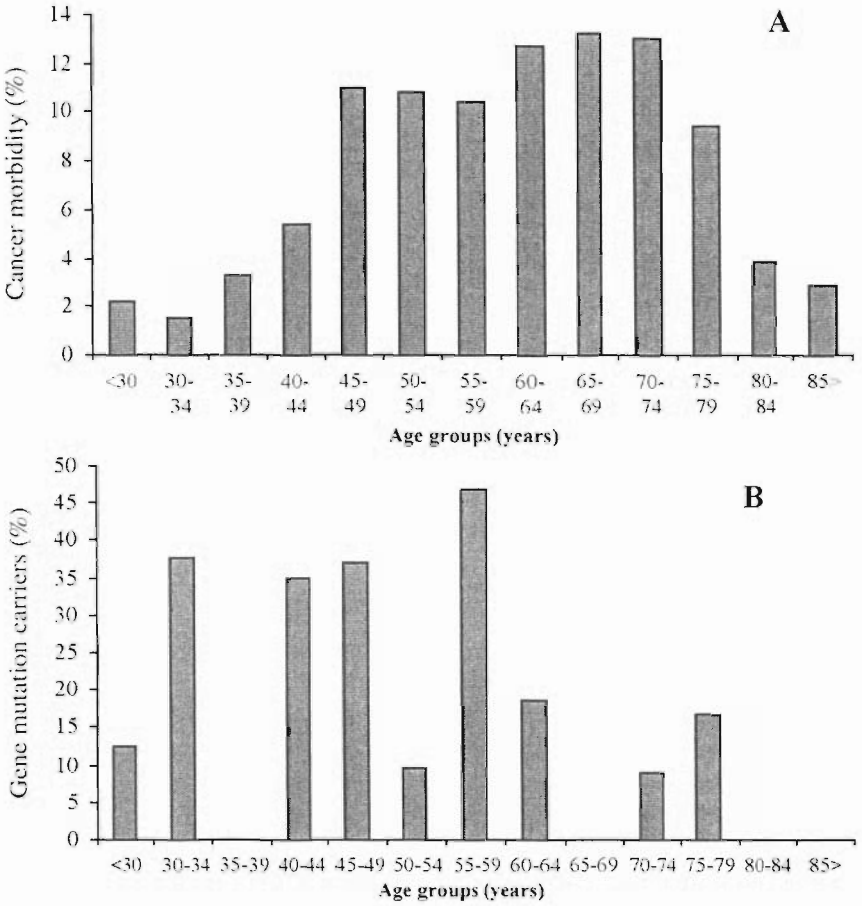

Fig. 1. Ovarian cancer patients and BRCA1 mutation carriers in different age groups. A, Ovarian cancer morbidity (%) in Latvia by age (average in 1999 - 2003). B, *BRCA1* gene mutation carriers (%) amongst ovarian cancer patients by age.

localised between 2401 and 4191 result in a higher proportion of ovarian cancer (Risch et al. 2001; Thompson et al. 2002). However, other genetic or environmental factors may function as modifiers of the risk of ovarian cancer (Phelan et al. 1996; Narod 2002). Our data indicate that a high proportion of mutations in the 3'-end of the gene can be detected among ovarian cancer patients. The screening for the most prevalent recurrent mutations resulted in detection of 17 carriers of the 4154delA mutation and 24 carriers of the 5382insC mutation and only two carriers of the 300T>G mutation were identified. Our results indicate that at least the specific mutation 5382insC localised in the 3'-end of the gene may be associated with ovarian cancer in a high proportion of cases.

If the recurrent mutations are not detected in a patient with early onset of disease and a strong family history of cancer, the analysis of entire *BRCA1* gene should be offered. An example of this strategy is the identification of the 962del4 mutation carrier. One ovarian cancer patient diagnosed at the age of 40 years had breast cancer at age of 36

and a positive family history of other cancers (colorectal and renal cancer). Recurrent mutations were not detected and the patient was involved in the analysis of the entire *BRCA1* gene. A protein-truncating mutation in the 5'-end of the exon 11 was detected.

Altogether 44 *BRCA1* pathogenic mutation carriers were identified in our study. The *BRCA1* gene mutations were implicated in a large proportion of ovarian cancer incidence in Latvia. The screening for specific mutation in their families would allow to identify unaffected relatives who are at elevated risk of cancer. Special surveillance programme favouring diagnostics at an early stage of disease then should be offered to *BRCA1* mutation carriers.

The results of this study are useful for genetic counselling and genetic testing in the Latvian population. Further studies would be useful to estimate the risk of disease and possibilities of prevention of cancer in healthy mutation carriers.

Acknowledgements

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Olnīcu vēzis Latvijā ir bieži saistīts ar mutācijām *BRCA1* gēnā

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Kopsavilkums

Iedzimto olnīcu vēzi raksturo agrīna saslimšana un bieža slimības atkārtošanās. Ir zināma tā saistība ar iedzimtām mutācijām krūts un olnīcu vēzi predisponējošos gēnos *BRCA1* un *BRCA2*. Darba mērķis bija novērtēt *BRCA1* gēna mutāciju nozīmi olnīcu vēža etioloģijā sievietēm Latvijā un raksturot Latvijā izplatīto mutāciju spektru. Pētījums veikts ar AML ētikas komisijas atļauju. 34 olnīcu vēža slimniecēm ar vēzi ģimenē vai agrīnu saslimšanu (vidējais vecums 43 g.) tika veikta pilna *BRCA1* gēna testēšana, izmantojot vienpavediena DNS konformācijas polimorfismu un DNS heteroduplekšu analīzi un atrasto ģenētisko variantu automātisko DNS sekvenēšanu. Izplatīto mutāciju skrīnings tika veikts dažādās pacientu grupās (161 sievietei). Kopumā, analizējot 195 slimnieču DNS, noteiktas 44 patogēno mutāciju nesējas, no tām divas mutācijas (5382insC un 4154delA) 41 slimniecei. Divu *BRCA1* gēna mutāciju bieža sastopamība (21 %) olnīcu vēža slimniecēm Latvijā dod iespēju viegli identificēt lielāko daļu to ģimeņu, kurās tiek pārmantota vēzi predisponējoša mutācija. Tālāka vienas konkrētas mutācijas testēšana veselajām radiniecēm šajās ģimenēs ļauj viegli identificēt lielāko daļu riska personu, kurām draud saslimšana ar krūts vai olnīcu vēzi.

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Tick-borne encephalitis – pathogen, vectors and epidemiological situation in Latvia 2002 - 2003

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Abstract

Tick-borne encephalitis (TBE) morbidity in Latvia declined significantly in 2002 compared to the previous decade, although in 2003 the number of TBE cases rose again. *Ixodes ricinus* tick activity observations in nature as well as from the records on seasonal tick numbers removed from patients in the Vaccination service for TBE virus (TBEV) tests pointed to a sharp rise in nymph numbers. TBEV prevalence shown with ELISA in ticks removed from humans was four times higher than in field collected ticks, but the total TBEV prevalence level decreased in 2003. Investigations of TBEV by RT-PCR was performed by means of two methods, targeting 5'NCR (non-coding) or NS5 (non-structural) sequences of the *TBEV* gene. The initial results of the newly adapted methods confirmed the validity of the developed RT-PCR and pointed at the necessity to improve and standardise the system of sampling, storage and transporting. Observations identified TBE as a continuing public health problem in Latvia requiring further research.

Key words: *Ixodes ricinus*, Latvia, nymphs, reverse transcription polymerase chain reaction (RT-PCR), tick-borne encephalitis virus (TBEV).

Introduction

Tick-borne encephalitis virus (TBEV) is a RNA flavivirus of the *Flaviviridae* family causing tick-borne encephalitis, a severe neurological disease in Eurasia (Kaiser 2002). Epidemiologically significant TBEV vectors are hard-bodied (*Ixodidae*) ticks among which *Ixodes ricinus* and *I. persulcatus* are common carriers of the disease in Latvia.

Three subtypes of the TBEV are known until now: Western subtype (W-TBEV), Far-Eastern subtype (FE-TBEV) and Siberian subtype. All known TBEV isolates from Europe belong to the W-TBEV subtype, while isolates from eastern Russia, China and Japan belong to the FE-TBEV subtype. The recently discovered Siberian subtype includes only three isolates – 'Vasilchenko', 'Aina' and 'Latvia 1-96'. All three subtypes are antigenically and phylogenetically closely related (Lundkvist et al. 2001). *I. ricinus* is known to be main transmitter for the W-TBEV subtype, and *I. persulcatus* for the FE-TBEV and Siberian subtype.

The severity and clinical course of tick-borne encephalitis are dependent on the TBE virus subtype causing the disease. The FE-TBEV subtype is characterised by more serious

damage of the central nervous system and a two-fold higher lethality in comparison with the W-TBEV subtype (Oschmann et al. 1999). Latvia belongs to the regions where all three virus subtypes are found (Mavchoutko 2000; Süss 2002).

I. ricinus has two activity peaks for both epidemiologically most significant developmental stages – adults and nymphs, this species is spread in the whole territory of Latvia but is rarer in its eastern regions where *I. persulcatus* dominates (Bormane 1999). *I. persulcatus* adults have only one activity peak in the spring. The majority of TBE cases each year occur in *I. ricinus* habitat regions of Latvia. Rather sharp annual changes were typical for TBE morbidity in Latvia: the highest peaks of TBE cases were registered in 1994 and 1995 (accordingly 1366 and 1341) while in 2002 there were 153 cases. However, in 2003, according to the statistical records, TBE morbidity rise again and 365 TBE cases were registered.

TBE virus is about 50 nm total diameter and has an infectious genomic single-stranded RNA. The length of the genome is about 11 000 nucleotides (10 927 - 11141 depending on the strain). TBEV has three main structural proteins: immunogenic envelope glycoprotein E, membrane-associated protein M and capsid protein C. Protein C, together with genomic RNA, forms the nucleocapsid; the protein coat consists mainly of glycoprotein E, and in mature virus particles – of protein M. Gene sequences coding structural proteins (E, M and C) are located in the first fourth of the TBEV genome, the rest is occupied by non-structural gene sequences (NS). Non-coding regions (NCR) limit the TBEV genome at the 5'- and 3'-terminations (Oschmann et al. 1999). Genetic analysis of E glycoprotein coding gene sequences has been used to determine TBEV subtypes.

The methods used world-wide for diagnosis of TBEV have been direct tests (RT-PCR reverse transcription polymerase chain reaction, electron microscopy, virus culture) and indirect tests for antibody detection – ELISA, immunoblot, hemagglutination inhibition test (HIT), virus neutralization test (NT) and complement-fixation reaction (CFR) (Oschmann et al. 1999).

ELISA, the most common diagnostic method in Latvia, was used for detection of IgG and IgM antibodies in human sera and cerebrospinal fluid, as well as to determine TBEV prevalence in questing *Ixodes* ticks collected from vegetation and in, to a more or less extent, engorged ticks removed from patients.

A recent development has been direct virus detection in ticks and human blood sera by RT-PCR by use of two methods: amplification of the non-coding (5'NCR) gene fragment and amplification of the non-structural (NS5) gene fragment. RT-PCR methods are known to be among the most sensitive tools of TBE virus detection (Schrader, Süss 1999). The aim of the present work was to assess the importance of the most significant factors likely affecting TBE morbidity in the period of 2002 - 2003 as well as to develop a direct and more sensitive PCR-based TBEV test method.

Materials and methods

Tick collecting

Questing tick seasonal activity changes were measured by flagging of all tick developmental stages in a permanent tick monitoring site for *I. ricinus* (in Riga region) from April to October. The number of ticks was counted two to three times monthly in permanent route lines.

A standard flannelette flag (100 × 90 cm) was used. Collected ticks were put into the vials with grass and preserved at 4 °C until determination of species.

TBEV prevalence was investigated in questing ticks collected from vegetation in 14 administrative districts of Latvia and also in ticks removed from patients. Patients bitten by ticks applied to the Vaccination service Ltd. in Riga to check the brought ticks for TBEV prevalence and to receive recommendations about prophylactic measures.

TBE virus (TBEV) detection in ticks by ELISA

Questing ticks from vegetation and ticks removed from patients were tested in the Laboratory of Virological investigations, State Agency "Public Health Agency" by the qualitative determination of tick-borne encephalitis virus antigen using the standard ELISA (Enzyme-linked immunosorbent assay) technique (Institute of Virology, Moscow).

The wells in the A, C, E and G lines were coated with IgG to TBEV. The wells in the B, D, F and H lines were coated with normal IgG. Every tick was smashed in 0.2 ml PBS and 0.05 ml was dispersed to wells with IgG to TBEV and with normal IgG. After incubation of samples with the first-layer IgG, horseradish-labelled IgG conjugate was added. Orthophenylene diamine solution was added and the enzyme activity was measured. The absorbency was recorded within 10 min after adding the stop-solution by a photometer (Multiscan Multisoft) at 492/620 nm. Samples with optical density twice more than the negative antigen control was considered as TBEV antigen positive.

TBE virus (TBEV) detection in ticks by RT-PCR

RNA purification and cDNA synthesis. RT-PCR was performed in Biomedical Research and Study Centre (BMC) University of Latvia by using two methods targeted at non-coding regions (NCR) gene or non-structural gene (NS) fragments. Individual questing ticks were stored at -70 °C before using for RNA extraction; removed tick homogenates and human blood sera were stored at -70 °C after delivering to BMC. Tick pools (five ticks) before RNA isolation were crushed in TE buffer (Tris-HCl, EDTA, pH 7.6).

TBEV RNA extraction was performed using a QIAamp Viral Mini Kit (QIAGEN GmbH).

cDNA synthesis was realized with a RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Lithuania). For the reverse transcription, first PCR set primers were used as described previously (Schrader, Süss 1999; Han et al. 2001).

PCR amplification. TBEV was analysed by two nested RT-PCR methods: 5'NCR-RT-PCR and NS5-RT-PCR. The 5'NCR-RT-PCR method was adapted from the one described by Schrader and Süss (1999). Briefly, the nested RT-PCR reaction was performed in a 50 μl reaction volume (reagents from Fermentas, Lithuania) with 10 μl cDNA and primers Ppl (5'-GCGTTTGCTTCGGACAGCATTAGC) and Pml (5'-GCGTCTTCGTTGCG GTCTCTTTCG) for the first amplification. A thermal cycler "Progene" (Techne, UK) was used for amplification at the following conditions: 2 min at 94 °C for initial denaturation, 40 PCR cycles with 30 sec at 94 °C and 30 sec at 66 °C. The final elongation was 5 min at 66 °C. For the second PCR set, 2 μl of the first amplification product, primers Pp2 (5'-TCGGACAGCATTAGCAGCGGTTGG) and Pm2 (5'-TGCGGTCTCT TTCGACACTCGTCG) were used for amplification at following conditions: 9 min at 95 °C for initial denaturation, 30 PCR cycles with 15 sec at 94 °C and 30 sec at 65 °C.

The final elongation was 10 min at 72 °C.

The NS5-RT-PCR method was similar to the one described by Han et al. (2001), except that the following primers for the first and nested reactions were used: first set primers I (5'-GAGGCTGAACAACTGCACG); II (5'-GAACACGTCCATTCCTGATCT) and second set primers III (5'-ACGGAACGTGACAAGGCTAG); IV (5'-GCTTGTTACC ATCTTTGGAG). The PCR programme was: 2 min at 96 °C for initial denaturation, 35 PCR cycles (for the first set) or 30 PCR cycles (for the second set) with 30 sec at 96 °C, 30 sec at 40 °C and 30 sec at 68 °C. The final elongation was 5 min at 68 °C.

For each PCR set, negative and positive control samples were included. Two kinds of positive control samples were used: Encepur Chiron Behring (Germany) containing the inactivated TBEV K23 strain and TBEV vaccine (Russia) containing the inactivated TBEV *Sofjyn* strain.

Amplicons were visualised on a 2.5 % agarose gel stained with ethidium bromide or 8 % PAAG gel, and documented by a Kodak image system.

Statistical methods

Statistical calculations were performed by using the mathematical paired Student's test method (Bailey 1959).

Results

Epidemiological data

The TBE morbidity level in 2002 was approximately the same as in the period of 1990 - 1992 before TBE morbidity peaks: with 153 TBE cases and an incidence rate of 6.52 it had reached its minimum during the last decade. However, in 2003 TBE morbidity increased again and number of cases doubled in comparison with the previous year: 365 TBE cases (15.66 per 100,000 inhabitants) were registered. In 2003 the highest number of TBE cases per month (101) was recorded in September, while in the previous year in that

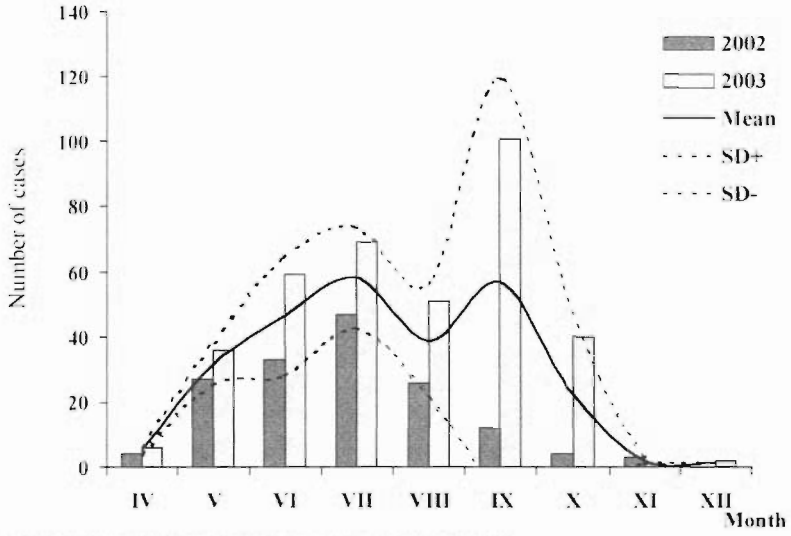

Fig. 1. Distribution of TBE cases by months (2002 - 2003).

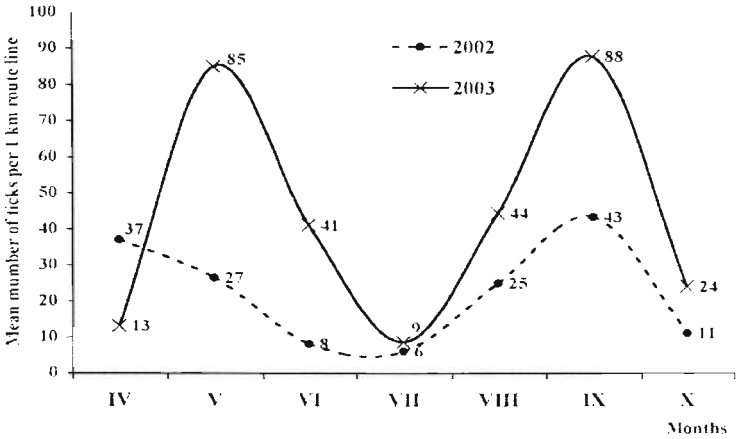

Fig. 2. Seasonal activities of *I. ricinus* nymphs (Riga region, 2002 - 2003).

month only a small number (12) of TBE cases was registered, and the seasonal morbidity peak was in July (Fig. 1)

Clinical data reveal that the majority (about 60 %) of TBE clinical cases in 2002 and 2003 were medium severity TBE meningeal form, about 30 % – febrile, and about 10 % of cases were the severest form – meningoencephalitis. TBE lethality in the period of 2002-2003 was 0.6 to 0.8 %: one lethal case in 2002 and three in 2003.

Tick number seasonal changes

Seasonal tick activity dynamics for *I. ricinus* adults registered in the monitoring site in Riga region (Tīreļi Forest Management District) indicated slight decrease in the tick activity in the season of 2003 in comparison with the previous year. The mean seasonal number of adult ticks per 1 km in 2003 (35.8 ticks per 1 km), but 1.45 times lower than the one in 2002 (24.6 ticks per 1 km) however statistically this difference was not significant ($p > 0.05$).

However, *I. ricinus* nymph activity increased significantly in 2003, especially in the first decade of May and third decade of September (Fig. 2). The mean seasonal number of nymphs per 1 km in 2003 (51.8) was two times higher than in the previous year (25.5 nymphs per 1 km).

In total, 2404 samples of ticks (predominantly nymphs and females from *I. ricinus* habitat regions) were removed from patients and brought to the Vaccination service in 2003, which was 3.4 times ($p < 0.05$) more than in 2002 (700 tick samples; Table 1). In several cases two to four ticks (almost only nymphs) bit patients simultaneously and the total number of collected individual ticks in 2003 was 2453, although only one sample per patient was tested for TBEV. The maximum number of ticks from patients in 2003 was collected by the Vaccination service in September, while a year before the peak number was registered in July. In 2002, 55 % (385/700) of removed ticks were adults (females), 43 % (303) – nymphs and 1.7 % (12) – larvae (Table 1). In 2003, nymphs contributed the highest numbers of removed individual ticks – 57.9 % (1420/2453), 41.1 % (1008) were adults and 1.0 % (25) – larvae (Table 2). In 2003, 14 % of patients indicated being bitten by tick in Riga, 37 % – in different parts of Riga region, but the majority (48 %)

Table 1. Number of ticks (samples) removed from patients for TBE tests in 2002 (Vaccination service, by dates of registration)

Month	Adult imagos	TBEV positive imagos	TBEV prevalence in imagos (% ± SD)	Nymphs	TBEV positive nymphs	TBEV prevalence in nymphs (% ± SD)	Larvae	TBEV positive larvae
April	10	2	20.0 ± 13.3	7	1	14.3 ± 14.3	0	-
May	72	37	51.4 ± 5.9	39	21	53.9 ± 8.1	0	-
June	101	41	40.6 ± 4.9	88	49	55.7 ± 5.3	6	4
July	125	31	24.8 ± 3.9	96	28	29.2 ± 4.7	3	2
August	47	16	34.0 ± 7.0	38	17	44.7 ± 8.2	3	0
September	35	10	28.6 ± 7.8	21	11	52.4 ± 11.2	0	-
October	5	2	40.0 ± 24.5	4	1	25.0 ± 25.0	0	-
total	395	139	35.2 ± 2.4	293	128	43.7 ± 2.9	12	6

of patients were likely to be bitten in the whole territory of Latvia (including eastern regions). About 1 % of patients received a tick bite abroad.

TBE virus investigations with ELISA

TBE virus prevalence in adult questing ticks collected from vegetation was 9.2 ± 1.5 % (34/369) in 2002 and 5.1 ± 1.2 % (17/336) in 2003. In 2002, the TBEV prevalence in *I. persulcatus* was higher than in *I. ricinus* (accordingly 29.0 ± 5.8 % and 5.2 ± 1.3 %), but in 2003 only one (0.9 ± 0.9 %) *I. persulcatus* tick (female) and 5.2 ± 1.8 % of *I. ricinus* ticks were TBEV positive. The results of both years indicate a higher TBEV prevalence in females of both species than in males. In 2002, the TBEV prevalence in *I. ricinus* females was 6.1 ± 1.9 % (in males – 4.2 ± 1.7 %); the TBEV prevalence in *I. persulcatus* females was 60 ± 9.1 % (in males – 0 %). In 2003, the TBEV prevalence in *I. ricinus* females was 13 ± 3.2 % (in males – 0.9 %); the TBEV prevalence in *I. persulcatus* females was 1.6% (in males – also 0 %). However, as the number of investigated ticks was low, the standard deviation was too high to show statistical significance.

The TBE virus prevalence rate in ticks (samples per patient) removed from patients in the Vaccination service was higher: in 2002, 35.0 % (136/385) of adults, 42.7 % (127/303) of nymphs and 6 of 12 larvae were TBE positive. However, in 2003, the TBEV prevalence in ticks was 23.9 % (240/1005) for adults, 14.8 % (203/1374) for nymphs and 12 % (3/25) for larvae. The TBEV prevalence in tested ticks changed during the whole tick activity season: the highest numbers of infected adults and nymphs brought to the Vaccination service were registered in the first part of season with the peak prevalence levels in June and July (accordingly 46 % to 59 % and 27 % to 28 %).

TBE virus investigations with RT-PCR

Twelve tick pools (five ticks per pool) and 22 tick homogenates from patients were investigated. Seventeen samples of 22 tick homogenates were previously identified as TBEV-positive by the ELISA test.

The RT-PCR targeted TBEV 5'NCR gene fragment pointed to TBEV prevalence in

Table 2. Number of ticks (samples) removed from patients for TBE tests in 2003 (Vaccination service, by dates of registration)

Month	Adult imagos	TBEV positive imagos	TBEV prevalence in imagos (% ± SD)	Nymphs	TBEV positive nymphs	TBEV prevalence in nymphs (% ± SD)	Larvae	TBEV positive larvae
April	6	2	33.3 ± 21.8	3	0	0	0	-
May	92	36	39.1 ± 5.1	63	5	7.9 ± 3.6	1	0
June	132	61	46.2 ± 4.4	173	46	26.6 ± 3.4	0	-
July	153	90	58.8 ± 4.0	295	83	28.1 ± 2.6	2	0
August	200	35	17.5 ± 2.7	213	25	11.7 ± 2.2	12	1
September	394	13	3.3 ± 0.9	595	38	6.4 ± 1.0	9	2
October	27	3	11.1 ± 6.2	32	6	18.8 ± 7.0	1	0
December	1	0	0	0	0	0	0	-
total	1005	240	23.9 ± 1.4	1347	203	14.8 ± 1.0	25	3

seven tick pools (of 12), but only in one sample of previously positive tick homogenates from patients. The RT-PCR targeted TBEV NS5 gene fragment pointed to TBEV prevalence in three tick pools (of 12).

The 5'NCR-RT-PCR method results indicated different PCR product lengths for the positive control-containing K23 TBEV strain and for the one containing the *Sofjyn* strain (accordingly ~178 bp and ~215 bp). The length of amplicone from the K23-containing sample was identical to that described in the literature (Han et al. 2001), but the PCR amplicone from the *Sofjyn*-containing sample was longer than theoretically expected (Fig. 3).

Lengths of amplification products of the NS5 gene fragment from the positive control-containing K23 TBEV strain was also identical to those given in literature but the positive control-containing *Sofjyn* TBEV strain did not show any positive results.

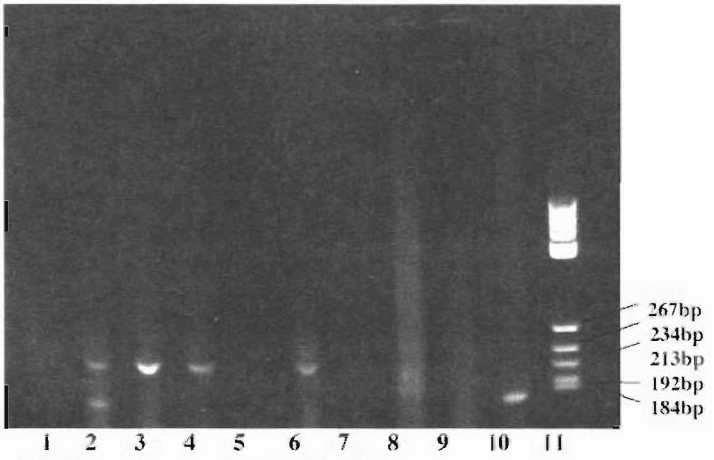

Fig. 3. Tick pools detected by nested 5'NCR-RT-PCR.

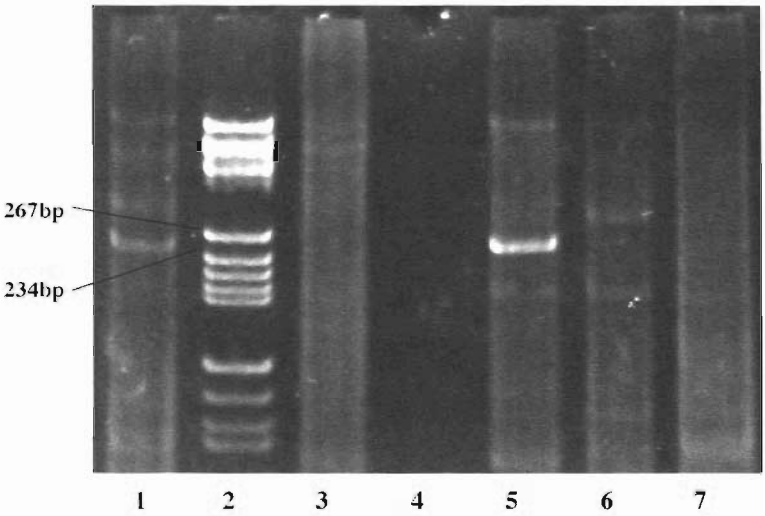

Fig. 4. Tick pools detected by nested NS5-RT-PCR.

5'NCR-RT-PCR tests in tick pools resulted in amplification products, the length of which (~220 bp) differed from that of the positive control-containing K23 strain, and was closer to the length of the positive control-containing *Soffyn* strain. For one of the positive samples, PCR resulted in two samples, one of which was identical to the positive control-containing K23 strain (~178 bp) and the second was ~220 bp. The amplification product with ~178 bp length was sequenced and the results confirmed the positive result.

NS5-RT-PCR tests in tick pools resulted in amplicon products the length of which (~252) were identical to the positive control-containing K23 TBEV strain (Fig. 4).

Sensitivity of the 5'NCR-RT-PCR method (determined with the antigen of TBEV K23 strain dilutions) was 9.8 fg TBEV RNA, but sensitivity of the NS5-RT-PCR method was only 900 fg TBEV RNA.

The 5'NCR-RT-PCR method as more sensitive was used to test for TBEV also in 13 TBE patient blood sera samples, previously tested and confirmed positive by the ELISA method (variable antibody titres were detected in samples). All samples failed to show any positive results.

Discussion

The TBE morbidity level has been influenced by different factors, among which the most important could be annual tick activity level, the immunisation level of the population, changes in recreation habitats due to weather conditions, TBEV prevalence in ticks, annual harvest of mushrooms and berries etc. Comparing the seasonal distribution of TBE cases in 2002 and 2003 and taking into consideration the common incubation period, it could be supposed that in 2002 most of the patients were bitten during the first part of summer (May, June). In 2003, massive exposure to ticks was likely to have occurred also in May - June, but this period lasted longer with a maximum tick bites in August and early September.

The monthly distribution of the number of ticks collected from patient reporting to

the Vaccination service indicates some similarity. The majority of ticks here were also collected in September (in 2002 – two months earlier). The correlation between seasonal distribution of TBE cases and numbers of ticks collected from persons in 2003 was rather high: for adults the correlation coefficient was 0.91 and for nymphs – 0.92 ($p < 0.05$). However, in 2002 the correlation was higher and more expressed for adults, not for nymphs: for adults the correlation coefficient was 0.98 and for nymphs – 0.95 ($p < 0.05$) (Fig. 5). This could indicate another significant factor besides tick activity influenced the TBE morbidity level in 2003. According to the personal observations of specialists of the Latvian Nature Museum, 2003 was a year with a very good mushroom harvest in woodlands started in autumn, in contrast to that in 2002. This may have helped to promote the frequency of visits of the Latvian population to forests.

Although the seasonal activity of adult questing *I. ricinus* registered in the tick monitoring site in the Riga region decreased in 2003 compared with the previous year, the nymph activity increased significantly especially in May and September. Similar differences were apparent from the Vaccination service data: in 2002 more than a half (55 %) of all collected ticks from patients were adults (predominantly females); while in 2003 the majority was nymphs (58 %).

In 2002 the mean TBEV prevalence level in questing adult ticks identified with the ELISA test method was 4.2 times lower than in ticks removed from patients. In 2003, this difference was similar (3.7 times), and a total decrease in the TBEV prevalence level had been noticed in both questing ticks and ticks removed from patients. In the same time, the number of tested ticks from patients in 2003 increased 2.6 times for adults but more significantly (4.5 times) for nymphs.

The seasonal dynamics of TBEV prevalence in ticks (adults or nymphs) brought by patients to the Vaccination service did not indicate a significant correlation with the seasonal distribution of TBE cases ($R = 0.11 - 0.50$, $p < 0.05$). This suggests that the tick population dynamics may be a more important factor influencing the TBE morbidity level than TBEV prevalence in tick population.

The results of the initial investigations of the TBEV virus with the direct test method

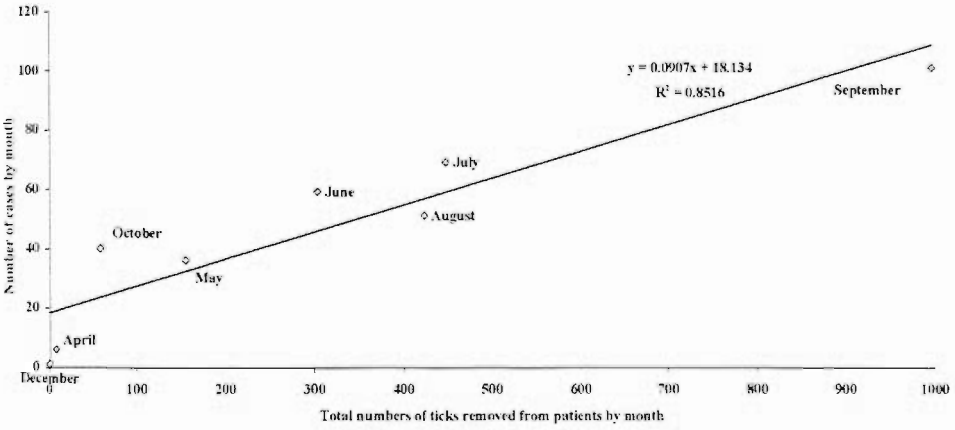

Fig. 5. Correlation (r) between monthly number of TBE cases and number of ticks removed from patients in 2003 (data from the Vaccination service).

– RT-PCR are still controversial. Higher results were obtained in TBEV investigations in tick pools. This may be explained by better storage conditions of individual ticks (after collecting from vegetation ticks were identified, immediately frozen and stored without interruption at -70 °C), which helped to avoid RNA degradation. Tick homogenates were frozen and thawed repeatedly while transported. Although the sensitivity of the RT-PCR method is high and it allows to differentiate TBEV subtypes, its use is limited by the instability of viral RNA, demanding an improved system of sampling, storage and transporting. Comparing the two methods, RT-PCR of the TBEV 5'NCR fragment was about 100 times more sensitive than RT-PCR of the TBEV NS5 fragment, therefore the former is more perspective to use in further investigations.

Differences in the seasonal distribution of TBE cases, activity of developmental stages of *I. ricinus* ticks, and tick registration data from the Vaccination service, suggest that one of most significant factors which was likely to have influenced the number of TBE cases in 2003 was the high number of nymphs. However, the total TBE prevalence in adult and nymphal ticks was lower in 2003. Therefore, another significant factor – visitation by the population to recreation sites, mainly woodlands, might have been much higher in 2003. Observations of TBE epidemiological situation, vector activity changes and TBEV investigations indicated TBE as a continuing public health problem in Latvia that requires further research.

Acknowledgements

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Ērču encefalīts – patogēns, pārnesēji un epidemioloģiskā situācija Latvijā 2002. - 2003. gadā

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Kopsavilkums

Ērču encefalīta saslimstība Latvijā 2002. gadā, salīdzinot ar iepriekšējo 10 gadu periodu, bija ievērojami samazinājusies, tomēr, 2003. gadā saslimšanas gadījumu skaits atkal pieauga. Gan *I. ricinus* ērču aktivitātes novērojumi dabā, gan arī cilvēkiem piesūkušos ērču uzskaite vakcinācijas kabinetā liecināja par ērču nimfu skaita krasu pieaugumu. Ar ELISA metodi noteiktā cilvēkiem piesūkušos ērču inficētība 2002. un 2003. gadā bija aptuveni četras reizes augstāka, nekā dabā savākto ērču inficētība, tomēr kopējais inficētības līmenis 2003. gadā bija zemāks. Ērču encefalīta vīrusa izpēte ar RT-PCR tika veikta, izmantojot uz nekodējošo 5'NCR un nestrukturālo NS5 vīrusa gēnu sekvencēm vērstas metodes. Sākotnējie rezultāti apstiprināja jaunattīstīto RT-PCR metožu ticamību un norādīja uz nepieciešamību uzlabot un standartizēt paraugu vākšanas, uzglabāšanas un transportēšanas sistēmu. Novērojumi apstiprināja, ka ērču encefalīts Latvijā turpina būt nozīmīga sabiedrības veselības problēma, kam nepieciešami tālāki pētījumi.

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Synthesis of all hepatitis B structural proteins in the Semliki Forest virus expression system

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Abstract

All structural genes of human hepatitis B virus (HBV), subtype *ayw*, have been expressed in the Semliki Forest virus (SFV) expression vectors pSFV1 and pSFV-C, allowing direct and SFV core-dependent expression of foreign genes, respectively. Three variants of HBV surface genes (large, middle and small), as well as the core gene were amplified by PCR technique as independent units and fusions with the SFV core protein gene, cloned in both SFV vectors and expressed in BHK-21 cell culture as single proteins or in different combinations. Maximal production of HBV proteins was achieved using pSFV/C vectors where target products were obtained after processing of SFV core-HBV protein fusions. After infection of the cultured BHK cells with recombinanat SFV-HBV viruses, the expression of all HBV structural genes was detected by immunocytochemistry. Electron microscopy revealed the formation of virion-like particles in the case of simultaneous expression of HBV surface and core genes. This efficient expression system of HBV subunits will be applied for the elucidation of molecular details of HBV assembly, structure and entry.

Key words: hepatitis B virus, Semliki Forest virus.

Introduction

Approximately 5 % of the world population is infected by the hepatitis B virus (HBV), which causes a necroinflammatory liver disease of variable duration and severity. Chronically infected patients with active liver disease carry a high risk of developing cirrhosis and hepatocellular carcinoma. HBV virions, or Dane particles, and attending macromolecular forms, the so-called 22-nm particles, are unique in their features, since the virions are formed by four structural proteins (for review, see Gerlich, Bruss 1993). One of them, the hepatitis B core antigen (HBc) encoded by viral gene C, forms an inner capsid packaging genomic partially double-stranded DNA, and three variants of the surface antigen (HBs) encoded by gene S form an outer envelope consisting of a phospholipid bilayer membrane with protein spikes. The major S gene product is the 226-amino acid-residue-long HBsAg, or S, or "small", or SHBs antigen; the two other HBsAg variants are N-terminally prolonged forms of the former, which contain an additional 55 amino acid residues (M, or "middle", or MHBs) and 119 or 108 amino acid residues for the two major HBV subtypes adw and ayw, respectively (L, or "large",

or LHBs). Ratios of superficially exposed epitopes of these three molecular variants of HBs determine in general the infectious capabilities of the virus and its extremely narrow host range (Berting et. al. 1995). The latter obstacle, together with the lack of efficient *in vitro* models for HBV replication, limit the possibilities for structural investigation of virus cell recognition, entry, self-assembly, and mutual protein-protein, protein-RNA and protein-DNA recognition. In the infected cell cytoplasm, virus assembly is directed by the structural proteins of virus in such a way that the capsid protein first complexes with the viral genome into a capsid structure. This then binds to viral spike proteins that have been inserted into the endoplasmic reticulum (ER) membrane, and buds into the ER lumen. The enveloped virus reaches the extracellular milieu via the secretory pathway. Virus entry into new host cells is directed by the spikes of the virus. These first bind to receptor structures on the host cell surface, and then subsequently mediate virus penetration, most likely via a process of virus membrane-host membrane fusion.

Direct analysis of the contribution of the HBV envelope proteins to virion assembly, by using infectious model and cultured hepatoma cells transfected with mutant HBV genomes bearing lesions in the envelope coding regions, was undertaken in the classical work of Bruss and Ganem (1991). Although the principal information about the role of S, M and L proteins in production of the virus was obtained, the low synthetic efficacy of the system, together with dependence on attendant viral processes, did not allow direct structural investigation of macromolecular protein structures. To reconstruct particles in high yields, simultaneous expression of S and C genes was undertaken in yeast (Shiosaki et. al. 1991) and in insect cells (Takehara et. al. 1988). Due to very long distances between the real HBV host and the used models, these attempts were not informative from the point of view of such intimate mechanisms as budding and self-assembly.

Recombinant plasmids based on the Semliki Forest virus (SFV) genome have been introduced to express foreign genes in a broad range of eukaryotic cells (Liljeström and Garoff 1991; Sjöberg et. al. 1994). The universal nature of the system made it ideal for accurate determination of HBV virion-like particles. The aim of this study was to adopt the SFV system for efficient expression of the above mentioned HBV structural genes, as well as to evaluate the possibility of synthesized recombinant proteins to form different kinds of HBV virion-like particles in transfected cells. This efficient expression system of HBV subunits could be very useful in studying the molecular details of HBV assembly, structure and entry.

Materials and methods

Construction of plasmids

To construct HBV-genes expressing plasmids, we used a plasmid pHB320 harbouring the complete genome of HBV, subtype *ayw* (Pumpen et. al. 1981) and two different variants of the SFV expression vectors – SFV1 (Liljeström, Garoff 1991) and SFVC-Bam HI (Sjöberg et. al. 1994). Restriction endonucleases, T4 DNA ligase and SP6 polymerase were purchased from Fermentas (Vilnius, Lithuania). DNA preparation, restriction enzyme cleavage, cloning of DNA fragments, bacterial transformation and growth were performed essentially as described by Sambrook et. al. (1989).

pSFV1/C. The oligonucleotides used for the HBc amplification were: 5'-GCGGATCCA-TGGACATTGATCCTTATA-3' and 5'-CGCCCGGGTAAAGTTTCCCACCTTATG-3'.

pSFV1/S. The oligonucleotides used for *S* gene amplification were: 5'-ATTGGGGTC-CTGCGCTGAACATGGAG-3' and 5'CGCCCGGGTTTAAATGTATACCCAAAG-3'.

pSFV1/M. The oligonucleotides used for the *M* gene amplification: 5'-ACGGATC-CTCAGGCCATGCAG-3' and 5'CGCCCGGGTTTAAATGTATACCCAAAG-3'.

pSFV1/L. The oligonucleotides used for the *L* gene amplification: 5'-GCCCCGGGAT-GGGGCAGAATCTTTCCA-3' and 5'-CGCCCGGGTTTAAATGTATACCCAAAG-3'.

pSFV-C/C. The terminal primers used for the amplification of the fused DNA were: 5'-G-ATGTATCTTCGAAGTCAAACACG-3' (the 5'-end primer) and 5'-CGCCCGG-GTAAAGTTTCCCACCTTAGT-3' (the 3'-end primer). The fusion primers used were: 5'-GAGGGGTCCGAAGAGTGGATGGAGAACA-TCACATCAGG-3' (HBV C-gene fragment primer) and 5'-CCTGATGTGATGCTTCTCCATCCACTCTTCGGACCCCT-CGG-3' (SFV C-gene fragment primer).

pSFV-C/S, pSFV-C/M and pSFV-C/L. The terminal primers used for the amplification of fused DNA fragments were the same for all three constructs: 5'-GACTGTATCTTCGAAGT-CAAACACG-3' (the 5' end primer) and 5'-CGCC-CGGGTTTAAATGTAGACCCAAAG-3' (the 3' end primer). The fusion primers used were: 5'-AGGGGTCCGAAGAGTGGA TGGAGAACATCACATC-3' (HBV S-gene fragment primer) and 5'-GATGTGATGTT CTCCATCCACTCTTCGGACCCCT-3' (SFV C-gene fragment primer) 5'-AGGGGTC CGAAGAGTGGATGCAGTGGAACTCCAC-3' (HBV M-gene fragment) and 5'-GTG GAGTTCCACTGCATCCACTCTTCGGACC-CT-3' (SFV C-gene fragment primer); 5'-CCGAGGGGTCCGAAGAGTGGATGGGGCAGAATCTTTCCACC-3' (HBV L-gene fragment primer) and 5'-GGTGGAAAGATTCTGCCCCATCCACTCTTCGGACCCCT CGG-3' (SFV C-gene fragment primer).

RNA transcription, electroporation and metabolic labeling of transfected cells

RNA transcripts were synthesized *in vitro* by SP6 RNA polymerase using SpeI or NruI-digested plasmids as templates. Reaction conditions for *in vitro* RNA transcription have been described previously (Liljeström, Garoff 1991). *In vitro* made RNA was transfected into BHK (Baby Hamster Kidney) cells (obtained from American Type Culture Collection) by electroporation. Confluent cell monolayers were trypsinized, washed once with complete BHK medium (GIBCO) supplemented with 5 % fetal calf serum, 20 mM HEPES (pH 7.3), once with ice-cold phosphate-buffered saline (PBS, without $MgCl_2$ and $CaCl_2$) and resuspended in 0.8 ml PBS. The cell suspension was mixed with *in vitro*-made RNA (20 ml of the reaction mix) and transferred to a 0.4-cm electroporation cuvette (Bio-Rad). In cotransfection experiments equal amounts (20 ml) of both RNA were used, except in the SFVC/C, SFVC/S, SFVC/M and SFVC/L cotransfection where half the SFVC/L RNA (10 ml) amount was employed.

Electroporation was carried out at room temperature by two consecutive pulses at 0.85 kV and 25 mF, using a Bio-Rad Gene Pulser apparatus (without the pulse controller unit). These conditions yielded virtually 100 % transfection efficiency. Electroporated cells were diluted into 18 ml complete BHK medium, transferred onto tissue culture plates and incubated at 37 °C. At 4 - 16 h post-electroporation, the culture medium was replaced with methionine-free minimal essential medium (GIBCO) supplemented with 10 mM HEPES. After 30 min at 37 °C, the medium was replaced with the same methionine-free medium containing 100 mCi of [^{35}S]methionine per ml and the cells were incubated at 37 °C for 30 min to 4 h (pulse). After an appropriate pulse time, the culture medium was collected and

clarified by centrifugation twice in an Eppendorf cenrifuge (5 min at 5.000 rpm at 4 °C). Cell monolayers were washed with PBS and solubilized with 1 % sodium dodecyl sulfate (SDS) or 1 % Nonident P-40 (NP-40) lysis buffers containing 10 mM iodoacetamide (Peränen et. al. 1988; Suomalainen et. al. 1990). Nuclei were removed from cell lysates by centrifugation in an Eppendorf centrifuge (5 min at 5.000 rpm at 4 °C).

Immunoprecipitation of proteins from cell lysates

HBV core antigen was immunoprecipitated from NP-40 cell lysate (in the case of pSFV1/C), or from SDS-solubilized cell lysate (in the case of pSFV-C/C) with a polyclonal rabbit anti-HBc antiserum (a kind gift of V. Tsibinogin, Rīga). HBVS, HBVM and HBVL proteins were immunoprecipitated from NP-40-solubilized cell lysates with polyclonal goat anti-HBs antiserum (V. Tsibinogin). Immunocomplexes were brought down with protein A-Sepharose (Pharmacia; 1:1 (v/v) slurry in 10 mM Tris-HCl, pH 7.5) using rabbit anti-mouse immunoglobulins (Dakopatts a/s, Denmark) as linking antibodies when necessary. Immunuprecipitates were solubilised in SDS-sample buffer (200 mM Tris-HCl, pH 8.8, 20 % glycerol, 5 mM EDTA, 0.02 % bromphenol blue, 4 % SDS, 50 mM ditiothreitol) by heating at 95 °C for 5 min. The samples were alkylated with an excess of iodoacetamide and spun for 2 min in an Eppendorf microcentrifuge at full speed before being analysed by SDS-PAGE. SDS-PAGE was performed in 12 % gels (Laemmli 1970). The gels were treated with 1 M sodium salicylate for 30 min at room temperature, then dried and exposed to Kodak XAR-5 film at -70 °C.

Construction of recombinant viruses and cell infection

For *in vivo* packaging of recombinant RNA into SFV particles, *in vitro*-transcribed RNA was electroporated into BHK cells together with SFV helper RNA (Fig. 1; Berglund et al. 1993) under the conditions described above. After 20 h, SFV particles were collected from the culture medium and frozen rapidly to be stored as virus stocks. The titres of SFV stocks were determined by infecting cells with serial dilutions of the stocks followed by indirect immunochemistry assay for the expressed proteins. The estimated titres varied from 1×10^7 to 5×10^7 viral particles per ml. The infection of BHK cells was carried out in serum-free medium with appropriate dilution of the virus stock to achieve 100 % cell infection.

Immunocytochemical detection of intracellular HBV antigens by monoclonal antibodies

BHK cells grown on sterile tissue culture chamber slides (Nagle Nunc International were infected with recombinant SFV, and incubated at 37 °C (5 % CO_2) for 20 h. After drying the slides at room temperature, the cells were fixed with ethanol/acetic acid (3:1) for 20 min, and rinsed thoroughly (3 ×) in distilled water. Then the slides were immersed in PBS for 10 min, then rinsed with PBS supplied with 0.25 % Triton X-100. Then slides were incubated for 24 h in a humidity chamber at 4 °C with the anti-HBs monoclonal antibody, or with anti-HBc monoclonal antibody (gift of Dr. I. Sominskaya, BMC, Riga) at a dilution 1:200 (in PBS + 0.25 %Triton X-100 + 0.25 % BSA), and after that rinsed in PBS + 0.25 % Triton X-100. Then cells were incubated with anti-mouse IgG conjugated with Alkaline Phosphatase (Sigma) at a dilution 1:200 in PBS + 0.25 % Triton X-100 + 0.25 % BSA at room temperature, in dark for 1 h, and subsequently rinsed with PBS.

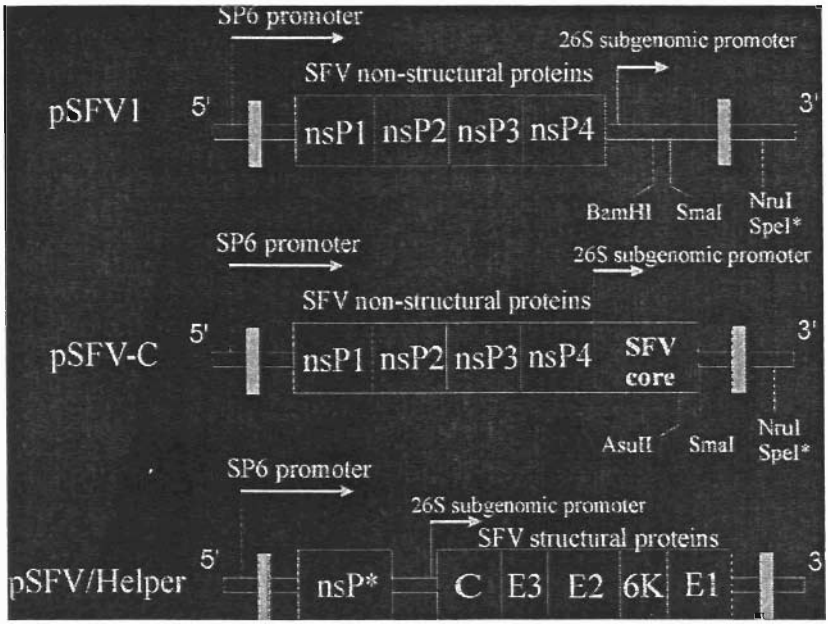

Fig. 1. General scheme of SFV vectors. Only SFV recombinant cassettes are shown. nsP, SFV non-structural proteins encoding replicases for transcription of subgenomic RNA from subgenomic 26S promoter. The pairs of cloning sites BamHI/SmaI (for pSFV1 vector) and AsuII/SmaI (for pSFV-C vector) were used for introduction of HBV structural genes (C, S, M, L). NruI, or SpeI is a plasmid linearization site. pSFV/Helper construct was used for production of recombinant SFV particles. The nsP* region, including the packaging signal, is deleted from the helper plasmid, which provides therefore the synthesis of SFV structural proteins only. The SP6 RNA polymerase promoter for *in vitro* transcription of recombinant RNA is indicated. Black boxes along the edges of the SFV cassettes denote sequences used by the SFV replicase for amplification of the SFV replicons.

Alkaline Phosphatase activity was developed by Sigma FAST reagent for 30 min at room temperature, where the Fast Red TR/Napthol AS-MX is the immunohistology substrate of choice for alkaline phosphatase conjugated antibodies, as it produces an intense red stain. The cells were then rinsed in deionized water; counterstained with hematoxylin; and mounted in glycerol gelatin (Sigma). The evaluation was conducted under a light microscope.

Electron microscopy

Electron microscopic analysis was performed on a JEM100C electron microscope at 80 kV accelerating voltage and screen magnification of × 100,000. The specimens were negatively stained in neutral 2 % phosphotungstic acid.

Results and discussion

Construction of expression plasmids

As a basis for the construction of HBV-genes expressing plasmids, we used a plasmid pHB320 (Pumpen et. al. 1981) that spans the complete genome of HBV (Bichko et. al.

1985). The HBV C-gene and three variants of HBV S-genes were amplified by PCR and, after appropriate treatment with DNA-modifying enzymes, introduced into two different variants of the SFV expression vectors: SFV1 (Liljeström, Garoff 1991) for direct expression of HBV genes and SFV-C (Sjöberg et al. 1994) to create fused chimeric proteins with the capsid protein of SFV (Fig. 1).

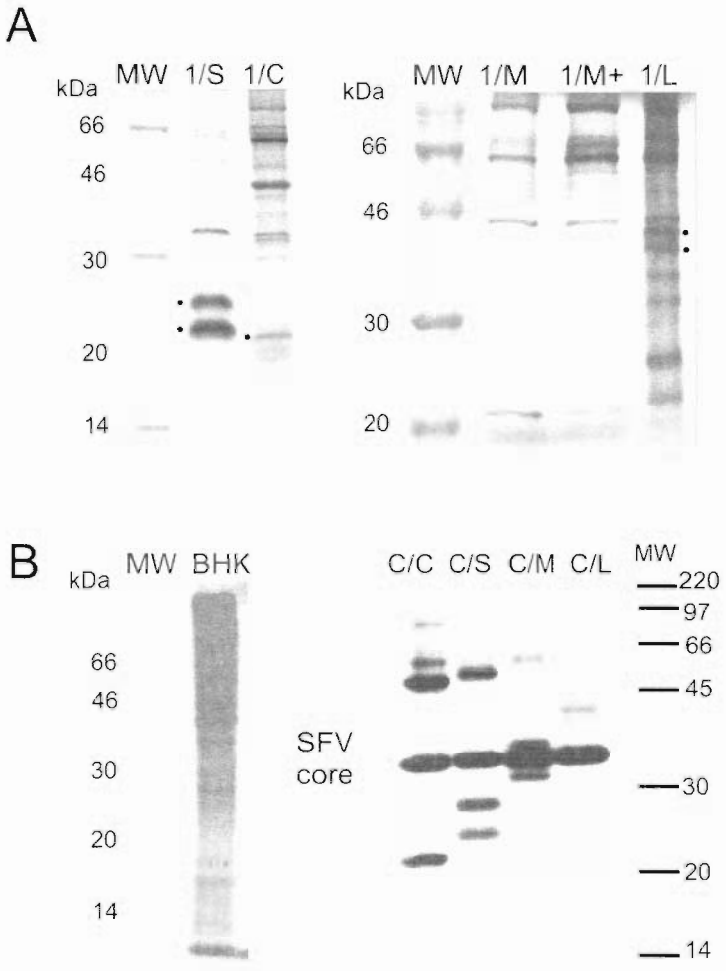

Fig. 2. Expression of HBV structural proteins in BHK cells upon RNA transfection. (A) Cells were transfected with RNAs of SFV1/S (1/S), SFV1/C (1/C), SFV1/M (1/M), SFV1/L (1/L), and pulse-labelled 2 h at 16 h post electroporation. In 1/M+ two times more of the RNA was used for electroporation. Equal amounts of NP-40 lysates were run on a 12 % gel. The bands of corresponding HBV proteins are marked with dots, except 1/M and 1/M+, where HBV specific bands were not identified. MW, rainbow [^{14}C] methylated protein marker (Amersham). The positions of protein size markers (in kilodaltons) are indicated on the left. (B) Cells were transfected with RNAs of SFV-C/C (C/C), SFV-C/S (C/S), SFV-C/M (C/M), SFV-C/L (C/L) and pulse-labelled 30 min at 6 h post electroporation. Equal amounts of NP-40 or SDS-lysates (C/C) were run on a 12 % gel. MW, High-Range Rainbow Molecular Weight Marker (Amersham) Non-transfected BHK cells were used as a negative control (BHK).

The genes for the HBV core (C) and three variants of HBV surface (S, M and L) proteins were engineered into the pSFV1 vector under the SFV subgenomic promoter (26S). For construction of pSFV1/C plasmid, the amplified HBV DNA fragment (602 bp) was cleaved with restriction enzymes BamHI and SmaI, and the 594bp-fragment was ligated with the SFV1 vector processed in the same way. To construct pSFV1/S and pSFV1/M plasmids, HBV DNA fragments corresponding to the HBV S (711 bp) and HBV M (875 bp) genes were cleaved with BamHI and SmaI restriction enzymes, and the appropriate fragments (700 bp and 862 bp) were inserted into the BamH1/SmaI-treated pSFV1 vector.

To construct the pSFV1/L plasmid, the HBV DNA-fragment coding for the HBV L gene (1187 bp) was inserted into the SFV1 expression vector using SmaI restriction sites.

The precise fusion of SFV C and HBV structural protein genes was carried out by PCR. The plasmid pSFV-C/C was constructed by ligating the 1026-bp AsuII-SmaI SFVC-HBVC-fusion fragment to pSFV-C vector treated by the same restriction enzymes. The amplified fused DNA fragments for HBV S (1140 bp), HBV M (1350 bp), and HBV L (1624 bp) were cloned in the SFV-C vector after cleaveage with AsuII and SmaI. The plasmid for analysis of HBV S protein synthesis was named pSFV-C/S, the plasmid for HBV M protein production was named pSFV-C/M and the plasmid for HBV L gene expression was named pSFV-C/L.

All synthesized constructs were confirmed by sequencing.

Expression of HBV proteins in SFV1 and SFV-C vectors

The resulting plasmids on the basis of the SFV1 vector (pSFV1/C, pSFV1/S, pSFV1/M and pSFV1/L) and SFV-C vector (pSFV-C/C, pSFV-C/S, pSFV-C/M and pSFV-C/L) were used as templates for *in vitro* transcription of the corresponding recombinant SFV genomes. The RNA of each construct was separately transfected into BHK cells. Fig. 2A, B shows an analysis of transfected BHK cell lysates. At 6 to 16 h post-infection cells were metabolically labeled with [^{35}S]-methionine for 2 h (SFV1 constructs), or for 30 min (SFV-C constructs), and lysed with NP-40 containing lysis buffer.

In contrast to the relatively inefficient synthesis of HBV proteins in the SFV1 system, especially in the cases of SFV1/M, the SFV-C system assured high level expression of all structural HBV proteins (Fig. 2B), where only a short pulse-labeling time was necessary. The expression of fused SFVC-HBV genes shut off completely the synthesis of cellular proteins, in contrast to the BHK control. All fused SFVC-HBV proteins were split correctly, although, in the case of C and S, traces of unsplit precursors were seen. Probably, processing was dependent on specific N-terminal amino acid sequences of added HBV protein. In contrast to the S, M and L products, the C product appeared as nuclear associated protein, since it was soluble only in SDS lysis buffer, but not in NP-40 lysis buffer. Products of S, M, and L genes appeared also in glycosylated forms in the same way as during HBV infection in men (Stibbe, Gerlich 1983). S products existed as non-glycosylated p24 and mono-glycosylated gp27 molecules. The M product showed three bands: non-glycosylated p31 (not detected during viral infection), mono- and double glycosylated forms gp33 (co-migrating with SFV core on the gel), and gp36. The L product showed non-glycosylated form p39 and mono-glycosylated form gp42.

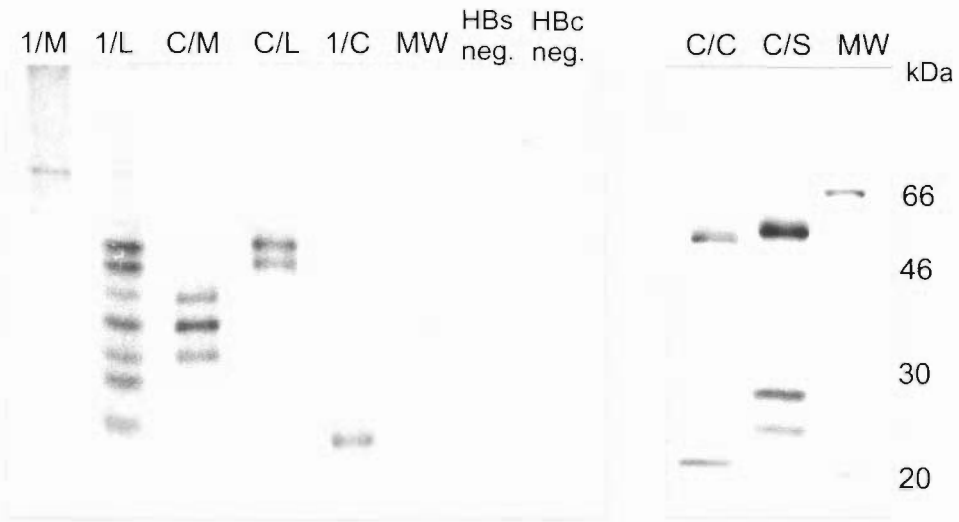

Fig. 3. Immunoprecipitation of intracellular products of BHK cells transfected with SFV1/M (1/M), SFV1/L (1/L), SFV-C/M (C/M), SFV-C/L (C/L), SFV1/C (1/C), SFV-C/C (C/C), SFV-C/S (C/S). Rabbit polyclonal anti-HBc antibodies were used for HBc protein immunoprecipitation, and goat polyclonal anti-HBs – for HBs immunoprecipitation. As negative controls were used the immunoprecipitations of the lysate of untransfected BHK cells incubated with the same rabbit anti-HBc antibodies (HBc neg.) and goat anti-HBs antibodies (HBs neg.). MW, rainbow [^{14}C] methylated protein marker (Amersham). The positions of protein size markers (in kilodaltons) are indicated on the right.

Immunological properties

Immunoprecipitation of the expression products with the appropriate antibodies demonstrated their immunological specificity (Fig. 3). Immuno-precipitation of the C product revealed a clear p22 band and an additional band which could be explained as SFV-C-HBc fusion protein. The same situation was observed in the case of HBV S gene expression, where additionally to p24 and gp27 bands, the possible SFV-C-HBs fusion protein was visible. Unfortunately, we did not detect M protein synthesis by the pSFV1/M construct. The reason of this is unclear, despite the strong confirmation of the nucleotide structure of this construct by sequencing.

Surprisingly, the pSFV1/L construct provided the synthesis of all variants of HBs proteins (L, M, S) in almost equal amounts, while the SFV-C dependent expression of L demonstrated only trases of M and S protein synthesis. We suppose that the reason for this phenomenon is the preferable translation of fusion protein while the distantly located AUGs of M and S proteins can not be reached by ribosomes.

The glycosylated nature of the three variants of HBV surface proteins was revealed by treatment with endoglycosydase H. After this treatment, all glycosylated forms were transferred to positions of the appropriate non-glycosylated forms on the gel (not shown).

Secretion of HBV gene S products

Analysis of intracellular and extracellular fractions revealed the successful secretion of

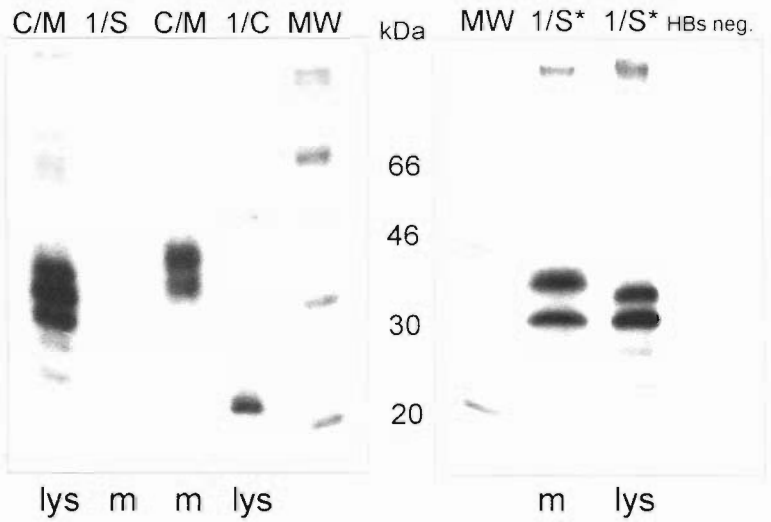

Fig. 4. Analysis of intracellular (lys) and extracellular (m) HBV proteins synthesized in RNA-transfected BHK cells. Cells were transfected with RNAs of SFV-C/M (C/M), SFV1/S (1/S), SFV1/C (1/C), and SFV1/S adw subtype (1/S*). Appropriate proteins were immunoprecipitated with goat polyclonal anti-HBs, or rabbit polyclonal anti-HBc antibodies. HBs neg., untransfected BHK cells incubated with goat polyclonal anti-HBs antibodies. MW, rainbow [^{14}C] methylated protein marker (Amersham). The positions of protein size markers (in kilodaltons) are indicated in the middle.

the M, but not S product (Fig. 4). However, later we found the S product secretion of *adw* subtype of HBV. These results are the focus for further investigation. As expected, the products L and C were not secretable. As seen on the figure, the secreted forms of HBs proteins are different from that of the intracellular forms, because of specific posttranslational modifications during transport.

Immunocytochemical analysis of BHK cells producing HBV proteins

As was shown above, we revealed the production of HBV structural proteins in SFV-driven expression by SDS-PAGE. More obviously these results were observed by immunocytochemical analysis of BHK cells infected with the appropriate recombinant SFV-HBV virus (Fig. 5). We showed that more than 90 % of tested cells were positive for HBV proteins. However, the intensity of staining was different and correlated with the levels of proteins detected by ELISA (not shown) and SDS/PAGE. The most intensive staining was found in all SFV core fused variants. Staining patterns showed a cytoplasmic, granular distribution of the HBV proteins in the cells. At the same time we observed mainly unipolar, perinuclear localization of HBc proteins in the case of SFV1/HBVC expression, and more homogeneous cytoplasmic distribution in the case of the SFV1/HBVS expression. All SFV core-dependent constructs demonstrated similar staining of cell cytoplasm with over-expressed HBV proteins.

The BHK cells, which were used in these experiments, are not natural host cells for HBV. However, they are optimal for infection with and production of recombinant SFV particles, allowing the highest yields of recombinant proteins. Beside the BHK cells, we established similar expression patterns of the HBV proteins for all studied constructs in

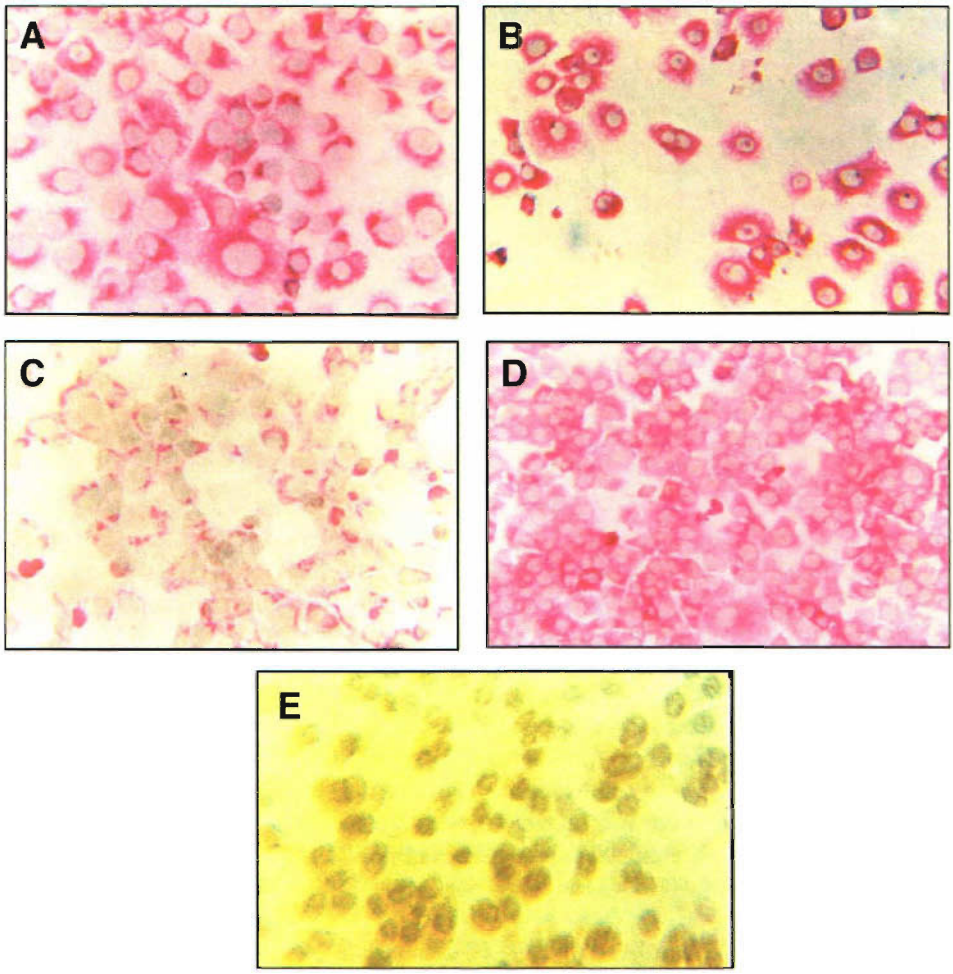

Fig. 5. Immunocytochemical detection of HBV proteins in BHK cells. A, SFV1/L antiHBs; B, SFV1/S anti HBs; C, SFV1/C antiHBc; D, SFV-C/M antiHBs; E, antiHBs/antiHBc neg. Cells were infected with the appropriate recombinant virus (SFV1/L; SFV1/S; SFV1/C; pSFV-C/M). At 20 h postinfection, the cells were fixed and processed as described in the Materials and Methods section. Proteins were detected with monoclonal anti-SHBs, or anti-HBc antibodies. Neg. control – uninfected BHK cells incubated with the same antibodies.

other cell lines (HuH-7, HepG2, COS-7), only the levels of production were lower (not shown).

Co-expression of HBV structural genes and secretion of HBV virion-like particles

Co-transfection of BHK cells with different combinations of RNAs led to efficient equimolar production of HBV structural proteins, also when all four genes were expressed simultaneously (not shown). Since L product was considered to be an inhibiting

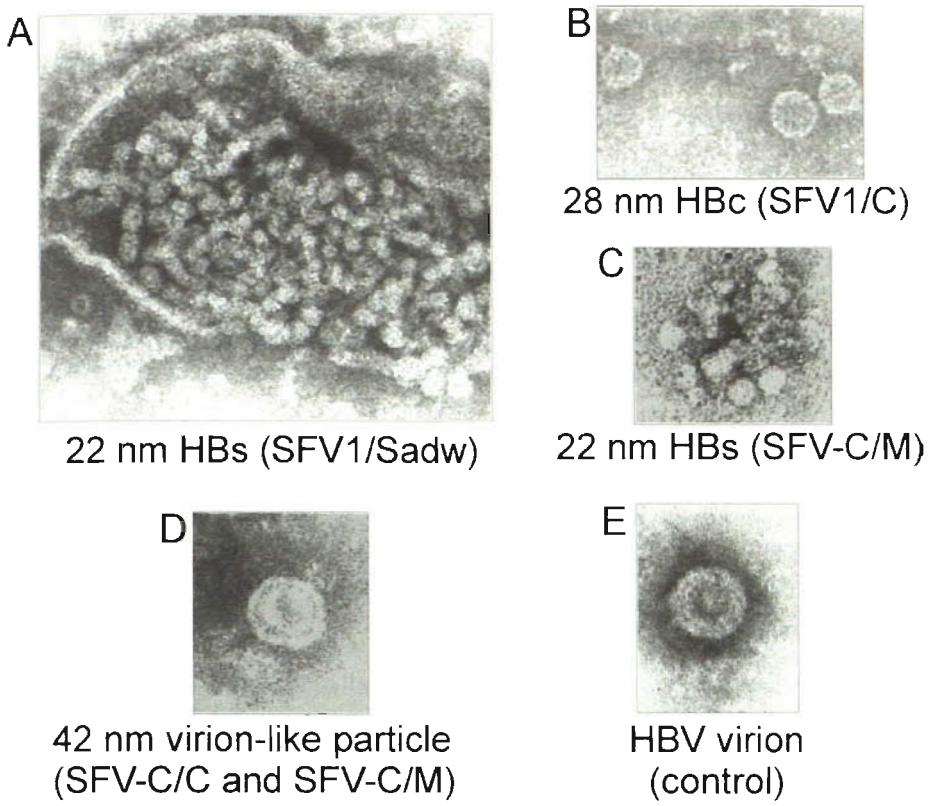

Fig. 6. Electron micrographs of HBV particles. A, HBsAg-22-nm-like particles in cryo-lysate of pSFV1/S-transfected (subtype *adw*) BHK cells. B, HBV core particles (HBc) in the NP-40 containing lysate of BHK cells transfected with pSFV1/C. C, Secreted HBsAg-22-nm-like particles in the medium of pSFV-C/M-transfected BHK cells. D, HBV virion-like particles in medium of BHK cells co-transfected with SFV-C/C and SFV-C/M. E, the native HBV virion.

secretion agent, we limited its production level by a two-fold decrease of its RNA for cell transfection. In the media, we found M products in the case of simultaneous expression of M and C genes. Unfortunately, we found no trace of C protein in the medium, probably due to insufficient sensitivity of method used (SDS-PAGE). Electron microscopy, as a more sensitive method, showed the presence of not only 22 nm HBsAg particles (Fig. 6C), but also virion-like particles similar to native Dane particles from human blood (Fig. 6 D, E). The separate expression of the Core particle formation was revealed by electron microscopy of the lysate of pSFV1/C transfected cells (Fig. 6B). The 22 nm HBsAg particles were seen in the cryo-lysates of pSFV1/S transfected cells (Fig. 6A). To enhance the productivity of the system and to broaden the possible host cell range, virus stocks for all four producers will be used to infect model cell cultures.

Therefore, high potential of simultaneous expression of all four HBV structural genes within SFV vectors in tissue culture cells was shown conceptually, in contrast to earlier attempts (Takehara et. al. 1988; Shiosaki et. al. 1991). The SFV system presents a unique capabilities to efficient model intimate mechanisms of HBV virion self-assembly and

turnover. The possible application of SFV-derived HBV virion-like structures as gene therapy tools and/or RNA vaccines is planned to be investigated fully in the future.

Acknowledgements

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Hepatīta B strukturālo proteīnu sintēze, izmantojot Semliki Meža vīrusa ekspresijas sistēmu

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Kopsavilkums

Visi Hepatīta B vīrusa (HBV) *ayw* subtipa strukturālie gēni tika ekspresēti Semliki Meža vīrusā (SFV) pSFV1 un pSFV-C vektoros. Labākais ekspresijas veids bija saistīts ar SFV *core* gēnu saturošo vektoru (pSFV-C sērijas) pielietošanu, kur ekspresējamos gēnus sapludināja ar SFV *core* gēnu un svešas daļas atbrīvošanās notika specifiskā procesinga rezultātā. Izmantojot endoglikozidāzes H apstrādi, parādīja glikozilēšanu visiem trim HBV virsmas proteīniem (LHBs, MHBs, SHBs). Intracelulārās un ekstracelulārās frakcijas analīze parādīja veiksmīgu M produktu sekrēciju, kas nebija novērota S produktam. Kā varēja paredzēt, L un C produkti nesekrētējas. BHK šūnu kotransfekcija ar dažādiem HBV gēniem ļauj efektīvi producēties HBV strukturāliem proteīniem arī tad, ja visi četri gēni ekspresējas vienlaicīgi. Izmantojot elektronmikroskopijas analīzi, parādīja 42nm HBV daļiņu veidošanos, kuras ir līdzīgas natīvam HBV virionam. Principā tika noskaidrota gēnu koekspresijas iespēja un jaukto vīrusveidīgo daļiņu veidošanās, tomēr pēdējās parādības izpratnei nepieciešami tālāki padziļināti pētījumi.

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Subcellular localisation of internally deleted HBV core proteins

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Abstract

Hepatitis B virus (HBV) variants with core internal deletions were isolated from renal graft recipients in whom an association with end stage liver disease was observed. The aim of this work was evaluation of intracellular localisation of these HBV core variants with internal deletions. Infection of baby hamster kidney cell culture BHC-21 C13 with recombinant Semliki Forest viruses encoding the deleted variant and undeleted wild type HBV core (HBc) genes led to the detection of HBc protein within the nucleus only in the case of deletion variants. Deleted HBc protein in all probability may be partially unfolded, hence unstable. As a consequence, deleted HBc proteins are not part of HBV nucleocapsids, but are left unassembled and are able to cross the nuclear membrane. We suggest that the presence of deleted HBc proteins within the cell nucleus may be of pathogenic importance.

Key words: deletion mutants, hepatitis B virus, HBV core protein, Semliki Forest virus, subcellular localisation.

Introduction

Hepatitis B virus (HBV) causes an inflammatory liver infection whose clinical course ranges from acute to chronic forms, which may lead to cirrhosis and hepatocellular carcinoma.

HBV has a small 3.2 kb DNA genome of four partially overlapping open reading frames. The 21 kDa HBV core (HBc) protein, encoded by the HBV C gene (precore-core), is the structural material for viral nucleoprotein capsids, which are composed of 180 or 240 HBc subunits and organised in T = 3 or T = 4 icosahedrons, respectively. The HBc protein consists of an amino-terminal self-assembly domain (amino acid residues 1 to 150) and a carboxy-terminal protamine-like arginine-rich domain. The latter contains a nuclear localisation signal (Eckhardt et al. 1991).

The HBc protein has been detected in chronic HBV carriers in both nucleus and cytoplasm (Mondelli et al. 1986). A nuclear distribution pattern was found to correlate

with mild hepatitis, whereas cytoplasmic localisation was found to be associated with chronic active hepatitis (Chu, Liaw 1987; Hsu et al. 1987). A positive correlation between the level of HBV DNA in serum and the degree of expression of HBc protein in the nucleus has been observed (Chu et al. 1997). It is supposed that the shift of intracellular HBc protein from the nucleus to the cytoplasm is caused by the regeneration of hepatocytes (Chu et al. 1995). Furthermore, an association of basal core promoter (BCP) mutations with cytoplasmic localisation of the HBc protein was demonstrated by Kawai et al. (2003). The precore sequence is considered to be essential for nuclear localisation of HBc protein (Aiba et al. 1997).

Subcellular localisation of the HBc protein is regulated by phosphorylation of serine residues within the nuclear localisation signal (Liao, Ou 1995) and is dependent on the cell cycle, with its nuclear localisation increased during the G1 phase (Yeh et al. 1993). Cytoplasmic HBc protein is phosphorylated, whereas nuclear HBc protein is unphosphorylated. Phosphorylated HBc protein binds to the nuclear pore complex (Kann, Gerlich 1994; Kann et al. 1999) to release the genome at the nuclear membrane; its uptake is mediated by viral polymerase, but is independent of HBc protein (Kann et al. 1997).

With a particle size of 30 or 34 nm for the T = 3 or T = 4 particles, respectively, HBc capsids exceed the functional diameter of the nuclear pore complex (Dworetzky, Feldherr 1988). Therefore, HBc capsids have to be dissociated for import of HBc protein into the nucleus. It has been shown in the transgenic mouse model that HBc capsids do not cross the nuclear membrane and that they are formed *de novo* within the nucleus (Guidotti et al. 1994).

Variants of the HBV gene C with internal deletions (CID) are frequently present in chronic hepatitis B carriers (Marinos et al. 1996). The internal deletion variants of the HBc gene were also isolated from renal graft recipients in whom an association with a severe course of the illness leading to end stage liver disease (ESLD) was observed (Günther et al. 1996). They are mostly in frame, located in the central part of the HBc protein and incompetent for autonomous replication (Günther et. al. 2000).

We cloned a set of HBV C gene variants with internal deletions into a Semliki Forest virus (SFV)-derived vector pSFV1 (Liljeström, Garoff 1991). The variants were derived from chronic carriers, mostly from patients with liver cirrhosis that led to ESLD, apart from one patient with mild hepatitis. The intracellular distribution of wild type HBc and CID variants was investigated by immonocytochemical methods in infected BHK and Huh7 cells. HBc protein was found in the nucleus of infected cells only in the case of HBc deletion variants and not in the case of wild type HBc.

Materials and methods

Cell cultures

BHK-21 C13 (Baby hamster kidney, Syrian or golden hamster, *Mesocricetus auratus*, ATCC CCL-10) were grown in BHK–21 medium (GIBCO-BRL) containing 5 % fetal calf serum, 10 % tryptose phosphate broth, 20 mM HEPES, and 2 mM glutamine, 100 U ml^{-1} of penicillin/streptomycin (final concentration). The human hepatoma cell line Huh7 (our laboratory stock) was grown in RPMI medium (GIBCO-BRL) supplemented with 2 mM L-glutamine, 2 % sodium selenite (GIBCO-BRL), 100 U ml^{-1} of penicillin/ streptomycin (final concentration) and 10 % foetal bovine serum. Cells were incubated in

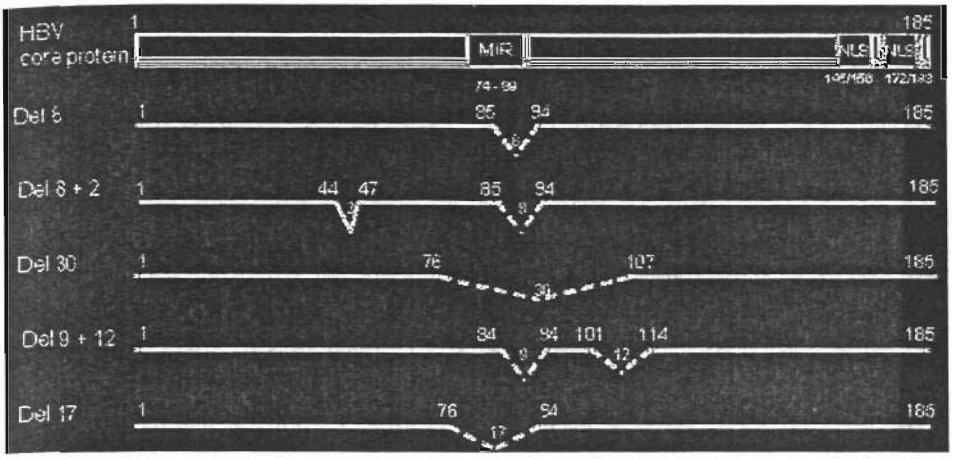

Fig. 1. Internally deleted variants of HBV core protein: the positions of the deletions are indicated by broken lines and the amino-acid positions are given. The numbers within the broken lines indicate the respective length of the deletions in amino acids (MIR, major immunodominant region; NLS, nuclear localisation signal).

a 5 % CO_2 atmosphere at 37 °C. For morphological evaluation and immunocytochemistry, cells were grown on sterile tissue culture chamber slides (Nagle Nunc International), infected with recombinant SFV and incubated at 37 °C (5 % CO_2). Cells were harvested at 4, 12, 24 and 36 h of growth or after infection for morphological evaluation and at 4 and 24 h for immunocytochemistry.

Plasmid construction

HBV core deletion variants were originally derived from serum of immunosuppressed renal transplant recipients (Günther et al. 1996). The HBc variants used in this work are presented in Fig. 1. All deletion variants were amplified by PCR using the following primers: 5'-GCGGATCCATGGACATTGACCCTTATA-3' to introduce a restriction site for BamHI and 5'-CGCCCGGGTAAAGTTTCCCACCTTATG-3' to introduce a restriction site for SmaI. The PCR fragments were cut out with SmaI and BamHI and ligated into the pSFV1 vector (Liljestrom, Garoff 1991) restricted with the same enzymes. Additionally a HBV pregenome (gene bank accession number: NCBI: 329640) of genotype D was cloned into the SmaI site of pSFVdelStNruI (gift of H. Garoff) after PCR amplification with the primers 3'-CCGGAAAGCTTGAGCTCTTCTTTTTCACCTCTGCCTAATCA-5', and 5'-CCGGAAAGCTTGAGCTCTTCAAAAAGTTGCATGGTGCTGG-3', restriction with Hind III and reconstruction of the blunt ends by T4 polymerase.

The pSFV helper 1 plasmid used for recombinant virus stocks preparation was a gift from H. Garoff.

Core genes

The following core genes were cloned into pSFV-1: (i) an 8 amino acid deletion, deleted from amino acid 86 to 93 (del 8); (ii) a variant comprising two deletions of eight and two amino acids in length from amino acid 45 to 46 and 86 to 93 (del 8+2); (iii) a 30 amino acid deletion, deleted from amino acid 77 to 106 (del 30); (iv) a variant comprising two

deletions of nine and twelve amino acids in length from amino acid 85 to 93 and 102 to 113 (del 9+12); (v) a 17 amino acid deletion, deleted from amino acid 77 to 93 (del 17) and (vi) an undeleted wild type core gene of genotype A (corewt). With the exception of variant del 9+12, which was derived from the serum of a patient with mild hepatitis, all variants were derived from the serum of patients suffering from liver cirrhosis that led to end-stage liver disease.

RNA transcription, generation of recombinant virus and cell infection

RNA transcripts were produced in vitro from 3 μg of SpeI-linearised pSFV1/del 8, pSFV1/del 8+2, pSFV1/del 30, pSFV1/del 9+12, pSFV1/del 17, pSFV1/HBVcorewt and NruI-linearised pSFV1delStNruI/HBVpgRNA, using SP6 RNA polymerase (Fermentas). For *in vivo* packaging of recombinant RNA into SFV particles, 5 - 10 μg *in vitro*-transcribed RNA was electroporated into BHK cells together with SFV helper 1 RNA at 850 V, 25 μF, pulsed twice (Bio-Rad Gene Pulser). Electroporated cells were diluted into 15 ml complete BHK medium, transferred into tissue culture flasks and incubated at 37 °C (5 % CO_2). After 20 h, SFV particles in the culture medium were collected and frozen rapidly to be stored as a virus stock. The infection of BHK cells was carried out in serum-free medium with an appropriate dilution of virus stock, which ensured that 100 % of the cells was infected. One hour after infection, BHK-21 medium containing 1 % fetal calf serum and 2 mM L-glutamine were added and incubation was performed overnight at 37 °C in an atmosphere of 5 % CO_2.

Cell lysis and ELISA (Enzyme-Linked Immunosorbent Assay)

At 16 to 20 h after infection, cell monolayers (on 3 cm ø plates) were washed with PBS, overlaid with 300 μl of lysis buffer (1 % Nonidet P-40, 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 2 mM EDTA, 1 μg ml^{-1} PMSF) and let stand on ice for 10 min. The lysates were transferred to microcentrifuge tubes and centrifuged (3000 × g for 5 min) to remove the cell nuclei. The undiluted supernatants were used for specific HBcAg immunodetection by a ELISA kit for HBeAg (Diasorin), as described by the manufacturer.

Panoptic staining (for morphological estimation of the cells)

The cells grown on chamber-slides, dried at room temperature were fixed in 0.1 % Leischman's solution in 96 % methanol for 3 min, rinsed and stained with freshly prepared solution of 0.1% azure II / 0.1% eosin K (10:30 v/v) for 15 - 20 min. The cell morphology was evaluated and cell number counted and calculated per 100 μm^2.

Immunocytochemical detection of intracellular HBc variants by an indirect method using monoclonal antibodies (Mabs)

BHK cells that were grown on sterile tissue culture chamber slides and infected with recombinant SFV after drying at room temperature, fixed with ethanol/acetic acid (3:1) for 20 min and rinsed thoroughly (3 ×) in distilled water. Then the slides were immersed in PBS for 10 min., rinsed with PBS supplied with 0.25 % Triton X-100 and incubated for 24 h in a humidity chamber at 4 °C with anti-HBc Mab at a dilution of 1:200 (in PBS + 0.25 % Triton X-100 + 0.25 % BSA), and after that rinsed in PBS + 0.25 % Triton X-100. Two types of Mab C1-5 (HBcAg epitope aa 78-82) and 10C-6 (aa 134-140) (Bichko et al. 1993) were used. The site of the antigen-antibody reaction was revealed by

application of alkaline phosphatase conjugated anti-mouse IgG (whole molecule, Sigma) at dilution 1:200 (room temperature for 1 h, in the dark). After rinsing with PBS, the alkaline phosphatase activity was developed for 30 min at room temperature by Sigma FAST where Fast Red TR/Naphtol AS-MX is the immunohistology substrate of choice for alkaline phosphatase conjugated antibodies as it produces an intense red stain.

The slides were mounted in glycerol gelatin (Sigma). The evaluation was done by light microscopy.

In the fluorescent variant the site of the antigen-antibody reaction was revealed by FITC-conjugated anti-mouse IgG (Fab specific, Sigma) and evaluated under a fluorescent microscope Leitz MPV 3 using the appropriate filters.

Results

Expression of HBc deletion variants

The analysed HBc deletion variants were originally derived from sera of immunosuppressed renal transplant recipients (Günther et al. 1996). HBc variants, which have been analysed earlier for their stability and self-assembly competence in *E. coli* (Preikschat et al. 1999), were cloned into the mammalian expression vector pSFV-1 (Liljeström, Garoff 1991). Five HBc variants (Fig. 1) and an undeleted wild type (wt) HBc gene of genotype A were expressed in BHK cells after infection with recombinant SFV viruses. Additionally, a whole HBV pregenome of genotype D (HBVpg) was cloned into vector pSFV1delStNrul.

Synthesis of HBc deletion variants in cultivated cells

The potential of the deleted HBc genes to synthesise variant HBc proteins was monitored by commercial HBe ELISA (Fig. 2). Variants with small deletions (del 8, del 8+2, del 17)

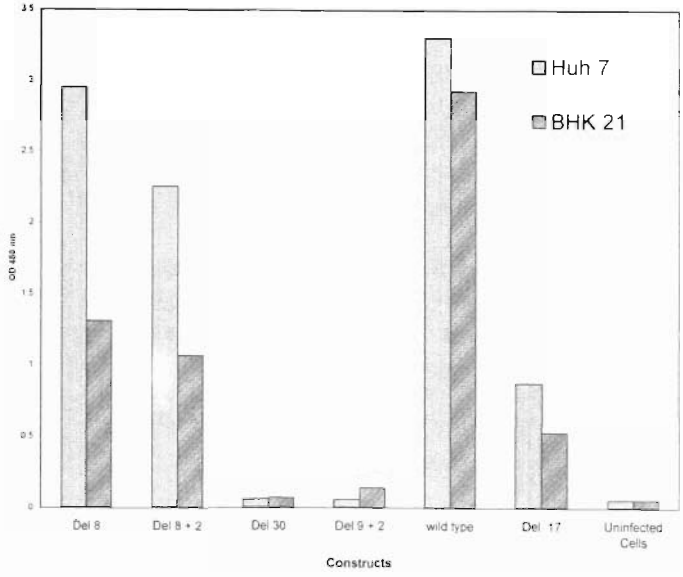

Fig. 2. Comparison of the expression level of different HBeAg variants. BHK-21 and Huh 7 cells were infected with recombinant SFV1/core viruses, and aliquots of cell lysates were analysed by ELISA (Diasorin) for HBeAg.

showed expression of variant HBc protein, albeit below the wt HBc level. Expression of HBc gene variants was not detected for mutants with larger deletions (del 9+12, del 30). Expression in cell lines BHK and Huh 7 did not reveal a difference in the relative expression level of the variants.

Intracellular localisation of HBc deletion variants

Attempts to detect the expressed proteins inside the BHK cells by indirect immunofluorescent assay with Mab 10C-6, whose epitope (aa 134-140) is preserved in all variants, led only to the detection of wild type HBc and of the highest expressed variant – HBc del 8.

Immunocytochemical analysis of HBc proteins with the same Mab by light microscopy, after reaction with anti-mouse IgG conjugated with alkaline phosphatase, showed the expression of all HBc deletion variants (Fig. 3). The expression of deletion variants del 8, del 8+2, del 9+12 and wild type was also confirmed by immunocytochemical staining with Mab C1-5. The epitope (aa 78 - 82) detected by Mab C1-5 was preserved only in these variants.

The numbers of stained cells and the intensity of staining varied greatly among HBc variants. The amount of stained cells was highest for the wt HBc construct, reaching values of 60 - 80 %, rather high (47 - 55 %) for del 8. The values for the other HBc deletion variants were lower. Only a few specifically stained cells were found in the case of the largest HBc deletion variant, del 30. Cytoplasmic staining was detected for all HBc deletion variants and wild type. Strong cytoplasmic staining was observed in the case of wt HBc, where HBc protein in form of densely packed granules accumulated mainly within the cell area around the nucleus or was dispersed throughout the cytoplasm. Cytoplasmic staining of the HBc deletion variants was much less intensive.

Further analysis of the intracellular distribution of the HBc variants revealed that a small amount of HBc protein was detectable in the nucleus of BHK cells only in the case of HBc deletion mutants (Fig. 3A). About 5 % of cells had HBc protein in the nucleus in the cases of the variants del 8 and del 8+2, less for variant del 30 and occasionally for variant del 9+12. The same expression pattern – cytoplasmic and nuclear staining – was observed for wt HBc protein expressed in the context of the HBV pregenome. Surprisingly, wt HBc protein expressed from SFV1/wt HBc showed strong cytoplasmic, but never nuclear staining.

Considering that the SFV expression system is cytopathic, a compromise has to be found between recombinant gene expression and cellular death. Therefore the synthesis of wt HBc and del 8 proteins was followed in a time course. The appearance of proteins

Table 1. Cell number per 100 μm^2 in 24 h BHK culture treated with HBc

Time of growth or after infection (h)	Control	SFV	wt HBc	Del8
4	170 ± 16	130 ± 13	130 ± 40	145 ± 13
12	358 ± 45	175 ± 28	155 ± 20	135 ± 50
24	425 ± 26	154 ± 27	96 ± 10	115 ± 38
36	549 ± 105	70 ± 20	92 ± 12	17 ± 14

could be noticed as early as 4 h after infection. Their level increased during the next 20 h. An insignificant decrease in the number of HBc-expressing cells 36 h after infection could either be connected with the injuring effect of SFV or with specific effects of the expressed HBc genes. To find out if any of the expressed HBc deletion variants exert an influence on cell growth potential or could provoke specific cytopathic effects on BHK cells, the latter were monitored for quantity and morphological changes starting 4 h after infection till 36 h after infection. The untreated BHK cell culture formed a monolayer at 24 h of growth and the cells showed similar morphology during the whole check-up period (4 h till 36 h): well structured cells with clear cell borders, basophile cytoplasm, fine reticular chromatin and prominent nucleoli (Fig. 4A). The cell amount per 100 μm^2

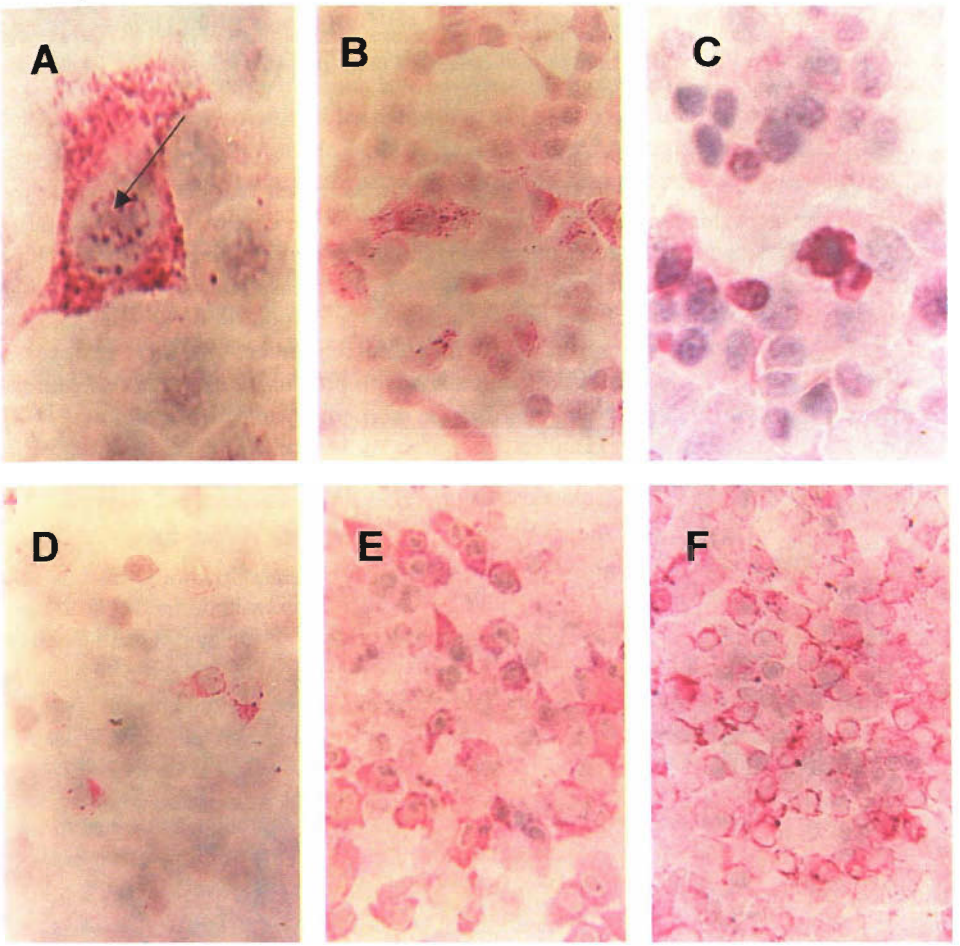

Fig. 3. Immunocytochemical detection of HBV core protein in cell cultures of BHK-21 cells that were infected with SFV1/HBV core variants and SFV1/HBV core wild type. HBcAg was detected after reaction with monoclonal antibody 10C-6. Del 8 (A) shows both nuclear (arrow) and cytoplasmic staining. The pictures show BHK-21 cells expressing the following HBV core variants: A, Del 8 (magnification × 270); B, Del 8 + 2 (× 108); C, Del 17 (× 108); D, Del 9 + 12 (× 108); E, Del 30 (× 108); F, wild type (× 108).

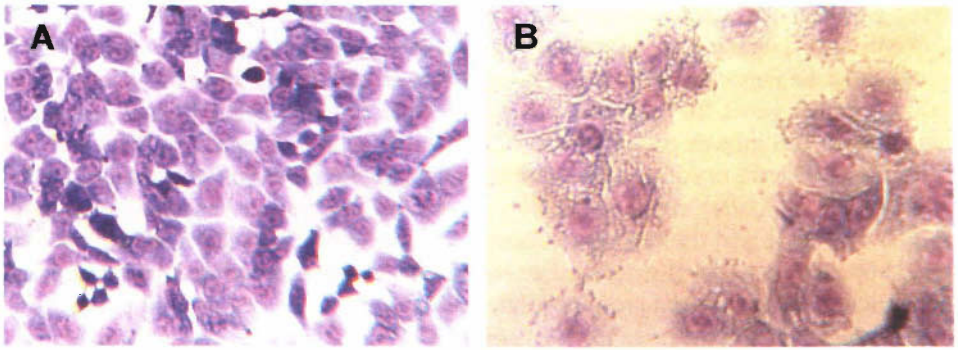

Fig. 4. The cell morphology after infection with recombinant viral particles of del 8 (B) shows signs of degeneration. The control (A) shows uninfected BHK cells (panoptic staining with Leischman-azure II-eosine). Magnification × 108.

gradually increased during the check-up period (Table 1). Cell morphology changes appear already 4 h after the infection: significantly increased basophily of cytoplasm, especially of the perinuclear cytoplasm, of most cells in the case of wt HBc and much rarely in Del 8. The above features could be regarded as a morphological manifestation of an increased ribonucleoprotein content. Evidence of increased intracellular formation of complexes between proteins and nucleic acids in the cytoplasm of wt HBc-infected cells was revealed also by staining of cells with fluorescent probe ANS together with EB (blue fluorescence if nucleic acids are complexed with proteins, data are not presented). The staining properties could be regarded as morphological manifestation of HBc accumulation/concentration on ribosomes in rough ER. As shown by the specific Mabs, the perinuclear cytoplasm is the main place where HBc is densely packed during the early period (4 h) after infection.

Besides the above observations, morphological signs of degeneration also were revealed even 4 h after infection in a small proportion of cells and in almost all cells 36 h after infection. Signs of degeneration such as pale nucleoli, not well defined cell borders, vacuolated cytoplasm, and, in most cases, reduced basophily of the cytoplasm were seen after infection with core deletion variants, as well as with the wt HBc and pSFV. At the same time, the nuclei showed well-structured chromatin without any signs of apoptosis (as shown by nuclear staining with Hoechst 33258 bis benzidine). Cell number per 100 μm^2 gradually diminished after infection with SFV alone as well as with wt HBc or the deletion variants (Table 1). Thus, no specific cytopathic effects were found apart from the effects typical for SFV infection.

Discussion

In the present work we studied the subcellular localisation of internally deleted HBV core proteins (CID). These HBV variants were originally derived from the serum of renal transplant recipients. An association with a severe course of the disease leading to liver cirrhosis and end stage liver disease was shown.

After infection of BHK cells with recombinant SFV particles encoding the wt and deleted HBc genes, HBc protein was detected in all cases in the cytoplasm of infected

cells by immunostaining with an anti-HBc Mab. The wt HBc protein showed a strong cytoplasmic staining, whereas cytoplasmic staining of the HBc deletion variants was much weaker. We have recently shown that HBc proteins with CID can be efficiently synthesised in an eukaryotic expression system *in vitro*, independently on the deletion size; *in vivo* however they are rapidly degraded by intracellular degradation pathways (in preparation). The weaker immunospecific cytoplasmic staining as well as the lower proportion of cells expressing HBc genes with CID, may reflect the effects of the ubiquitin-proteasome machinery in the degradation of misfolded HBc proteins.

Only deletion variants of HBc protein, but not undeleted wt HBc protein showed nuclear as well as cytoplasmic localisation. We suggest that the decisive factors for subcellular localisation are the ability of the expressed wt HBc protein for self assembly and the stability of the assembled HBc capsids. As the diameter of HBc particles exceeds the functional diameter of the nucleopores (Dworetzky, Feldherr 1988), HBc particles can not be transported through the nuclear membrane (Guidotti et al. 1994) and dissociation of the HBc capsids is required for the uptake of HBc protein into the nucleus. Full length wt HBc protein, synthesised in BHK cells is stable and assembles into HBc particles, as demonstrated by electron microscopy (not shown). The observed HBc deletions appear mostly in a region that hardly tolerates mutations (Koschel et al. 1999), probably because it is mostly α-helical (Wynne et al. 1999), and deletions lead to a deformation and at least partial unfolding of the HBc protein.

The question remains whether these results reflect the natural situation where HBc wt and its variants coexist in the cell. In this case, the CID HBc might be stabilised in mixed HBc particles composed of wt and deleted HBc protein. The ability of HBc proteins with CID to form mixed HBc particles was shown for the 17 amino acid deletion variant del 17 in *E. coli* expression (Preikschat et al. 2000). In the yeast two hybrid system, the del 17 variant showed weak interaction with protein of the same variant type and intermediate interaction with wt HBc; only variant del 8 showed strong interaction with both wt and analogous del 8 protein. All other variants however showed no interaction with wt and in the case of del 8+2 and del 9+12 only weak interaction with protein of the same type (R. Krenzer, MD thesis). Therefore, in the natural environment in the infected hepatocytes, formation of mixed HBc particles seems not very likely, at least in the case of variants del 8+2 and del 30. This means that HBc proteins with deletions are not part of the HBc capsids and can be translocated through the nuclear pore complex. In the case of the variant del 8, it is possible that the HBc variant is included into mixed HBc particles, but due to its variant folding it can be assumed that an unknown amount of it is left unassembled.

When BHK cells are infected with recombinant SFV particles encoding the HBV pregenome, wt HBc protein is present within the nucleus. This is not necessarily a contradiction. During the natural course of infection the dissociation of HBc particles has to occur when the HBV genome is imported into the nucleus (Kann et al. 1997). Kann et al. (1994, 1999) suggested that phosphorylation of HBc protein (Liao, Ou 1995) serves as a maturation signal that is concomitant with DNA synthesis and induces a conformational change within the HBc protein in a way that the carboxyterminal nuclear localisation signal becomes exposed and can serve as a signal for nuclear targeting. Only phosphorylated HBc particles bind to the nuclear pore complex (Kann et al. 1999). As binding of nucleic acid stabilises the HBc capsids (Birnbaum, Nassal 1990), phosphorylation renders HBc

particles less stable and favours dissociation. We suppose that HBc protein that is derived from an SFV-encoded HBV pregenome is assembly competent and encapsidates HBV pregenomic RNA, which appears in the SFV system as subgenomic RNA, and serves also as mRNA for the synthesis of HBV polymerase. After encapsidation of pregenomic HBV RNA alone with HBV polymerase, DNA synthesis proceeds, which leads eventually to the dissociation of HBc capsids during the nuclear import of the HBV genome. Due to this dissociation, HBc protein is detectable within the nucleus.

The function of HBc protein within the nucleus is not very clear. It does not seem to be necessary for the import of the HBV genome (Kann et al. 1997). Liao and Ou (1995) speculate that it may bind to supercoiled DNA and thereby suppress viral mRNA transcription as a step in establishing a persistent infection.

Our results do not support the hypothesis that nuclear localisation of HBc protein coincides with milder forms of hepatitis. All but variant del 9+12 were isolated from patients with liver cirrhosis and ESLD. As we observed nuclear localisation for HBc proteins with CID for variants for which an association with ESLD was shown, it is tempting to speculate that their nuclear localisation might play a role in pathogenesis. Several mechanisms might be involved. HBc protein inhibits the expression of the beta interferon (IFN) gene (Whitten et al. 1991) and could be instrumental in the establishment of viral infection and inhibition of the CTL response. Recently it has been shown that the HBc gene suppresses the tumour suppressor gene p53 (Kwon, Rho 2003). Furthermore it downregulates the human MxA protein, an IFN-inducible GTPase with antiviral activity, both by affecting the IFN-stimulated response elements and by direct interaction with the MxA promoter (Fernandez et al. 2003). Therefore the detailed elucidation of the putative role of HBc and its variants with CID mutations in the HBV pathogenesis should be a subject of further study.

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Iekšējo HBV *core* proteīna delēcijvariantu lokalizācija šūnās

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Kopsavilkums

Hepatīta B vīrusa (HBV) variantus ar iekšējām HBV *core* (HBc) proteīna delēcijām (CID) izolēja no pacientiem ar nieru transplantātu un galēji smagu aknu bojājumu. Darba mērķis bija noteikt šo HBc variantu lokalizāciju inficētā šūnā. Izmantoja kāmju nieru šūnu kultūru BHK-21 C13. Kultūras infekcija ar rekombinantiem Semliki meža vīrusiem, kas kodē kā delēcijas saturošus, tā arī nedeletētos savvaļas tipa HBc gēnus, izraisīja HBc proteīna parādīšanos šūnu kodolā tikai HBc delēcijvariantu, bet ne savvaļas HBc gadījumā. Visticamāk, ir izmainīta HBc delēcijvariantu telpiskās struktūras izveidošanās, jeb foldings, un tas izraisa to nestabilitāti šūnās. Šādi HBc delēcijvarianti nespēj pašasociēties par pilnvērtīgām HBc kapsīdām, atrodas šūnā neasociētā veidā un tāpēc ir spējīgi pārvarēt kodola membrānu un nokļūt šūnas kodolā. Mēs uzskatām ka šādu HBc delēcijvariantu klātbūtne šūnu kodolā var būt saistīta ar HBV patoģenitāti.

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Hepatitis C virus molecular epidemiology in Latvia

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Abstract

The aim of this study was to identify the hepatitis C virus (HCV) genotypes distributed in Latvia and to estimate their prevalence in various risk groups. HCV genotypes of 65 isolates were estimated using amplification and direct sequencing of PCR fragments from the core region of HCV genome. Phylogenetic analysis of the genotypes was conducted. Genotype 1b was identified in about 85 % of cases, genotype 3a in about 10 %, and HCV genotypes 1a and 2c were present only in some isolates. A similar epidemiological distribution is typical for the former Soviet Union Republics. Our study suggests that contaminated blood products may be main route of HCV infection in Latvia.

Key words: genotypes, HCV, prevalence.

Introduction

Hepatitis C virus (HCV) is the major agent of parenterally transmitted non-A, non-B hepatitis worldwide. Although representative prevalence data are not available from many countries, it is known that about 3 % of the world's population are infected with HCV. It is estimated that 170 million people worldwide are at risk of liver cirrhosis and hepatocellular carcinoma due to chronic infection with HCV (Cohen 1999). HCV causes 20 % of acute hepatitis cases, 70 % of all chronic hepatitis cases, 40 % of all cases of cirrhosis of the liver, 60 % of hepatocellular carcinomas, and 30 % of liver transplants in Europe (European Association for the Study of the Liver 1999).

Molecular characterization of HCV revealed the existence of a positive sense RNA genome of approximately 9400 bases in length. The complete genome sequence has been determined in different HCV isolates worldwide, which indicated substantial nucleotide sequence variability throughout the viral genome (Choo et al. 1991). HCV exhibits enormous genetic diversity: the comparison of published sequences of HCV has led to the identification of distinct HCV genotypes that may differ from each other by as much as 33% over the entire viral genome (Okamoto et al. 1992). The variability within the HCV genome has formed the basis for several genotyping systems. The current, most commonly used classification system has been proposed by Simmonds et al. (1994). HCV is classified into six major types (genotypes 1 to 6) and numerous subtypes (e.g., genotype 1a, 1b), which differ in diversity, geographical distribution, and transmission routes. Genotypes 1 to 3 are distributed widely around the world, while others have a more restricted distribution. For example, types 5 and 6 are only found in specific geographical (Simmonds 1999).

The genotypes of HCV appear to differ in serological reactivity and in treatment, although their role in variation of disease progression remains unclear (Poynard et al. 2003). Any successful HCV vaccination or control strategy, therefore, requires an understanding of the nature and variability of the epidemic behavior among subtypes.

The aim of this study was to identify hepatitis C virus genotypes distributed in Latvia and to estimate their prevalence in various risk groups.

Materials and methods

Patients

In total, 65 anti-HCV positive sera were included in this work: 23 sera from patients with chronic HCV infection, 18 sera from patients after kidney transplantation, 5 sera from patients undergoing dialysis and 19 sera from patients from the pediatric oncology ward. All sera were tested for the presence of anti-HCV antibodies using a second generation ELISA kit (Abbott Laboratories, Chicago, IL).

Amplification of fragments of HCV and HBV genomes

HCV-RNA was extracted from 100 μl of serum with a commercially available DNA/RNA isolation kit based on phenol/chloroform extraction ("Litech", Moscow, Russia). Amplification of HCV core region 476-725 nt fragment (numbering according to Takamizawa et al. 1991) was performed by "in house" nested RT-PCR. cDNA synthesis for amplification of core sequences was carried out using primer AS1 (5'ATGTACCCC ATGAGGTCGGC3'). Primers 2S (5'TAGATTGGGTGTGCGCGCGA3',) and 1AS were used for the first round PCR, whilst primers 3S (5'CGCGCGACTAGGAAGACTTC3'), 4S (5' TGTGTGCGCGACGCGTAAA3') and 5AS (5'GCAYGTRAGGGTATCGATGA CYT3') for the second.

Sequencing of the PCR fragments

Products of the PCR were excised from the agarose gel and purified using the DNA Extraction Kit (MBI Fermentas, Vilnius, Lithuania). Purified fragments were subjected to direct sequencing in both directions using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, USA), and electrophoregrams were obtained using an ABI Prism 377 sequencer (Applied Biosystems). PCR primers 3S and 5AS served as sequencing primers.

The sequences were edited manually by BioEdit Sequence Alignment (Hall, 1999) and subsequently aligned in the FASTA format (http://ngfnblast.gbf.de/docs/fasta.html). The phylogenetic tree was constructed using the DNA-distance algorithm and the neighbor-joining method in the PHYLIP package (Felsenstein 1989).

Results

Phylogenetic analysis of HCV core region nucleotide sequences showed that 57 isolates belonged to genotype 1b, six isolates to genotype 3a, one isolate to genotype 1a and one isolate to genotype 2c (Fig. 1). Distribution of HCV genotypes in isolates from different patient groups are shown in Table 1.

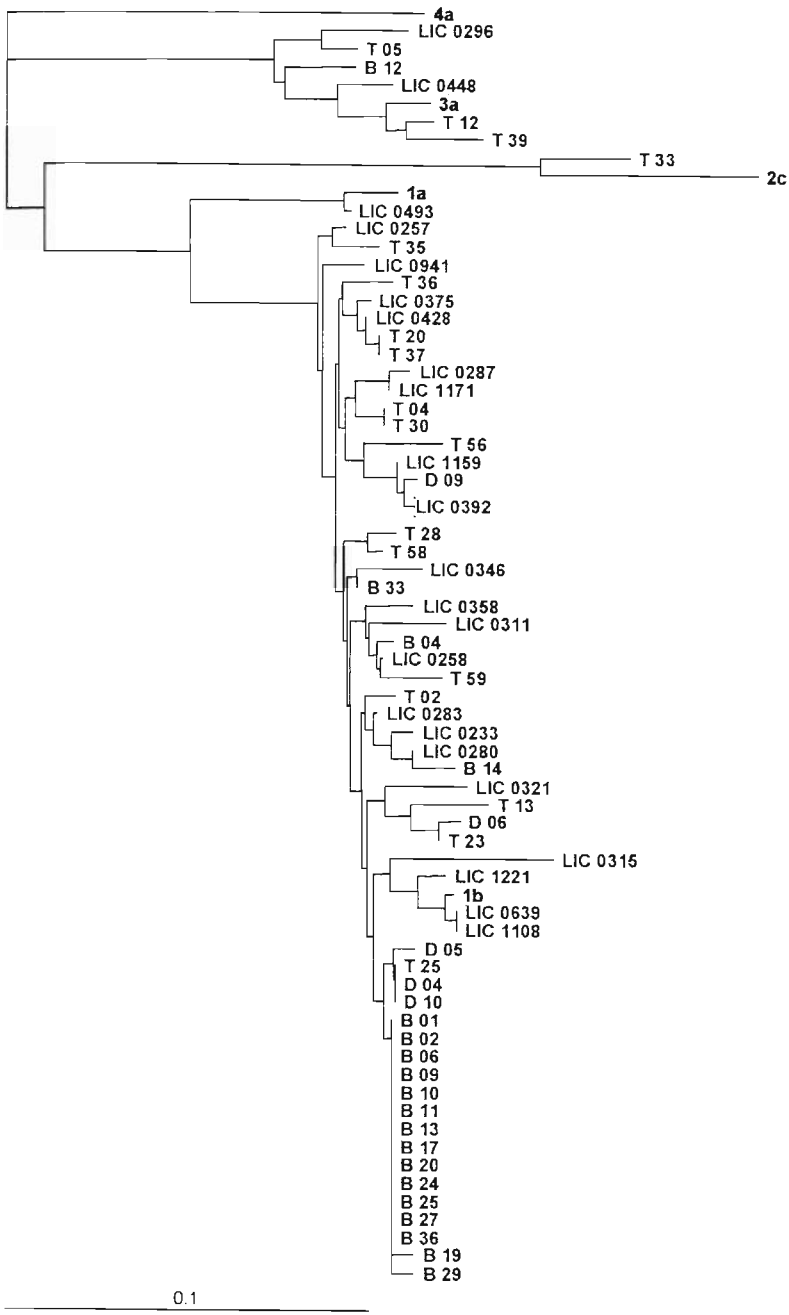

Fig. 1. Phylogenetic tree of 70 HCV core region sequenced fragments. Sequences from patients with chronic HCV infection have the prefix LIC, sequences from patients after kidney transplantation have the prefix T, sequences from patients ongoing dialysis have the prefix D and sequences from patients from the pediatric oncology ward have the prefix B. Reference sequences representing published genotypes 1a, 1b, 2c, 3a and 4a are included.

Table 1. Distribution of HCV genotypes in different groups of patients

Group of patients	1b	1a	2c	3a
Patients with chronic HCV infection	20	1	–	2
Patients after kidney transplantation	14	–	1	3
Patients undergoing dialysis	5	–	–	–
Patients from the pediatric oncology ward	18	–	–	1
Total	57	1	1	6

Discussion

The obtained results showed a strong predomination of genotype 1b in all groups of patients: 87 % in cases of chronic HCV infection, 78 % in posttransplantation, 100 % during dialysis treatment and in 95 % of cases in patients from the pediatric oncology ward. Not all of the analyzed groups are equally representative. As it was shown earlier (Dumpis et al. 2003), most of the examined patients from the pediatric oncology ward were infected in an outbreak. According to phylogenetic analysis, 13 of the 19 patients were infected with the same HCV genotype 1b isolate. On the other hand, the rate of evolution to chronicity after acute exposure to HCV was 92 % in patients exposed to HCV genotype 1b infection, compared with 33 % to 50 % in patients exposed to other genotypes (Zein 2000). The distribution of the HCV genotypes in patients undergoing dialysis and after kidney transplantation is similar to the distribution among non-renal patients in the same country (Fabrizi et al. 2001). Taking all of these obstacles into consideration, about 85 % of HCV isolates present in Latvia may belong to genotype 1b. Genotype 1b is seen more often in patients who acquired HCV through blood transfusion of unscreened blood products and medical procedures (Zein 2000), and unfortunately it shows relatively poor response to treatment by traditional anti-viral drugs (Poynard et al. 2003).

The genotype 3a may be present in about 10% of the Latvian patients infected by HCV. According to published data, this genotype is most common among intravenous drug users and shows a good response to interferon therapy (Zein 2000).

In a contrast with West-Europe countries (Zein 2000), HCV genotypes 1a and 2 are not common in Latvia. A similar epidemiological distribution was also described in other former Soviet Union Republics and some of Asian countries (Lvov et al. 1996; Viazov et al. 1997; Kurbanov et al. 2003). We believe that, at present in eastern neighbour countries, the main transmission pattern of HCV epidemic in Latvia is 1b genotype infection that spreads through blood transfusion and medical procedures.

Thereby, complete and proper screening of blood products, improving conditions needed for sterilisation of medical instruments, and using disposable syringes on the one hand, and management of measures targeted to users on the other, are two main directions that are important for limiting HCV transmission and the prevention of new outbreaks in Latvia.

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Hepatīta C vīrusa molekulārā epidemioloģija Latvijā

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Kopsavilkums

Darba mērķis bija identificēt Latvijā izplatītus hepatīta C vīrusa genotipus, kā arī novērtēt to izplatību dažādās riska grupās. Pielietojot HCV genoma *core* reģiona fragmenta PCR amplifikāciju, sekvinēšanu un iegūto sekvenču filoģenētisko analīzi, HCV genotipu noteica 65 paraugiem. 1b genotipu atrada apmēram 85 % gadījumu, 3a genotipu – apmēram 10 % gadījumu, HCV genotipus 1a un 2c atrada tikai dažos paraugos. Līdzīga epidemioloģiskā situācija ir raksturīga bijušajām PSRS valstīm. Mūsu pētījumi ļauj secināt, ka Latvijā HCV izplatās ar inficētiem asins materiāliem.

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Graft union formation in elepidote rhododendrons

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Abstract

Light microscopy was used to study the graft union formation of splice graft in the elepidote rhododendrons cultivar 'Cunningham's White'. The first visible reaction after grafting was the appearance of a necrotic layer between graft partners consisting of fragmented and compressed cells. Callus was visible six days after grafting and was formed from recent cambial derivatives and phloem rays cells. New cambium and tracheary elements were differentiated from callus cells. Newly differentiated cambium was slightly curved or had an *S*-shape, and it depended on rootstock and scion tissue matching. When initially the gap between the graft partners remained wide, callus produced more new cambiums: one of them emerged between pre-existing cambium of graft component and an uncommon one – in the pith region. The possible function of this uncommon cambium is discussed.

Key words: callus development, cambium, grafting, rhododendron, vegetative propagation.

Introduction

Rhododendron can be propagated either by seed or by vegetative methods. One of the most widely used vegetative propagation method is propagation by grafting. Grafting is essential for propagation of many woody plant species, cultivars, and hybrids because of existing difficulties in their propagation by cuttings and by micropropagation.

The development of graft union is a process of forming a functional unit through the interaction of organs, tissues or cells from the same or different plants (Shanfa 2000). A number of detailed studies have been made about graft union formation with woody and herbaceous plants (Hartman et al. 1997). The anatomical changes that occur during graft union formation are in the approximate order of occurrence, following grafting in many plants. These include the death of layers of cells at the graft interface, cohesion of scion and rootstock, generation of callus and establishment of vascular continuity and a new stem centre (Miller, Barnett 1993). According to Moore (1984), the development of a compatible graft includes three events: cohesion of rootstock and scion, proliferation of callus cells at the graft interface, and vascular differentiation across the graft interface. However, insufficient information is available about graft union formation of elepidote rhododendrons. Detailed information about this process is essential for the propagation of difficult-to-root cultivars. The elepidote rhododendrons cultivar 'Cunningham's White' is widely and successfully used as a rootstock for rhododendron grafting, because it has good grafting union formation potential (Cox, Cox 1988). The objective of this study was to investigate the structural development of graft union formation in this cultivar.

Materials and methods

Plant material

Investigation was carried out in December, 2001 and 2002, using the elepidote rhododendrons cultivar 'Cunningham's White' [*Rh. caucasicum* × *Rh. ponticum* var. *album*] both as rootstock and scion. The plant material was obtained from the Botanical Garden of the University of Latvia and the Experimental Nursery of Rhododendron Breeding "Babite", University of Latvia. Rootstocks for grafting were 2-year old plants propagated by cuttings. Dormant scion material – new shoots, were collected from shrubs of rhododendron shortly before grafting. Scions were splice grafted to the rootstock. During the grafting procedure a diagonal cut was made at the top of rootstock and an appropriate cut at the base of the scion. The scion and rootstock were fitted together and wrapped with rubber grafting tape (Albrecht, Sommer 1991). The grafted plants were covered with a polyethylene tent and kept at 23 °C during the day and 20 °C at night, with a 16-h photoperiod in the growth chamber.

Histological analysis

Collections of five graft unions were made 3, 6, 9, 12, 15, 18, 25, 32, 39 days after grafting. Transverse sections in the middle of graft unions were cut by manual microtome and a razor. The sections were stained with safranin-astra blue, rinsed in water, dehydrated in an ethanol-xylol series and permanently embedded in Canadian balsam (Braune et al. 1999).

Sections were examined with an Olympus CH30RF200 light microscope and photographed using a Leica DMLS light microscope by digital camera Canon Power Shot S40.

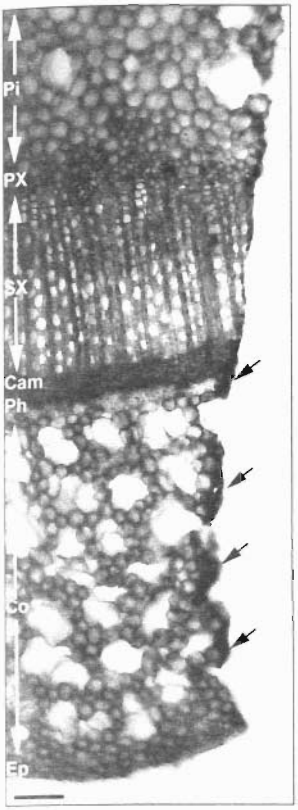
Results and discussion

The anatomical changes that occur during graft union formation of elepidote rhododendrons are comparable to graft formation of other woody and herbaceous plants. Graft union development can be varied temporally between each of the grafts. It depends not only on the physiological condition of graft partners, but also on the success of the grafting technique.

The rootstock and scion anatomy was similar, consisting of epidermis, cortex, phloem, cambium, secondary xylem, primary xylem and pith (Fig. 1). A detailed stem anatomy of the rhododendron cultivar 'Cunningham's White' was described in our previous investigation (Kondratovics, Megre 1999).

The first visible reaction after grafting was the appearance of a necrotic layer between graft components consisting of fragmented and compressed cells, observed three days after grafting (Fig. 1). Subsequently the necrotic layer became thinner and eventually disappeared, presumably being absorbed by the developing callus. A similar observation was described by Stoddard and McCully (1980).

There are different opinions regarding the origin of callus in different species. All living undamaged cells – parenchyma cells from pith to cortex and also cambium – are capable of callus formation. Usually, callus is formed from various tissues, but with different intensities of cell division (Dormling 1963). The cambial region was observed to play



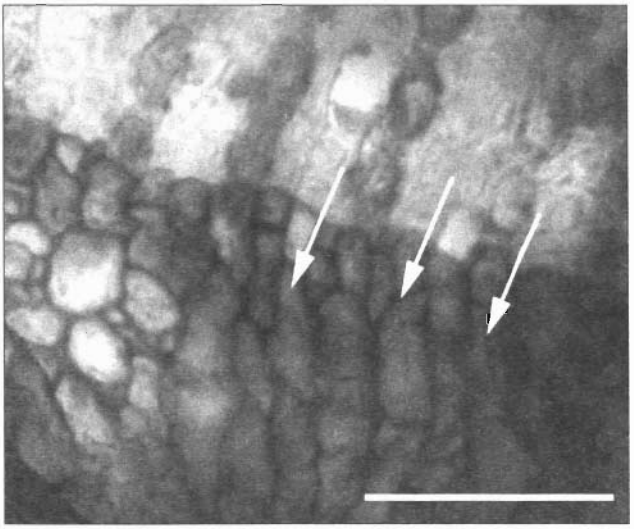


Fig. 2. Callus cell formation from recent cambial derivatives (arrows) in a 6-day-old graft union. Bar = 50 μm.

Fig. 1. Transverse section through a 3-day-old graft union shows scion anatomy and layer of necrotic tissues (black arrows). Ep, epidermis; Co, cortex; Ph, phloem; Cam, cambium; SX, secondary xylem; PX, primary xylem; Pi, pith. Bar = 100 μm.

an essential role as a callus producer in elepidote rhododendrons. Callus was produced from recent cambial derivatives (Fig. 2) and phloem ray cells (Fig. 3), and became visible six days after grafting. Callus formation from phloem ray cells is characteristic for many plants, such as *Pseudotsuga menziesii* (Copes 1969), *Picea sitchensis* (Miller, Barnett 1993), *Lycopersicon esculentum* (Fernandez-Garcia et al. 2004), but there are different views regarding the importance of the cambium as a callus producer. As in our work, Fuji and Niko (1972) suggested that the cambium has an essential role in callus formation, while other observations have suggested that cambium contributed little to early callus formation (Copes 1969) or not at all (Sharples, Gunnery 1933 cited in Larson 1994). Callus formation from xylem ray parenchyma cells has been observed in *Pinus sylvestris* (Dormling 1963) and in apple (Soumelidou et al. 1994). However, our study showed that xylem rays are not capable of producing callus in elepidote rhododendrons. In the subsequent days the callus growth was so abundant that it became impossible to determine the origin of any individual cell or group of cells. Callus continued to proliferate and to fill the space between scion and rootstock.

Tracheary elements were formed from callus and they could be recognized by the presence of reticulate secondary walls (Fig. 4). In *Picea sitchensis* graft union, such cells might assist in transport of water across the graft interface (Weatherhead, Barnett 1986), but it is possible that these cells formed merely as a result of the influence of auxin and nutrients on the callus, with no specific function (Soumelidou et al. 1994).

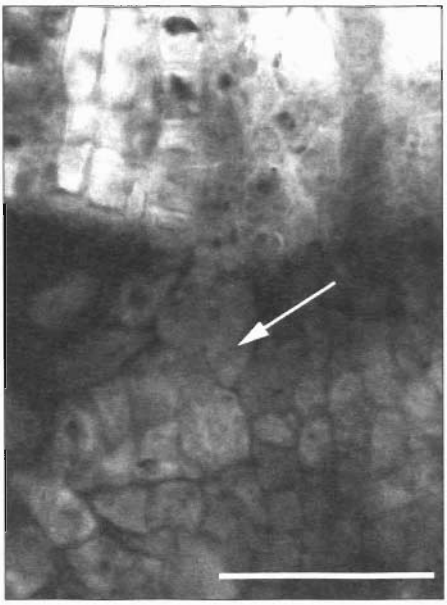


Fig. 3. Callus cells formation from phloem ray cells (arrow) in a 6-day-old graft union. Bar = 50 μm.

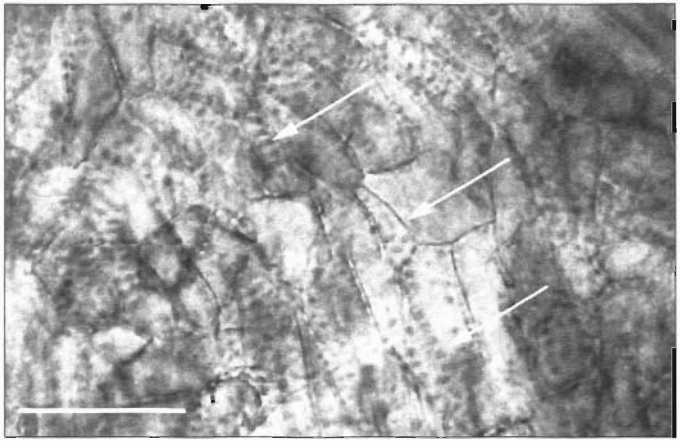


Fig. 4. Randomly arranged tracheary elements (arrows) by direct differentiation of callus cells in a 25-day-old graft union. Bar = 50 μm.

An important stage of graft union formation is the differentiation of callus cells by forming new cambium between pre-existing graft partner cambium. Differentiation of callus cells to form cambium between the cut ends of bud and rootstock cambium involves the formation of new cells aligned at right angles to the pre-existing cambium, as if in response to a stimulus flowing horizontally through the callus from one cambium to the other (Soumelidou et al. 1994). The new cambium was first recognizable 18 days after grafting (Fig. 5). New cambium shapes were variable, depending on rootstock and

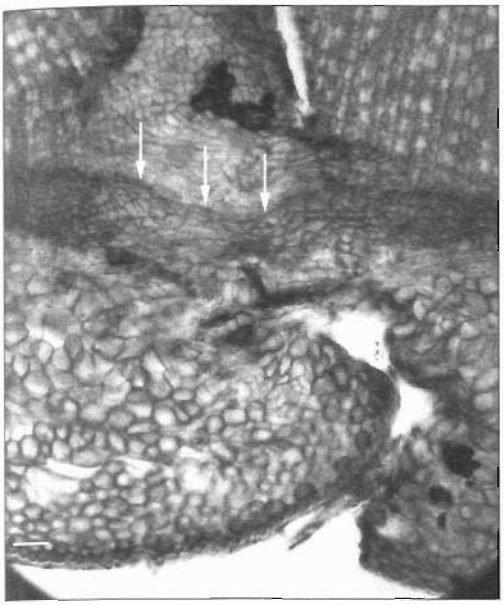


Fig. 5. Transverse section through a 18-day-old graft union shows a slightly curved newly formed cambium (arrows) between pre-existing cambium of the graft partners. Bar = 50 μm.

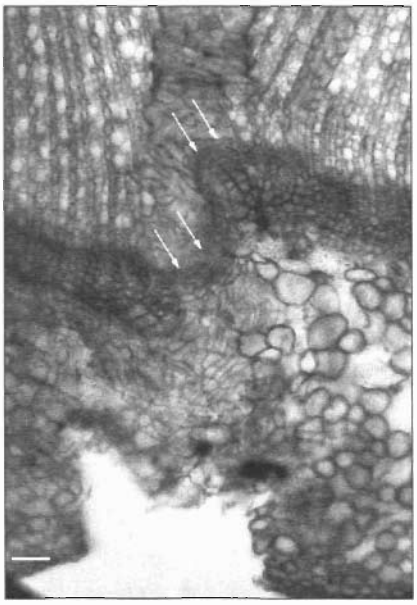
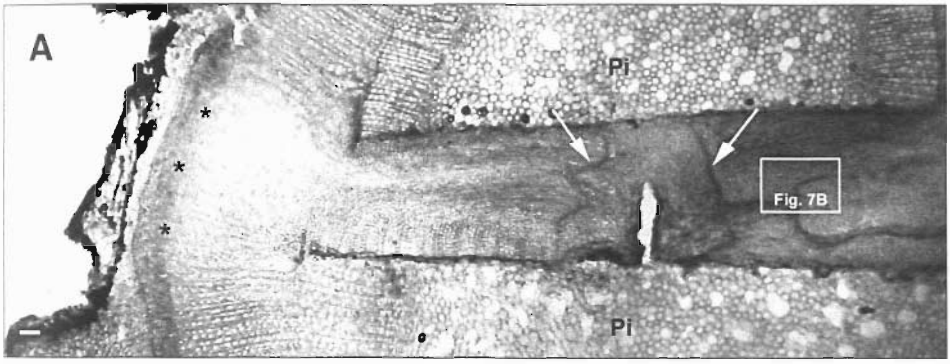


Fig. 6. Transverse section through a 25-day-old graft union displays a newly formed *S*-shape cambium (arrows) between pre-existing cambium of the graft partners. Bar = 50 μm.



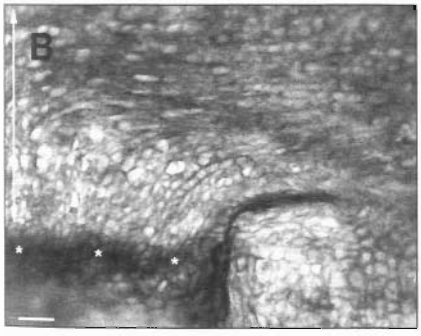


Fig. 7. Transverse section through a 39-day-old graft union. A. New cambium formation between pre-existing cambium (*) and between rootstock and scion piths (arrows). Pi, pith. Bar = 100 μm. B, newly formed cambium (*) and tracheary elements (arrows). Bar = 50 μm.

scion tissue matching. If graft partners were well matched, their pre-existing cambiums joined forming a slightly curved (Fig. 5) or *S*-shape (Fig. 6).

If physical pressure from binding tape, which is used to hold the graft partners together, is insufficient, a wide gap between the graft partners remains. Cambium development between pre-existing cambiums of graft partners was observed, as well as an uncommon cambium development between pith of rootstock and scion (Fig. 7A). Tracheary elements, which were formed partly by the differentiation of callus cells and partly by uncommon new cambium cells, were visible outward from the uncommon cambium (Fig. 7B). These elements may provide water transport, but it is not clear why they are formed in pith region. On the other hand, tracheary elements can also increase the mechanical endurance, which is essential for development of graft union. The functions of the uncommon cambium are yet unclear.

The graft union development of the elepidote rhododendrons cultivar 'Cunningham's White' is consistent with the graft union formation of other woody plants. Further study is required to determine the influence of uncommon cambium in graft union development.

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Mūžzaļo rododendru potējumu saaugšanas gaita

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Kopsavilkums

Izmantojot gaismas mikroskopiju analizēta mūžzaļo rododendru šķirnes 'Cunningham's White' potējumu (vienkāršā kopulēšana) saaugšanas gaita. Pirmā pamanāmā izmaiņa pēc potēšanas bija nekrotiska slāņa izveidošanās uz griezumu virsmas. Kalluss tika konstatēts sestajā dienā pēc potēšanas un tas veidojās no lūksnes staru un kambija šūnām. Koksnes elementi un jaunveidotais kambijs veidojās, diferencējoties kallusa šūnām. Jaunajam kambijam var būt dažāda forma – nedaudz izliekta vai *S* veida un tā ir atkarīga no potcelma un potzara kambiju savietošanas. Ja sākotnējā telpa starp potcelmu un potzaru ir plata, tad veidojas vairāki kambiji – viens starp potējuma partneru kambijiem un otrs, kura funkcija pagaidām nav skaidri zināma, – serdes rajonā.

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Distribution of the freshwater pearl mussel *Margaritifera margaritifera* (Linnaeus 1758) in Latvia in relation to water quality

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Abstract

The aim of the present work was to update the distribution of the freshwater pearlmussel *Margaritifera margaritifera* in Latvia. From 1999 to 2003 a total length of about 610 km was surveyed in 163 rivers. The distribution of the species in Latvia is about 40 km of river-parts in eight rivers, with a mussel population of about 25,000 individuals. In six rivers only shells and parts of shells were found indicating that populations occurred here previously and had become extinct. All the Latvia's pearl mussel populations are aging, but the pearl mussels are healthy and able to reproduce. Chemical analyses of water samples showed that the quality of water in Latvian pearl mussel rivers is considerably worse than in other countries where viable populations are found. Therefore, we can expect a rapid population decline and loss during the next 10 - 50 years. The populations could be maintained if the conditions in the rivers were improved for the survival of young pearl mussels.

Key words: ecology, distribution in Latvia, *Margaritifera margaritifera*, water quality.

Introduction

The freshwater pearl mussel *Margaritifera margaritifera* is probably the most abundant bivalve worldwide (Araujo, Ramos 2000). The freshwater pearl mussel has a holarctic circumpolar distribution. In Europe it is now largely restricted to the northern highland zone, to 71° N in Norway (Kerney 1999). It is distributed in the northern part of Asia and the northeastern part of North America (Zadin 1952).

M. margaritifera is a typical oligotrophic water species. Changing the environmental conditions usually endangered the most highly specialized species. *M. margaritifera* is decreasing not only in Latvia but also in its whole geographical distribution (Bauer 1988). In Europe it occurs only in mountain regions, but in the lowlands between agricultural lands and in urbanized areas all of the populations have disappeared (Kinkor et al. 1996; Erikson et al. 1998; Araujo, Ramos 2000).

In spite of rather good survival strategy of *M. margaritifera*, its complicated cycle of development, in which the glochidia must go through a phase of parasitic development, and highly specialized adaptation for living in oligotrophic conditions, make pearl mussel populations especially sensitive against changes in the environment. Therefore, *M. margaritifera* is a very important bioindicator of the general level of pollution (Bauer 1988; Cimdins et al. 1995; Hruska, Bauer 1995).

Already in 1855, E.Wahl (Wahl 1855) wrote that in Latvia's pearl mussel rivers, economic activities on and around the rivers will lead to, if not complete extinction of the species, then certainly to restriction in the pearl mussel to population size, as in ancient pearl fishing times. During the1920s and 1930s the number of pearl mussel populations had decreased because of intensive pearl mussel extraction during the previous centuries (Kawall 1872; Eke 1925; Meder 1925; Pētersons 1933; Schlesch 1942). An action plan for *M. margaritifera* in Latvia and a strategy for conservation of this species was prepared in 1999 (Rudzīte 2001), but full data about its distribution was not clear.

The goal of the work was to update the distribution of the pearl mussel in Latvia.

Materials and methods

The method used was total survey of the whole riverbed and total counts of all mussels. The river were investigated under suitable weather conditions – periods of low water in the river, sunny or partly cloudy weather. The parts of rivers were surveyed in the whole length by walking on the riverbed. Special attention was paid to the deepest parts of the river and disturbance of the surface water layer was avoided as much as possible. Additionally, polaroid glasses and optical underwater tubes were used. In this way the whole riverbed can be observed and all pearl mussels could be found.

From 1999 to 2003 the distribution area of pearl mussel populations and rivers where pearl mussels could be expected were surveyed. At present, two thirds of the areas where pearl mussel populations were possible, according to historical data (Fischer 1791; Groschke 1805; Kawall 1872; Braun 1884; Riemschneider 1908; Eke 1925; Meder 1925; Pētersons 1933; Schlesch 1942), have been checked. Previous investigations by students at the Department of Zoology and Animal Ecology of the Latvian University (V. Bernards, K. Krišāns, I. Maksimova, A. Tukiša, unpublished data) and collections at the Museum of Zoology of the Latvian University were summarized.

All rivers in the Gauja National Park were surveyed in 2000, the Northern Vidzeme Biosphere Reserve in 2002, and in part of the Daugava basin in 2003 (Table 1, Table 2). Data in the Table 2 differ from those published earlier (Rudzīte 2001) due to additional

Table 1. Research conducted in the rivers frm 1999 to 2003. Information is available from a total 610 km length of rivers surveyed during 1999 - 2003

Year	Territory	Surveyed parts of rivers (total km)	Counting of pearl mussels (total km)
1999	Separate places in previously known rivers and surroundings	~ 35	~ 16
2000	Gauja National Park	~ 63	0.3
2001	Investigation of pearl mussel populations and river basins in known areas	~ 87	~ 36
2002	North Vidzeme Biosphere Reserve	147 (59 rivers)	-
2003	A part of the Daugava basin rivers	93 (28 rivers)	~ 12

Table 2. Number of pearl mussels and their shells in populations surveyed from 1999 to 2003. *, data is incomplete, the number of pearl mussels may be larger

Basin	River	Number of pearl mussels and found shells	Years of research
Gauja river basin	Ludze	20 000	1999 - 2001
	Rauza	3 000	1999 - 2001
	Pērļupe (tribulary of Amata)	570	1999 - 2003
	Dadžupe	200	2000, 2003
	Dzirnupe (tribulary of Amata)	20	2000, 2003
	Strīķupe	0	2000, 2001
	Meļļupīte	0	2000
	Lenčupe	Fragments of shells	1999
	Abuls	0	2000
	Pērļupe (tribulary of Gauja)	0	2000
Daugava river basin	Tumšupe	1200	1999, 2001, 2003
	Pededze	30*	1999
	Mergupe	7*	2002, 2003
	Vedze	Shells	1996
	Paparze	Fragments of shells	1999
	Veseta	Shells	2003
	Zaube	Shells	2003
Salaca river basin and Northern Vidzeme rivers of Gulf of Riga	Aģe	Shells	2002
	Korģe	0	2002
	Iģe	0	2002
	Pērļupe (tribulary of Svētupe)	0	2002
	Ķīšupe	0	1985
	Pēterupe	0	1985

survey since 2001.

From 1999 to 2003 a total length of about 610 km of rivers was surveyed.

The distribution area of pearl mussels is about 40 km river length. In some places counting was repeated, therefore the actual distance where counting was conducted was greater (64 km) than the length of the river parts with pearl mussel populations. Systematically and completely 108 rivers were survived, and information was summarized about fragmentary investigations in 55 rivers.

The following maps were used in describing the distribution: Latvia Republic Satellite map (scale 1:50,000); Soviet Union army topographic maps (scales 1:50,000 and 1:10,000). Information on water chemistry was obtained from the Regional Environmental Boards in Valmiera and Lielrīga.

Results and discussion

According to the literature (Fischer 1791; Kawall 1872; Braun 1884; Riemschneider

1908; Eke 1925; Meder 1925; Pētersons 1933; Schlesch 1942) the former pearl mussel distribution in Latvia included Vidzeme and the northwestern part of Latgale. There is only one report about a pearl mussel in Kurzeme, in the river Durbe (Groschke 1805).

From all the surveyed rivers, 23 were chosen (Table 2), which correspond at least to one of the following conditions: there is a pearl mussel population in the river at present; there are no live pearl mussels found, but only shells or there is information from the literature or shells in museum collections that confirm that pearl mussels have been there. The most important collection used was collected by R. Kampe during the 1920s and 1930s, which is currently housed in the Museum of Zoology of the Latvian University. River were not included, if a large part of the river was straightened, destroying the natural habitats where pearl mussels could live, even if the river was mentioned in the literature.

At present, pearl mussels in Latvia have been found in eight rivers (Table 2). In six other rivers shells and fragments of shells have been found (Fig. 1).

In the Gauja river basin, local populations occur in five rivers: Dadžupe in about 800 m, Dzirnupe in about 200 m, Pērļupe (tributary of Amata) in about 2 km, Rauza in about 24 km, Ludze in about 7 km. In Daugava basin *M. margaritifera* occurs in Tumšupe in about 4 km, and about 1 km of Mergupe.

The total distribution of the species in Latvia is about 40 km of river-parts, with total number of mussels of about 25,000. Of the 108 totally surveyed rivers, only eight still contain pearl mussel populations. Only shells and parts of shells found in six rivers proved the populations now are extinct. In about nine other rivers there is enough evidence to conclude that populations did occur and that they have became extinct (Table 2). In the remaining 85 surveyed rivers, there is no evidence of the species, but nevertheless these rivers lay in the described pearl-extraction area (Fischer 1791; Kawall 1872; Braun 1884; Riemschneider 1908; Eke 1925; Meder 1925; Pētersons 1933; Schlesch 1942). Probably, the populations became extinct long ago and therefore even parts of shells can not be found.

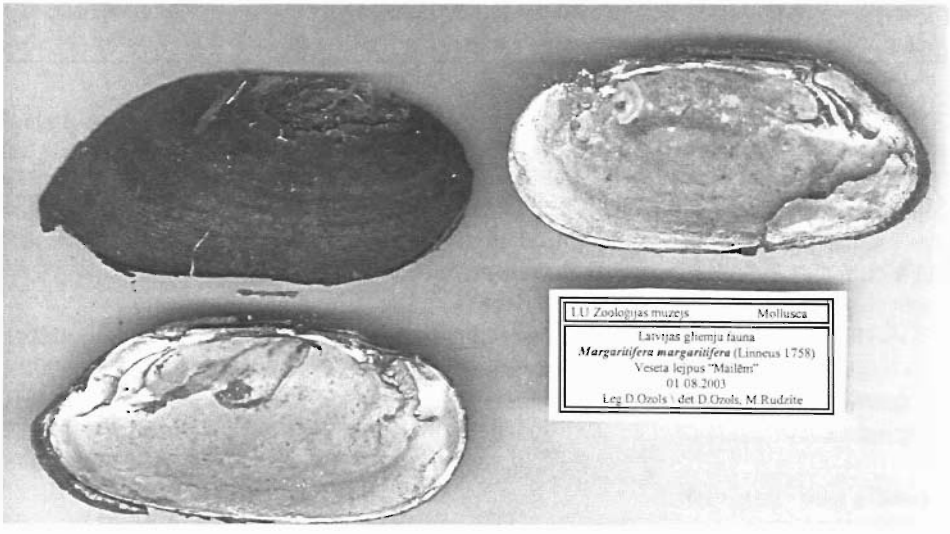

Fig. 1. Pearl mussel shells found in 2003 in the river Veseta.

Table 3. Nitrogen and phosphorus concentrations in river water in different pearl mussel populations in Latvia and Europe. Data obtained from the Latvian Regional Environmental Boards in Valmiera and Lielrīga in 2001 and published literature from Ireland, Norway and Austria (Lande, Lande 2000; Moorkens et al. 2000). n.d., not determined

Populations	Dissolved oxygen (% sat.)	Conductivity (μS cm^{-1})	Oxidised nitrogen (mg l^{-1})	Total ammonia (mg l^{-1})	Total nitrogen (mg l^{-1})	Ortho-phosphate (mg l^{-1})
Ireland	min 9.0	max 1.7	max 1.7	max 0.1	n.d.	max 0.12
	(mg l^{-1})	65 - 129	0.04 - 1.3	0.015 - 0.03		0.005 - 0.06
Norway	n.d.	15.5 - 271	n.d.	n.d.	0.21 - 0.52	0.002 - 0.1
Austria	98 - 131	91 - 110	0.9 - 1.4	< 0.01	n.d.	0.009 - 0.014
Ludze	87 - 95	336 - 367	max 0.007	max 1.17	max 1.38	max 0.024
			med 0.0065	med 0.82	med 0.99	med 0.022
Pērļupe	89	325	0.083	2.07	2.19	0.023
Rauza	77 - 97	368 - 399	max 0.015	max 1.21	max 1.22	max 0.058
			med 0.001	med 0.6	med 1.00	med 0.043
Tumšupe	n.d.	n.d.	max 0.039	max 1.43	max 4.76	max 0.018
			med 0.037	med 1.35	med 3.83	med 0.013

In a previous study of the age structure of the population (Rudzīte 2001) it was found that younger age classes were not represented in any of the populations, meaning that all populations were in the phase of aging. However, the pearl mussel glochidia larva phase has been observed on the gills of young salmonids (Rudzīte, unpublished data).

Pearl mussel health can be estimated by the ability to push out a water trickle, when taken out of water (Bischoff et al. 1986; Baer 1995), as this means pearl mussels are healthy and able to reproduce. This was monitored in all the surveyed populations.

In researche conducted in Germany, it was found that young pearl mussel survival requires a low concentration of nitrogen in the water (Buddensick 2001). In Norway populations with young pearl mussels were found only in rivers with a very low nitrogen concentration (Lande, Lande 2000). The optimal pearl mussels water quality has been estimated: "Towards a margaritiferid water quality standard" (Moorkens et al. 2000).

Comparing the chemical parameters in rivers of Latvian populations with those in Ireland, Norway and Austria (Table 3, Lande, Lande 2000; Moorkens et al. 2000) where the young pearl mussel survival is normal, in Latvia there are significantly increased ammonium, total nitrogen, and phosphorus concentrations and conductivity (Table 3). The amount of dissolved oxygen in water is also low (Table 3).

Thus, water quality in the Latvian pearl mussel rivers is substantially worse than necessary for the survival of pearl mussels.

Aging of populations has been observed not only in Latvia but also in Europe (Kinkor et al. 1996; Erikson et al. 1998; Araujo, Ramos 2000; Moorkens et al. 2000). According to the present age structure of populations (Rudzīte 2001), we may forecast a rapid population decline and loss during the next 10 - 50 years. However Latvia's pearl mussels are not so old to exclude reproduction. Populations could be established and maintained if the conditions in the rivers were improved for the survival of young pearl mussels.

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Margaritifera margaritifera (Linnaeus 1758) izplatība Latvijā saistībā ar ūdens kvalitāti

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Kopsavilkums

Pētījuma mērķis bija noskaidrot ziemeļu upespērlenes *Margaritifera margaritifera* izplatību Latvijā. Laika periodā no 1999. gada līdz 2003. gadam apsekoti upju posmi 163 upēs ar kopējo garumu 610 km. Sugas areāls Latvijā aizņem nedaudz vairāk par 40 km upju posmu astoņās upēs. Uzskaitēs konstatētais gliemeņu kopskaits ir ap 25 000 eksemplāru. Sešās upēs konstatētas čaulas un to fragmenti, kas ir pierādījums tam, ka populācijas tajās ir bijušas, bet iznīkušas. Visās Latvijas pērleņu atradnēs konstatētas populācijas novecošanas stadijā. Ūdens ķīmisko analīžu salīdzinājums parāda, ka Latvijas pērleņu upēs ūdens kvalitāte ir ievērojami sliktāka kā vietās, kur dzīvo populācijas, kas nav novecošanas stadijā. Latvijas upespērlenēm draud iznīkšana tuvāko 10 - 50 gadu laikā. Tomēr populācijas varētu atkal atjaunoties, ja upēs būtu pērleņu mazuļu izdzīvošanai labvēlīgi dzīves apstākļi.

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The predatory mite (Acari, Parasitiformes: Mesostigmata (Gamasina); Acariformes: Prostigmata) community in strawberry agrocenosis

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Abstract

Altogether 37 predatory mite species from 14 families (Parasitiformes and Acariformes) were collected using leaf sampling and pit-fall trapping in strawberry fields (1997 - 2001). Thirty-six were recorded on strawberries for the first time in Latvia. Two species, *Paragarmania mali* (Oud.) (Aceosejidae) and *Eugamasus crassitarsis* (Hal.) (Parasitidae) were new for the fauna of Latvia. The most abundant predatory mite families (species) collected from strawberry leaves were Phytoseiidae (*Amblyseius cucumeris* Oud., *A. aurescens* A.-H., *A. bicaudus* Wainst., *A. herbarius* Wainst.) and Anystidae (*Anystis baccarum* L.); from pit-fall traps – Parasitidae (*Poecilochirus necrophori* Vitz. and *Parasitus lunaris* Berl.), Aceosejidae (*Leioseius semiscissus* Berl.) and Macrochelidae (*Macrocheles glaber* Müll).

Key words: agrocenosis, diversity, predatory mites, strawberry.

Introduction

Predatory mites play an important ecological role in terrestrial ecosystems and they are increasingly being used in management for biocontrol of pest mites, thrips and nematodes (Easterbrook 1992; Wright, Chambers 1994; Croft et al. 1998; Cuthbertson et al. 2003). Many of these mites have a major influence on nutrient cycling, as they are predators on other arthropods (Santos 1985; Karg 1993; Koehler 1999).

In total, investigations of mite fauna in Latvia were made by Grube (1859), who found 28 species, Eglītis (1954) – 50 species, Kuznetsov and Petrov (1984) – 85 species, Lapiņa (1988) – 207 species, and Salmane (2001) – 247 species. There are more than 300 predatory mite species that have been recorded in Latvia currently.

The predatory mite fauna in strawberry agroecosystems has not been investigated previously with the exception of Eglītis (1954) who mentioned *Bdellodes lapidaria* (Kram.) (Bdellidae) on cultivated strawberry, and Kuznetsov and Petrov (1984) who mentioned 6 species: *Amblyseius subsolidus* Begl., *Euseius finlandicus* Oud., *Phytoseius salicis* Wainst. et Arut. (Phytoseiidae), *Lorryia armaghensis* Baker, *L. reticulata* (Oud.) (Tydeidae), and *Anystis baccarum* L. (Anystidae).

The aim of the research was to determine the structure of fauna of insects and mites, abundance and dominance of particular species in Latvian strawberry agroecosystems. The structure, diversity and dominance of the predatory mite fauna is important as the predatory arthropods have an important role in sustainable agriculture.

Materials and methods

Studies of the injurious and beneficial mite (Acari, Parasitiformes, Acariformes) communities in strawberry agrocenosis were carried out during 1997 - 2001 in Latvia. The main material was collected generally from cultivated strawberry fields (about 6 ha) in the Pūre Horticultural Research Station (PHRS; Tukums District, northwest Latvia), but also on privately-owned strawberry fields (Rīga, Dobele and Limbaži Districts). Strawberries were planted in rows with 30-cm distances between plants and 100-cm distances between rows on the PHRS strawberry fields. The PHRS is located on calcareous podzolic sandy loam soil on dolomite bedrock. The private field in the Rīga District was on sandy soils and in the Limbaži District on loamy soils.

The investigations were carried out on fields where chemical treatment was used once per season every two years. Direct observations, collections from leaves, and pit-fall trapping were used. Four to six leaf samples were collected monthly from each field during 1997 - 2000. One leaf sample consisted of 200 leaves (100 folded and 100 unfolded). A method described by Petrova et al. (2000a) was used for sampling, collecting and calculating of mites.

The pit-fall trapping was carried out in 2001. Investigations started in April 9 and ended in October 30. Traps were laid between strawberry plants in rows in triennial strawberry fields at the PHRS. Glass cups (500 cm^3) with a diameter of 70 mm were used for this purpose. For preservation 6 % acetic acid and detergent added were used. Six pit-fall traps were used to catch arthropods living on soil surface. Checking of traps was conducted twice per month. Mites were removed under a stereoscopic microscope and mounted in Berlese medium.

Results

Predatory mite fauna

The predatory mite fauna of cultivated strawberries consisted of 37 species from 27 genera and 14 families (Table 1): Aceosejidae (6 species), Ameroseiidae (1), Antennoseidae (1), Anystidae (1), Bdellidae (1), Cheyletidae (1), Cunaxidae and Eviphidae (2), Laelaptidae (2), Macrochelidae (1), Parasitidae (6), Phytoseiidae (12), Tydeidae (2), Veigaiaidae (1). The highest species richness was found for Phytoseiidae, Aceosejidae and Parasitidae.

During the 5 years of sampling, we found 32 species of Parasitiformes (86.5 % of the total number of species recorded), and 5 species of Acariformes (13.5 %). The occurrence of mites during the period of investigations is shown in Table 1. Unfortunately not all species were identified, especially for the Cunaxidae and Tydeidae.

Thirty-six predatory mite species were recorded for the first time in strawberry fields in Latvia. Two species *Paragarmania mali* (Oud.) (Aceosejidae) and *Eugamasus crassitarsis* (Hal.) were new for the fauna of Latvia.

Leaf samples

In total, 21 predatory mite species of Parasitiformes from 10 families (Aceosejidae (2), Eviphidae (1), Macrochelidae(1) and Phytoseiidae (12) and Acariformes from 4 families (Anystidae (1), Bdellidae (1), Cheyletidae (1), Tydeidae (2)) were found on strawberry leaves. The number of Parasitiformes and Acariformes mites significantly varied among

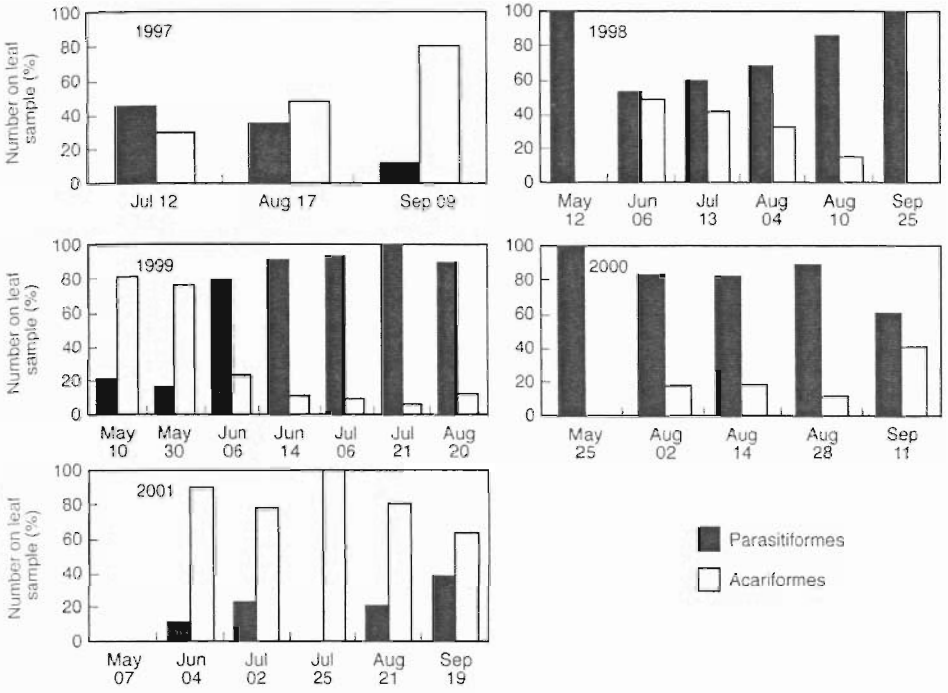

Fig. 1. Comparison of the numbers (%) of predatory mites from families *Parasitiformes* (mainly *Phytoseiidae*) and *Acariformes* (*Anystidae*, *Bdellidae*, *Cheyletidae*, *Cunaxidae*, *Tydeidae*) captured from strawberry leaf samples during the 1997 - 2001.

years (Fig. 1). The abundance of those mites varied on strawberry leaves depending on the age of strawberry plants, population densities of strawberry pests, meteorological conditions and the survival of predatory mites during the winter period. Species of the families Tydeidae, Anystidae, Cunaxidae, and Phytoseiidae were observed on leaves annually. Predatory mites of the remaining families were less frequent.

Phytoseiidae were the most abundant family, and had the highest number of species (12). All of them were found for the first time on strawberry leaves in Latvia. *Amblyseius barkeri* (Hug.) was recorded as a new species for the fauna of Latvia (Petrova et al. 2000c). The most frequent were *Amblyseius cucumeris* Oud., *A. herbarius* (Wainst.), *A. aurescens* A.-H., and *A. bicaudus* Wainst. The abundance of phytoseiid species varied among years and months. *A. cucumeris* was most abundant species in 1997, *A. aurescens* in 1998, and *A. bicaudus* in 1999. *A. cucumeris* and *A. aurescens* was most abundant species in 2000 (in equal proportions), *A. marginatus* (Wainst.) in 2001. In May (1998 - 1999) the most abundant were *A. herbarius* and *A cucumeris*, in June (1998 - 1999) – *A. aurescens*, and *A. cucumeris*, in July (1997 - 1999) – *A cucumeris* and *A. agrestis* (Karg), in August (1997 - 2000) – *Typhlodromus rademacheri* Dosse and *A. agrestis*.

Proctolaelaps bickleyi (Bram) (Aceosejidae), *Paragarmania mali* (Oud.) (Aceosejidae), *Alliphis siculus* Oud. (Eviphidae), *Macrocheles glaber* (Müll.) (Eviphidae), and *Poecilochirus necrophori* Vitzt. (Parasitidae) were recorded as less abundant species on strawberry leaves.

Acariformes mites were represented by Anystidae (1), Bdellidae (1) Tydeidae (2), and Cheyletidae (1). The dominant species was *Anystis baccarum* L. (Anystidae.). It was observed on strawberry foliage annually and the most abundantly in May and June. Tydeidae and Cunaxidae were observed annually on folded and unfolded strawberry leaves among phytophagous mite (*Tetranychus urticae* Koch, *Phytonemus pallidus* Banks) colonies. *Bdellodes longirostris* (Herm.) (Bdellidae) and *Cheletomorpha lepidopterorum* (Schaw) (Cheyletidae) were less abundant.

The predatory mite fauna of the small strawberry fields was represented by six species: *Amblyseius aurescens*, *A. cucumeris*, *A. herbarius*, *A. reductus* Wainst., *T. rademacheri* Dosse (Phytoseiidae), and *A. baccarum* (Anystidae). As rule, these agroecosystems had the least pest mite infestation and predatory mite occurrence (Table 2).

All species of Parasitiformes and Acariformes, with exception of *A. baccarum*, recorded on strawberry foliage were found on strawberries for the first time in Latvia.

Pit-fall traps

In total, 24 mite species were found during the period of pit-fall trapping (Table 1). The highest species diversity was found for Parasitidae (6) (Table 3). Of the Parasitiformes, the family Parasitidae had the highest number of species and the highest number of individuals, and was the most abundant in 2001. Parasitidae mites were found during the whole investigation period of 2001 with an increase in October. *Poecilochirus necrophori* Vitz. and *Parasitus lunaris* Berl. (Parasitidae) were the most abundant in 2001. *Eugamasus crassitarsis* (Hal.) was recorded for the first time in Latvia.

The second most diverse family was Aceosejidae (5 species). *Leioseius semiscissus* (Berl.) was the most abundant species. Aceosejidae were recorded in April, May, and June, but in April and May they were the most abundant.

The family Phytoseiidae took the third position in species richness – four species with dominance of *A. zwoelferi* and *A. aurescens*. The family Phytoseiidae was the most abundant in June.

Table 1. List of the predatory mites collected in strawberry agroecosystems in 1997 - 2001. *, collected from leaves; **, collected from pit-fall traps

Family, Species	1997*	1998*	1999*	2000*	2001*	2001**
Parasitiformes						
Aceosejidae						
Cheiroseius necorniger (Oud., 1903)						×
Leioseius bicolor (Berl., 1918)						×
Leioseius minutus (Hal., 1915)						×
Leioseius semiscissus (Berl., 1892)						×
Paragarmania mali (Oud.)		×				×
Proctolaelaps bickleyi (Bram, 1956)			×			
Ameroseiidae						
Epicriopsis horridus (Kram., 1876)						×
Antennoseiidae						
Antennoseius sp.						×

(continued)

Family, Species	1997*	1998*	1999*	2000*	2001*	2001**
Eviphidae						
Alliphis siculus (Oud., 1905)					×	×
Iphidosoma fimetarium (Mull., 1859)						×
Laelaptidae						
Laelaps agilis (C.L. Koch, 1836)						×
Hypoaspis lusisi (Lapina, 1976)						×
Macrochelidae						
Macrocheles glaber (Mull., 1860)					×	×
Parasitidae						
Eugamasus crassitarsis (Hal.)						×
Gamasodes spiniger (Trāg., 1910)						×
Parasitus lunaris (Berl., 1906)						×
Pergamasus vagabundus (Karg, 1968)						×
Pergamasus crassipes (L., 1758)						×
Poecilochirus necrophori (Vitzt., 1930)					×	×
Phytoseiidae						
Amblyseius agrestis (Karg, 1960)	×	×	×	×		
Amblyseius aurescens (A.-H., 1961)	×	×	×	×	×	×
Amblyseius barkeri (Hug., 1948)		×	×	×		
Amblyseius bicaudus (Wainst., 1962)	×	×	×	×		
Amblyseius cucumeris (Oud.,1933)	×	×	×	×	×	
Amblyseius herbarius (Wainst., 1960)	×	×	×	×	×	
Amblyseius marginatus (Wainst., 1961)					×	×
Amblyseius reductus (Wainst., 1962)	×					
Amblyseius zwoelferi (Dosse, 1957)	×	×			×	×
Anthoseius sp.					×	
Proprioseiopsis okanagensis (Chant, 1957)		×	×			
Typhlodromips rademacheri (Dosse, 1958)	×	×				
Veigaiaidae						
Gamasolaelaps excisus (C.L. Koch, 1879)						×
Acariformes						
Anystidae						
Anystis baccarum (L., 1758)	×	×	×	×	×	×
Bdellidae						
Bdellodes longirostris (Herm., 1804)			×			
Cheyletidae						
Cheletomorpha lepidopterorum (Schaw,1794)		×			×	×
Tydeidae	×	×	×	×	×	×
Tydeus californicus (Banks, 1904)		×				
Tydeus kochi (Oud., 1928)		×				
Cunaxidae		×	×	×	×	×

Table 2. Pest mite and predatory mite infestation and population density in the small strawberry fields

Location	Season	Area	Pest	Infestation (%) pest / predat.	Population density (No. ind. leaf^{-1}) pest / predat.
Rīga District	July 1997	0.02 ha	*T. urticae*	0	0
			P. pallidus	4.2 / 2.0	0.2 / 0.1
Limbaži District	July 1998	0.02 ha	*T. urticae*	6.8 / 3.1	2.4 / 0.1
			P. pallidus	12.5 / 4.4	9.7 / 0.3
Dobele District	July 1999	0.5 ha	*T. urticae*	15.2 / 2.7	1.3 / 0.1
			P. pallidus	0	0

The other families – Ameroseiidae, Antennoseiidae, Eviphidae, Laelaptidae, Macrochelidae, Veigaiaidae – were represented by one species.

Acariformes mites found in pit-fall traps were represented by two species: *A. baccarum* (Anystidae) and *Ch. lepidopterorum* (Cheyletidae). *A. baccarum* was the dominant species. *Ch. lepidopterorum* was found in July.

In total, 23 species of predatory mites from pit-fall traps were recorded for the first time in strawberry agroecosystems in Latvia.

Discussion

The present sampling in Latvian cultivated strawberry fields recorded 32 species of Parasitiformes and five species of Acariformes. Predatory Gamasina mites (Parasitiformes) are known from a wide range of habitats, mainly as free living and mobile predators, and they are often used as bioindicators (Karg 1993; Koehler 1999). The mite species recorded by the authors in strawberry fields are common inhabitants of Latvian agroecosystems (Kuznetsov, Petrov 1984; Lapina 1988; Petrova et al. 2000a,b; Salmane 2001). They are important predators of Nematoda, Acari, Collembola and insect larvae (Karg 1993; Koehler 1999), and they can influence the population growth of the other soil organisms. The predatory mites of Phytoseiidae, Anystidae, Bdellidae, Cheyletidae, Cunaxidae, Tydeidae (Parasitiformes, Acariformes) living on plants may efficiently control small arthropods like mites and thrips (Livschits, Mitrofanov 1975; Karg 1993; Koehler 1999). The Phytoseiidae mites are the most studied group, as they are effective and specialised predators on numerous plant pests (Salmane, Petrova 2002).

The Phytoseiidae community and dominance structure of species associated with cultivated strawberries varies among countries. In Russia, of 12 species, the dominant were *A. herbarius*, *A. reductus* Wainst., and *A. zwölferi* Dosse (Meshkov 1996). In Finland, among 12 species, dominant were *Anthoseius rhenanus* Oud. and *Amblyseius tenuis* (West.) (Tuovinen 1995). In South Sweden, 5 phytoseiid species (Steeghs et al. 1993) have been recorded. In Latvian strawberry fields, the phytoseiid fauna consisted of 15 species with dominance of *A. aurescens*, *A. bicaudus*, *A. cucumeris*, and *A. herbarius*.

According to the results of the authors and data from the literature, the Latvian predatory mite fauna of cultivated strawberry agroecosystems consists of 43 species

Table 3. The number of predatory mite species and specimens from families *Parasitiformes* and *Acariformes* collected in pit-fall traps in strawberry fields in 2001

Family	Number of species	Number of specimens	Specimens (%)
Parasitiformes			
Aceosejidae	5	14	6.0
Ameroseiidae	1	1	0.4
Antennoseiidae	1	1	0.4
Eviphidae	2	3	1.3
Laelaptidae	2	2	0.9
Macrochelidae	1	34	14.9
Eviphidae	2	3	1.3
Laelaptidae	2	2	0.9
Parasitidae	5	99	43.4
Phytoseiidae	4	16	7.0
Veigaiaidae	1	3	1.3
Acariformes			
Anystidae	1	54	23.7
Cheyletidae	1	1	0.4

from 27 genera and 14 families (Aceosejidae, Ameroseiidae, Antennoseidae, Anystidae, Bdellidae, Cheyletidae, Cunaxidae, Eviphidae, Laelaptidae, Macrochelidae, Parasitidae, Phytoseiidae, Tydeidae, and Veigaiaidae).

Thirty-six species of the predatory mites were recorded for the first time in strawberry agroecosystems. Two predatory mite species were new for the fauna of Latvia: *P. mali* (Aceosejidae) and *E. crassitarsis* (Parasitidae).

Phytoseiidae and Anystidae were the most abundant families. The dominant Phytoseiidae species were *A. cucumeris*, *A. aurescens*, *A. herbarius* and *A. bicaudus*; the dominant Anystidae species was *A. baccarum*.

In the material collected from the pit-fall traps the most abundant families were Parasitidae (43.4 %) and Anystidae (23.7 % from total). Four Gamasina species – *Poecilochirus necrophori* Vitz. (37.5 %), and *Parasitus lunaris* Berl. (23.5 %) (Parasitidae), *Macrocheles glaber* Müll. (11.4 %) (Macrochelidae) and *Leioseius semiscissus* Berl. (5.4 % from total) (Aceosejidae) – were the most abundant species.

Acknowledgements

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Plēsīgo ērču (Acari, Parasitiformes: Mesostigmata (Gamasina); Acariformes: Prostigmata) fauna zemeņu stādījumos

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Kopsavilkums

Plēsīgo ērču (Acari, Parasitiformes, Acariformes) pētījumi (1997 - 2001) tika veikti kultivēto zemeņu agrocenozēs Latvijā. Materiālu ievāca ar zemeņu lapām un augsnes lamatām. Pavisam konstatētas 37 plēsīgo ērču sugas, no tām 36 sugas zemeņu stādījumos atrastas pirmo reizi Latvijā. Divas Gamasina (Parasitiformes) sugas *Paragarmania mali* (Oud.) un *Eugamasus crassitarsis* (Hal.) (Parasitidae) ir jaunas sugas Latvijas faunā. Visvairāk ērču uz zemeņu lapām tika atrasts no sekojošām dzimtām (sugām): Phytoseiidae (*Amblyseius cucumeris* Oud., *A. aurescens* A.-H., *A. herbarius* Wainst., *A. bicaudus* Wainst.) un Anystidae (*Anystis baccarum* L.). Ar augsnes lamatām ievāktajos paraugos visvairāk ērču tika atrasts no dzimtām Parasitidae (*Poecilochirus necrophori* Vitz., *Parasitus lunaris* Berl.), Aceosejidae (*Leioseius semiscissus* Berl.) un Macrochelidae (*Macrocheles glaber* Müll).

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Origin of globins and a mystery of myoglobin codon root symmetry

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Abstract

The amino acid sequence 49-94 of myoglobin involved in heam binding has 74 % symmetric codon roots – the second codon letters. The search for the reason of the symmetry revealed that the ancestor of globin genes was not formed by exon shuffling, but by multiplication of a 21-membered nucleotide and that it was a regular polynucleotide. The repeat unit pile of contemporary myoglobin codon root sequence posesses an inversion centre indicating that symmetry of codon roots evolved after the origin of globin gene. Its cause and possible biological functions is a mystery. A model of coding of the globin ancestor gene is suggested to be formed of identical repeat units separated by 23 introns.

Key words: multiplication of nucleotides, old ancestral introns, origin of introns, repeat units of genes and proteins.

Introduction

The primary structures of angiotensin and bradykinin contain some symmetrically located amino acids posessing certain common physico-chemical and structural features. It is supposed that the partially symmetric structures of these tissue hormones evolved for optimal adaptation to cell receptor binding sites (Beddell et al. 1977). How widespread is the internal symmetry of peptide chains and what is it true functional significance are unknown.

The physico-chemical properties of amino acids correlate with the structures of amino acid codon roots – the second codon letters (Pelc 1965; Sjostrom, Wold 1985). During evolution, codon root structures are much more conservative and undergo less changes than amino acids. Complementarity of the codon roots A/T (U) and G/C determine the polar component of coded amino acid inter- and intramolecular interaction during protein folding and complexformation reactions (Chipens 1996). The codon root plays the most important role in the codon; its substitution is the most critical for protein structure and biological functions. Therefore, to study protein and gene internal symmetry we chose the method termed comparative amino acid codon root analysis (CAACRA; Chipens, Ievina 1994), and elaborated a new screening test – backward (reverse or invert) autoscanning of protein and gene sequences. This simple method revealed covered symmetry of myoglobin primary structure: in the middle part of the peptide chain, amino acids encoded by codons with identical codon roots (termed "common-root" amino acids) are located

symmetrically. Search for the causes of this phenomenon led to a new theory of genes and intron emergence (Ievina, Chipens 2003), but the functions of myoglobin codon root dyad symmetry is still a mystery.

Materials and methods

In accordance with the method of backward autoscanning, identical symbols (amino acids and/or codon roots) are counted and registered graphically or in a form of a table where the amino acid sequence of a peptide chain or a chain of gene exons (codon root sequence) is moved backward over themselves step-by-step i.e. symbol-by-symbol. At each step the overlapping symbols are compared, and identical symbols are counted and registered (Fig. 1A, B). The position of the symmetry axis shows the ordinal number of the step with the highest number of identical symbols.

The primary structures of genes and proteins were taken from the GenBank via Internet.

Results and discussion

Cover symmetry of myoglobin

The backward autoscanning graph of the sperm whale myoglobin central fragment His 49 - His 94 (since the main object of our studies is the regularity of gene and protein structures, we enumerate also myoglobin methionine in position 1) has a well expressed

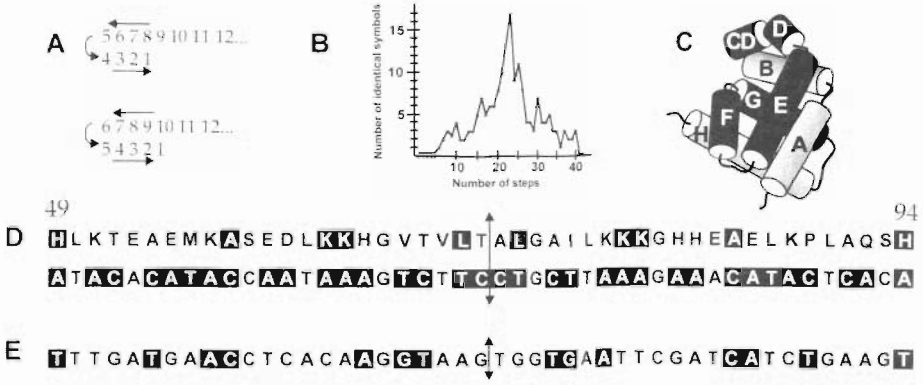

Fig. 1. Analysis of the sperm whale myoglobin codon root symmetry. A, principle of backward autoscanning shown by two steps of analysis (the step 4 and 5) of a number sequence. Arrows indicate direction of chain movement. At each step opposite numbers (i.e. corresponding symbols of amino acids and/or their codon roots) are compared and the identical are counted and registered when a chain is moved backward over itself step-by-step, i.e. symbol by symbol. Parameters are correlated graphically with the ordinal numbers of steps. Maximums show local or basic positions of symmetry axes. B, a backward autoscanning graph of myoglobin fragment 49-94. C, location of amino acids having symmetric codon roots in myoglobin 3D-structure (shown by black colour). D, symmetric disposition of amino acids and amino acid codon roots (the second letters) revealed by analysis of sperm whale myoglobin fragment 49-94. Symmetric symbols are shown against a black background. Two head arrow denote basic symmetry axis. E, accidental positions of symmetric codon roots in randomized sequence of myoglobin 49-94.

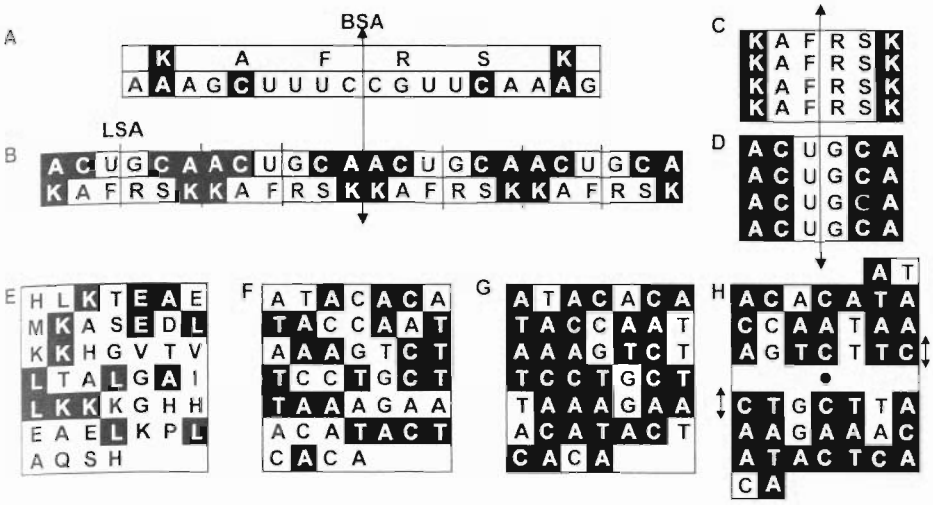

Fig. 2. Repeat unit piles (RUP) of a model peptide and the central fragment of the sperm whale myoglobin 49-94. A, a model of a symmetric repeat unit containing four symmetric codon roots and two amino acids shown against a black background. B, a multimer of the model "A" repeat unit. A small part of a long multimer chain is shown containing four repeats. The local symmetry axes (LSA) crosses repeat unit boundaries as well as repeat unit centres. Symmetric symbols are shown against a black background. BSA, the basic symmetry axis. C, D, the model multimer of repeat unit piles (6RUP) of amino acid and codon root sequences are bilateraly symmetric (symmetric symbols are shown against a black background. E, F, repeat unit piles (7RUP) of amino acid and codon root sequences of the myoglobin central part 49-94 are not symmetric. Identical RUP symbols in vertical lines are shown against a black background. G, Symbols of myoglobin 49-94 codon roots which are mirror-symmetric in linear structure (Fig. 1D) are not mirror symmetric in 7RUP structure . H, the same structure of 7RUP as in "G" subdivided into two parts in accordance to the position of the basic symmetry axis (shown by small two-headed arrows between codon roots C (Thr71) and C (Ala72), see also Fig.1D) reveal symmetric structure with an inversion centre (shown as filled circle).

maximum (Fig. 1B) which corresponds to the basic symmetry axis (BSA) crossing the peptide chain between Thr 71 and Ala 72 (Fig. 1D). The analysed myoglobin fragment contains 74 % common-root amino acids (Table 1), among them 22 % identical amino acids. In the 3D-structure of myoglobin (Fig. 1C), this fragment forms α-helices D, E, F and G (partially) and takes part in haem binding (Kendrew et al. 1960). Accidental amino acid sequences with the same amino acid composition, generated by the repeated Monte Carlo simulation (Dorit et al. 1990) resulted in only 30 % symmetric codon roots (the highest value, obtained from 20 separate simulations; Fig. 1E). Thus, the symmetry of myoglobin codon root symbols is not accidental and it is interesting to understand how it arose.

Modelling experiments reveal that long symmetric polynucleotide chains can be formed by multiplication of oligonucleotides. If the nucleotide contains some symmetric elements (e.g. codon roots; Fig. 2A), then these elements after multiplication are distrubuted symmetrically along the full length of the polynucleotide chain, which in such case have many local symmetry axes (LSA) – between every two repeat units or

Table 1. Groups of potentially equifunctional, tantamount or related common-root amino acids

Group name	Codon structure	Group composition
Adenine (A) group	NAN	D, E, H, N, Q, Y, K
Uracil (U) group	NUN	F, L, M, V, I
Cytosine (C) group	NCN	T, A, P, S
Guanine (G) group	NGN	C, G, R, S, W

between repeat unit centres – and one as the main or basic symmetry axis (BSA) through the geometric centre of the chain (Fig. 2B). Discovery of myoglobin covert symmetry and modelling experiments of nucleic acid formation by oligonucleotide multiplication reactions indirectly pointed to the possibility that periodic nucleic acids could be a raw material for emergence of living mater (ancestors of genes) and that multiplication of nucleotides may be a universal common mechanism of genes origin. This concept has been generally accepted in our later studies (Ievina, Chipens 2003). However, it is necessary to note that covert symmetry of myoglobin represents a unique case, because it arose during evolution after formation of the globin gene ancestor by multiplication reactions of nucleotides.

A repeat unit of globins

For investigation of myoglobin codon root symmetry, it was necessary to determine exact size of the globin repeat unit. For this purpose we use the method of common prime multipliers of gene numerical parameters, such as exon dimensions, intron co-ordinates, size of the gene coding part, etc. measured by numbers of nucleotides. Prime multipliers characterize the internal regularity of the numbers themselves. If the globin gene family has common ancestor, members of the family must have identical size of repeat units. The identity of repeat dimensions in turn must be reflected in exon sizes in cases when exon size and intron co-ordinates numerical values are determined by a whole number of repeats. In the given case we use the dimensions of three globin gene exons selected from a number of different globin exons, i.e. the human β-globin exon 3 (126nt), the soybean *Glycine max* symbiotic globin exon 2 (105nt) and the protozoa *Paramecium caudatum* β-globin exon 1 (189nt). The dimensions of all these exons have common internal regularity. The product of common prime multipliers (3 × 7, framed):

$$
\begin{array}{lllllll}
105 & = & & \boxed{3} & \times 5 \times & \boxed{7} & = 5 \times 21 \\
126 & = & 2\times & \boxed{3} & \times 3 \times & \boxed{7} & = 6 \times 21 \\
189 & = & 3\times & \boxed{3} & \times 3 \times & \boxed{7} & = 9 \times 21
\end{array}
$$

indicates that the potential size of the globin primary repeat unit is 7RU or 21nt/7aa. The number before the abbreviation of repeat unit (RU) indicates its size – the number of amino acids (aa) or codon roots in the repeat. Symbols 7RU and 21nt/7aa are equivalent.

In accordance to our new model of gene origin (Ievina, Chipens 2003), immediately after emergence of gene precursors, exons and introns were formed of repeat units (RU) that were identical in size and sequence. The coding parts of genes contained a whole number of RU, but introns possibly were located after every RU or micro-exon in the borders of repeats, termed the gene knot points. It is necessary to note that multiplication reactions from only symmetric exons with all introns in phase zero, but the real structures of contemporary genes indicate that during evolution introns can change their positions

and phases and be partially or completely eliminated.

Intron positions in modern globin genes more or less correspond to the calculated size of the primary RU. Multiple alignement of 91 globins and globin-related proteins reveal 12 intron positions that are represented in seven genomic sequences. Among these, the soybean globin intron 71-0 (Stoltzfus et al. 1994) with the co-ordinates 210nt (intron co-ordinate is a nucleotide ordinal number of exon row just befor the intron) crosses the globin gene knot point and is a real candidate for an ancestral intron still sitting in the birth position (old "immobile" introns, OII). Multiple alignment of globins is a difficult problem because their sequences are very diverse and they can not be aligned reliably (Stoltzfus et al. 1994). More precise data can be obtained by comparison of the relative positions of introns in the frames of separate α-helices of globins. For example, the distance between introns B5/B6 and B12-2 is 20nt, between F3-1 and F9/10 – 21nt, between F8-1 and E14/E15 – 20nt (Hankeln et al. 1997). In these cases introns are separated by one repeat (21nt) with small deviations determined only by a intron phase change during evolution. This indicates the possibility that introns of the globin gene ancestor indeed were situated after every RU (a micro-exon) and that during evolution a massive loss of introns took place. Interestingly, the exons of several globin genes are formed of a whole number of repeats, which supports the above thesis, e.g., the 3'-terminal exons of myoglobin (the third exon, 49 codons, 7×7 RU) and soybean symbiotic globin C_2 (the fourth exon, 42 codons, 6×7 RU), the third exon of soybean globin C_2 (35 codons, 5×7 RU), the first exon of *Paramecium caudatum* β-globin (63 codons, 9×7 RU), etc.

The comparison of symmetric codon root sequences of model peptides formed of symmetric repeats (Fig. 2A, B) with the symmetric myoglobin codon root sequence (Fig. 1D) reveal some differences in the pattern of symmetric symbols. In a model of a repeat unit multimer, each repeat is symmetric and symmetric elements are repeated regularly. In myoglobin, codon root sequence does not possess such a regularity – the myoglobin sequence as a whole is symmetric. The difference is well seen in the form of repeat unit piles (RUP). For this purpose, peptide chains are cut into fragments corresponding to the repeat unit size and then are laid out horizontally in stacks to form RUP. Repeat unit piles (Fig. 2C, D) formed of a multimer chain containing symmetric repeats (Fig. 2B) are symmetric also relative to the symmetry axis going through the middle of the pile. In contrast to this, the 7RUP structure formed of the central part of sperm whale myoglobin is not mirror- or bilateraly symmetric, it does not contain isosteric symmetric amino acids or amino acid codon roots (Fig. 2E, F). This is better seen when codon root symbols, which are mirror-symmetric in a linear myoglobin codon root sequence (Fig. 1D), are shown in the 7RUP structure against a black background (Fig. 2G). Transformation of this pile structure by subdividing it into two parts (in accordance with the position of BSA) reveals a RUP possesing an inversion centre (Fig. 2H). At the inversion centre,any two points can interchange (in our case – any two symbols shown against the black background, because the codon root symmetry is not complete, but only 74 %) located at the same distance from the inversion centre along the line going through the centre. Thus, the highly expressed symmetry of myoglobin codon roots in the sequence of the central part of the chain (Fig. 1D) is not a direct result of chain formation by the multiplication reaction of a mirror-symmetric repeat, but is a consequence of evolution of the myoglobin sequence after the emergence of the gene ancestor. If the globin gene ancestor repeat unit itself and the corresponding product of multiplication has been bilaterally symmetric,

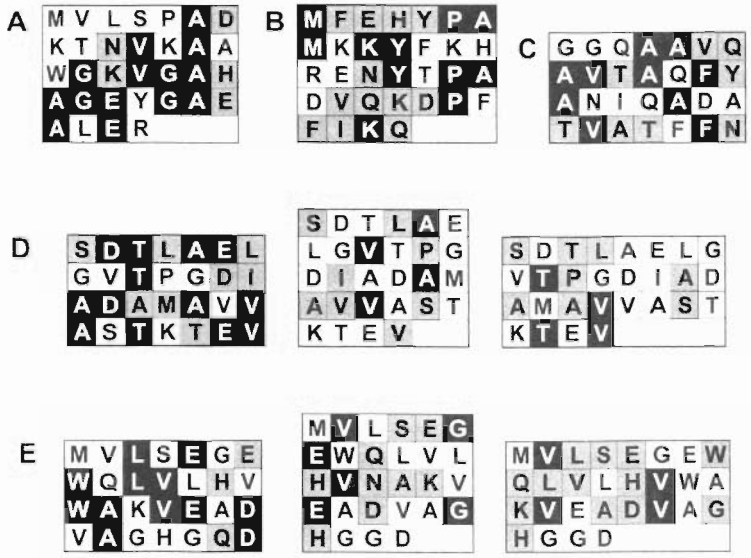

Fig. 3. Repeat unit piles 7RUP of different globin fragments. A, the human α-globin 1-32 (f = 59/44); B, the *Pseudoterranova decipiens* globin 51-82 (f = 69/34); C, the *Paramecium caudatum* globin 8-35 (f = 64/36); D, the *Chlamydomonas eugametos* globin 131-158 (7RUP f = 75/46; 6RUP f = 43/14; 8RUP f = 43/14); E, the sperm whale myoglobin 1-28 (7RUP f = 53/43; 6RUP f = 53/21; 8RUP f = 53/14). Identical symbols of amino acids in vertical lines are shown against a black background, and common-root amino acids – against a grey background.

then during evolution this symmetry was disrupted and excahnged by symmetry with the inversion centre. The driving force for this change may have been formation of structural peculiarities necessary to ensure some possible yet unknown important functional mechanism of myoglobin. In any case, if the structure of a protein is not necessary for a specific function, during evolution it is gradually disrupted by mutations.

Some parts of myoglobin and other primary structures of globin (Fig. 3) however have retained rudimentary regularity characteristic for ancestor globin repeat units containing seven symbols (codon roots or common-root amino acids). The regularity of repeat unit pile (RUP) structure may be characterized as the percentage ratio of common-root (CR) and identical (I) amino acids dominating in the vertical lines of RUP. This ratio we show as a fraction and it is termed the f-factor, f = CR/I (%). The f-factor usually is sensitive to small RU size changes, e.g., by one symbol. The Chlamydomonas eugametos globin sequence 131-158 in the form of 7RUP shows an f = 75/46 (there is a very small possibility to form such a high regularity by chance), but only f = 43/14 in the form of 6RUP or 8RUP (Fig. 3D). RUPs of myoglobin sequence 1-28 also show similar regularity changes (Fig. 3E).

A model of the globin gene organisation

The framework of gene structural organisation is determined by the dimension of RU and disposition of the gene knot points, which are formed by borderlines of RU. In the globin exon row, the knot points are probably situated after each 21nt, and they indicate

the potential positions of the splice sites of introns as well as the potential positions of local (including basic) symmetry axes. Theoretically, the basic symmetry axis (BSA) in the ancestor gene structure must cross the knot point in the centre of chain and coincide with the central intron, or this intron potential position if the intron itself has been lost during evolution. The psotion of myoglobin BSA determined by backward autoscanning (Fig. 1A, B) does not cross the middle point of the myoglobin peptide chain as a result of truncation during evolution of the gene 5'-terminus encoding signal peptide. Contrary to this, the COOH-terminal half of myoglobin peptide chain does not contain indels, because the size of the exon 3 is 49 codons (including the termination codon), that is 7×7RU, but the distance from the position of BSA to the 3'-terminus of the gene coding part also contains a whole number of 7RU, i.e. 84 codons (12×7) or 252nt (12×21 nt; Fig. 4C). If our concept of nucleotide multiplication reactions is correct, then the length of the globin ancestor gene coding part was 84×2 = 168 codons or 504nt (24×21 nt). We suppose that in this way we are virtuallu measuring the coding part of gene existing before divergence of plants and animals about 1,500 millions years ago (Anderson et al. 1996; Hardison 1996).

The calculated theoretical length of the globin gene ancestor coding part allows to design a model of globin structural organisation. The nucleotide chain containing 504nt

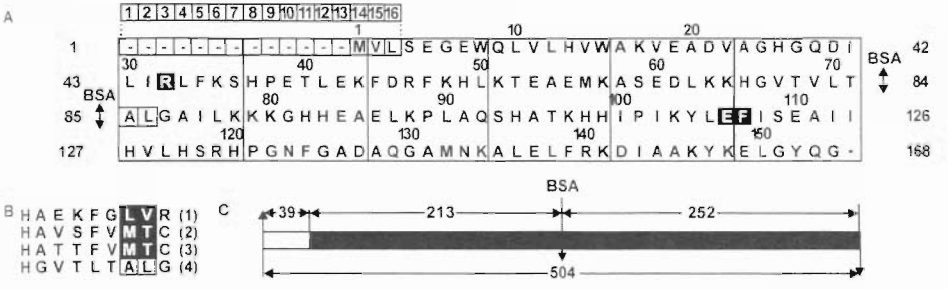

Fig. 4. A model of structural organisation of the globin family protein ancestor. A, the sequence of sperm whale myoglobin is written in a frame of 7-membered repeat units grouped in four lines in such a way that the symmetry axis BSA of myoglobin coincide, with the geometric symmetry axis of the linear 168-membered peptide chain. The BSA is shown as two headed arrows between amino acid symbols in positions 84 and 85. The amino acid sequence has double numeration: in accordance to the model of globin ancestor structure (numbers in both flanks of lines) and natural myoglobin (above the symbols). The myoglobin intron N.1 in the phase 2 crossess the arginine 32, but intron N.2 crosses separate codons 106/107. Corresponding amino acid symbols are shown against a black background. The position of the central intron (lost in myoglobin structure) is between alanine 85 and leucine 86 (symbols are framed). The potential position of the myoglobin ancestor signal sequence 1-16 is shown separately (cells with numbers). An asterisk denotes the termination codon (position 168). B, determination of a potential central intron position in myoglobin by alignment of the E-helices of globins: the symbiotic *Glycine max* globin C2 62-70 (1); the *Glycine max* nonsymbiotic globin 69-77 (2); the *Parasponia andersonii* globin 70-78 (3), and myoglobin 65-73 (4). Intron positions (all in the phase-0) separating codons of corresponding amino acids are marked by a black colour. C, a sheme illustrating the determination of globin ancestor dimension using the revealed (Fig. 1) co-ordinates of the basic symmetry axis, BSA. Numbers show length, measured by numbers of nucleotides. The disposition of the sperm whale myoglobin sequence coding part is shown in black.

can be formed by 24 primary repeats 7RU (21nt/7aa). The calculated size of globin peptide chain encoded by 168 codons (504nt) accounts for 157 amino acids and a stop codon and is comparable with the size of natural globin peptide chains, e.g. the plant *Parasponia andersonii* globin (162aa), the midge *Chironomus thummi* globin encoded by the gene *ctt-XI* (167aa), the nematode *Ascaris* and *Pseudoterranova decipiens* globins (the first domain – 167aa) and others.

Writing of the sequence of the sperm whale myoglobin in the structural frames of the above described model in such a way that the basic symmetry axis BSA of myoglobin (between Thr71 and Ala72; Fig. 1) coincides with the geometric symmetry axis of the model (crossing the centre of the chain between codons 84/85) reveals an interesting picture (Fig. 4A): (i) the termination (stop) codon of the myoglobin gene coding part takes the extreme position of the model corresponding to the codon 168 (in accordance to our symmetry analysis and calculation data); (ii) the second (central) intron of the globin gene family is in a close neighbour position to myoglobin BSA. The central intron of myoglobin has been lost during evolution, but the alignment of myoglobin and plant globin sequences in the region of E-helices (Fig. 4B) indicates that the central intron of plant globin is only 3nt aside from myoglobin BSA; (iii) in the N-terminal part of the model there is a place for the globin signal peptide containing 16aa (Andersson et al. 1996). We suppose that the leader sequence of globins during evolution has been lost later (as in a case of myoglobin) or changed by mutations (as in a case of plant globins).

Up to 55 % of the mammalian genome consists of different kinds of repetitive and regular nucleic acids (satellites, mini- and micro-satellites, short and long interspersed elements, etc.; Cavalier-Smith 1985). The remaining half of non-repetitive DNA in several until up to now analysed genes after translation to codon root sequences also show residual regularity (Ievina, Chipens 2003). Consequently, possibly there is only one basic mechanism of nucleic acid (including the genome DNA) formation, in good accordance with the concept of biochemical universiality (Dayhoff 1972).

The discovery and study of internal covert symmetry and regularity of myoglobin for the first time bring to light the true mechanism of emergence of the very first gene ancestors by multiplication of nucleotides.

According to the exon theory of genes (Crick 1979; Gilbert 1987) the DNA sequence that codes for the globin ancestor did not begin to evolve as a single uninterrupted strech of DNA. Instead, it evolved from three distinct exons which already existed (evolved earlier) and were brought together in the genome by random shuffling. However, (i) the identical size of repeats of globin exons i.e. 7RU or 21nt/7aa; (ii) the identical internal regularity of several globin exons e.g. 5×7RU, 6×7RU, and 7×7RU; and (iii) the same regularity of soybean symbiotic globin C_2 "old immobile" intron co-ordinate (210 nt or 10×7RU) in our view do not support exon shuffling in this case.

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Globīnu izcelšanās un mioglobīna kodona sakņu simetrijas noslēpums

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Kopsavilkums

Mioglobīna fragmentam, kas piedalās hēma saistīšanā, 74 % aminoskābju sekvences 49-94 kodē nukleotīdu tripleti, kuru kodonu saknes (otrie burti) ir simetriskas. Simetrijas cēloņu meklējumi ļāva secināt, ka globīna gēnu priekštecis neveidojās eksonu pārneses rezultātā, bet bija 21 locekļa nukleotīda multiplicēšanās galaprodukts – regulārs polinukleotīds. Mūsdienu mioglobīna kodona sakņu sekvences atkārtojuma vienību grēdai ir inversijas centrs, kas norāda, ka kodonu sakņu simetrija attīstījusies pēc globīnu gēnu rašanās. Simetrijas cēlonis un iespējamās bioloģiskās funkcijas pagaidām nav zināmas. Ir izveidots hipotētisks globīna gēnu priekšteča kodējošās daļas modelis, kas sastāv no identiskām atkārtojuma vienībām, kuras atdala 23 introni.

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Elevated artificial nest sites for Mallard *Anas platyrhynchos* in Latvia

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Abstract

The use of elevated covered nest sites for Mallard *Anas platyrhynchos* was studied in 1999 - 2003 on coastal lakes and ponds of different origin. Within the 5-year period, a total of 723 checks of nest sites were made. Nest site occupancy by Mallard was much higher on ponds (46.8 %) than on large lakes (3.3 %). Occupancy of nest sites increased by years, likely due to increased experience by both managers and ducks. Nest sites appearing more natural seemed to be preferred by ducks. No significant difference was found in occupancy between two-entrance nest sites (hay cylinders, wooden boxes) and one-entrance nest sites (53.4 % and 50.0 %, respectively) in 2003. Nesting success within a pond was higher in elevated nest sites with predator guards (87.7%) than in those without them (67.6 %). In the nest sites with predator guards nesting success averaged as high as 89.4 % (all locations combined) which is much higher than recorded in natural nests during the same period (L. Engure – 23.3 %, L. Kanieris – 54.3 %, ponds – 25.0 %). In the nest sites with predator guards, 7.6 % were abandoned nests and 2.9 % were predated (only American mink *Mustela vison*). No predation by Marsh Harrier *Circus aeruginosus*, Hooded Crow *Corvus corone cornix* and Raven *Corvus corax* was observed in the elevated nests. The best results were achieved in the fifth year: 55 nest sites, 35 occupied, and 27 successfully hatched in a 10 ha pond.

Key words: American mink, artificial nest sites, Latvia, Mallard, predators, nesting success.

Introduction

The decline of waterfowl populations in at least several hundreds of years has been documented in the Northern Hemisphere. It is widely agreed that the main cause of the decline is habitat loss for breeding, moulting, staging during migration and wintering but it is clear that also other factors such as human-caused mortality due to overshooting, lead-poisoning, oil-spills and by-catch have played a certain role. Recently, many studies of ducks have recorded very low nesting success below the threshold level (15 - 20 %) believed necessary to maintain a population (Covardin et al. 1985; Klett et al. 1988).

Breeding populations of ducks have declined also in Latvia, especially during recent decades. The decline has affected nearly all species including also the Mallard *Anas platyrhynchos*, the most common duck species, which was considered stable about 10 or 20 years ago (Strazds et al. 1994). Recently, in Lake Engure, where in the early 1990s the breeding population of Mallard was estimated as 1200 pairs (Blums et al. 1993), it decreased to 500 pairs in 1999 (Viksne et al. 2000). During the same time, the nesting success declined from 71 % in the 1989 - 1993 period to 48 % in 1994 - 1998, to 23 % in

1999 - 2003, and was even as low as 10 % in 2002. According to our observations during the 1999 - 2003 period the nesting success of Mallard was very low also on ponds – about 25 %, and was comparatively higher (52.3 %) only in some places as Lake Kanieris.

The low nesting success of Mallard and other ducks is caused by heavy nest predation. Besides the traditional nest predators such as Marsh Harrier *Circus aeruginosus*, Hooded Crow *Corvus corone cornix* and Raven *Corvus corax*, which are common depredators of Mallard nests on ponds and lakes in Latvia (Vīksne et al. 2000), American mink *Mustela vison* became a new alien mammalian predator in the 1970s. This species was widely reared in farms for fur, and escaped specimens established a wild population exceeding 19065 individuals in 2003 (unpublished data by the State Forest Service of Latvia; the real number is believed to be much higher). American mink predates duck nests destroying clutches and killing incubating females. Although Mallard suffers less from mink predation in comparison with some other duck species, in specific habitats such as mats of emergent vegetation and small islets, nest loss due to mink predation is quite significant. According to our observations in 1999 - 2003 on ponds, at least 70 % of the total Mallard nest predation could be accounted for by American mink.

The idea to protect Mallard nests from predators by mounting elevated artificial nest sites is known at least since the 17th century (Eley Game Advisory Station 1969, cit. by Doty et al. 1975) and since then such attempts (less often regular practice) have appeared in different countries in Europe (Bjärnvall 1970; Majewski, Beszterda 1990). Elevated nest sites have been widely used in North America since the 1960s, where they gradually developed from open nest baskets (protect nests from climbing mammals) to covered structures which quite effectively protect duck nests also from avian predators (Bishop, Barrat 1970; Doty 1979; Haworth, Higgins 1993; Eskowich et al. 1998). Good occupancy of these artificial nesting structures by Mallard and its high nesting success stimulated us to launch the present study, the purpose of which was:

(i) to test different types of covered artificial nest sites for suitability for Mallard nesting in Latvia, in relation to the wetland;

(ii) to establish artificial nest sites for Mallard, which are optimal in our conditions considering the availability of materials, costs, occupancy and duck nesting success;

(iii) to promote as wide as possible implementation of artificial nest sites in Latvian wetlands to protect nesting Mallards from avian and mammalian predation.

Materials and methods

The study was carried out in 1999 - 2003 on different Latvian wetlands, including shallow freshwater coastal lakes and ponds of different origin. In the coastal lakes Engure (41 km^2) and Kanieris (11.4 km^2), which are well known bird lakes, Ramsar sites since 1995, both rich in emergent vegetation and islets, breeding populations of Mallard in 1999 - 2003 were estimated correspondingly as 500 and 300 pairs (Vīksne et al. 2000). More detailed descriptions of these lakes are available in other publications (Vīksne 1997; Vīksne et al. 2000). The strongly overgrown Lake Sarnate (ca. 1.5 km^2; Ventspils region) was lowered in 1970s, then restored in 2001 but is still almost completely covered with continuous reed.

Ponds used for experiments with artificial nest sites were: specially created by hunters mostly for waterfowl, fish-ponds abandoned long ago and recently partly covered with

emergent vegetation, and Beaver *Castor fiber* ponds. In some cases nest sites were erected on fish-ponds used also recently for intensive fish-breeding. The size of ponds averaged 12.2 ha (4 - 30). Unfortunately, we have no information about the number of nesting Mallards on the ponds before erection of the artificial nest sites. As a rule, single or some pairs of Mallards attempted to nest there also before, except perhaps in some newly created ponds.

As no special funding was obtained for this study in 1999 - 2002, we attempted to involve volunteers – landowners and hunters. Beginning in January 1999, articles advertising mink-safe nest sites for Mallard were published in the Latvian hunters' journal MMD (e.g. Vīksne 1999; 2000). Appeals were made to hunters to try different types of artificial nest sites and they were requested to inform the authors on the wetland location, number and type of nest sites, the number of occupied and the number of successful clutches. After receiving the above information nearly all of the locations were visited by the authors, the nest sites were checked repeatedly, and recommendations were given to owners regarding the construction and placement of nest sites, identification of successfully hatched and predated nests, etc. In 2003, information was obtained about 27 wetlands in which no less than 350 artificial nest sites were erected. However, the number of both localities and nest sites erected was higher – many hunters who erected several nest sites did not inform the authors.

In 1999 - 2003, a total of 723 checks were made of available nest sites. Several types of artificial nest sites were used (Table 1, Fig. 1): (i) two-entrance (Fig. 1A, 1B, 1C, 1D) and (ii) one-entrance nest sites (1E), both mounted on wooden poles supplied with predator guards, and (iii) nests made on black alder *Alnus glutinosa* stumps (1F) which were impossible to supplement with predator guards. Hay cylinders, which were widely used in North America, served as a prototype for two-entrance nest sites (Doty 1979; Lewis 1998), both according to materials and dimensions (welded wire fencing, fine hay, roofing felt, fine hay; 80 cm long and 30 cm in diameter). About 20 % of hay cylinders were fashioned differently – with a wooden floor and an arched hay roof. Both from the inside and outside they were similar to the hay cylinder, and therefore both these types were combined for analysis. Other two-entrance nest sites followed the same idea – to give a chance for incubating duck to escape predator which could appear at either entrance. They differed only according to the external material – wood, their dimensions

Table 1. Numbers of available artificial nest sites of different types in Latvia, 1999 - 2003

Type of nest site	Available	Designation on Fig. 1
Hay cylinders	301	A
Wooden boxes	82	B
Hollowed round timbers	12	C
Wigwams	11	D
Two-entrance nest sites on poles, total	*406*	
One-entrance nest sites on poles	260	E
Nest sites on poles, total	*666*	
Stump nests	57	F
Total	*723*	

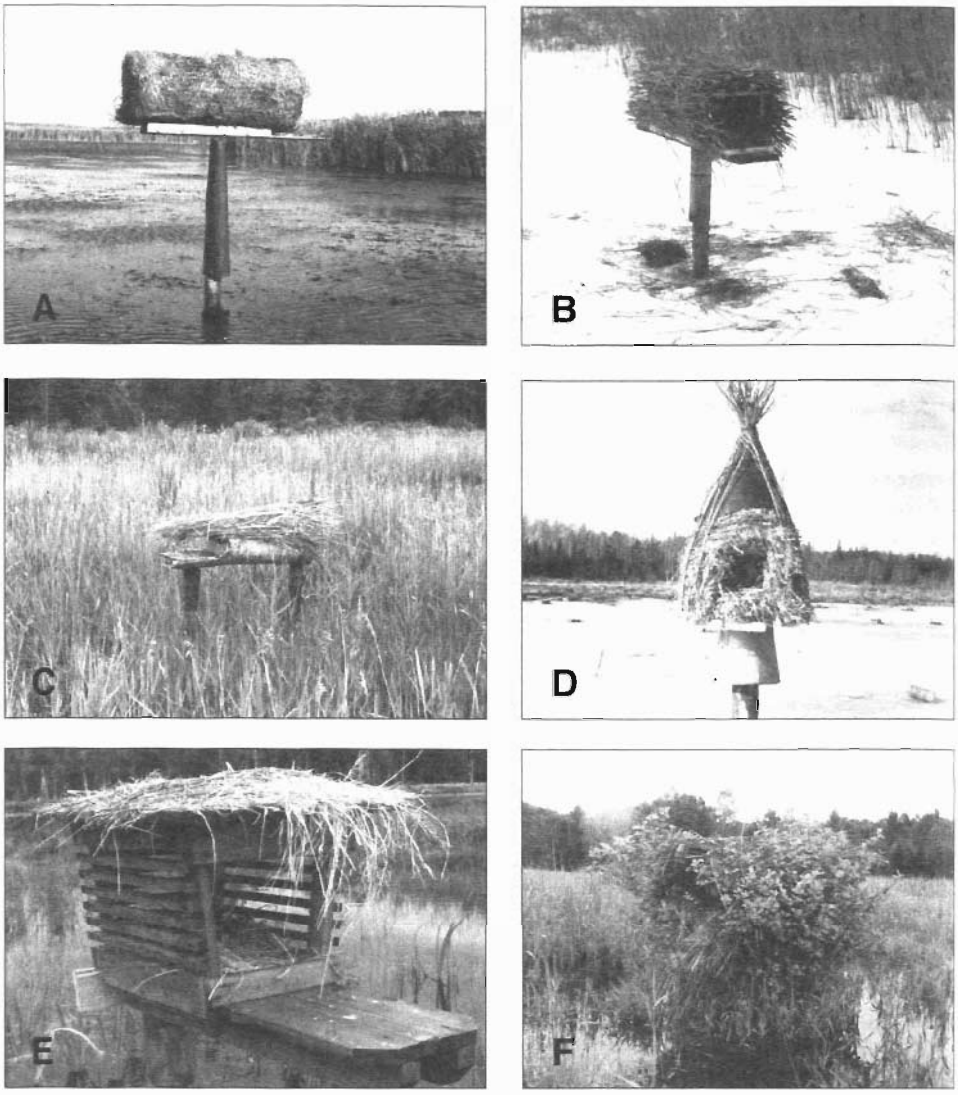

Fig. 1. Types of artificial nest sites used: A, hay cylinder; B, wooden box; C, hollowed round timber; D, wigwam; E, one-entrance nest site; F, stump nest.

were similar to that of hay cylinders (wooden boxes – 80-cm-long, ca. 28-cm-wide and high; hollowed round timber – 80 cm long, inside diameter 30 cm). Wigwams had a wooden floor 50 × 50 cm, two entrances and cone-shape roof consisting of shrub stems, hay, roofing felt, hay and stems. The same materials were used to cover stump nests. One-entrance nest sites (Wandel 1994) had an inside floor 50 × 30 cm, inside ceiling 50 × 45 - 50 cm, 35 cm high, racky walls and a 50-cm long landing platform ahead of entrance.

Beginning with 2000, two-entrance nest sites were supplemented with ca. 20-cm long and 15 - 20 cm wide wooden landing platforms in front at one or both entrances to facilitate exploring of the nest site by a female. Wooden boxes and hollowed round timber

nest sites, in recent years also hay cylinders, as a rule were covered with reed forming a roof over the landing platforms. Side walls of wooden boxes usually were also covered with reed. The purpose of this was to give wooden nest sites a more natural appearance and to make the landing of Marsh Harrier on the platform in front of the entrance more difficult.

All nest sites were mounted on wooden poles 0.6 - 1.0 m above water, iron pipes were used only in some places. Poles as a rule were supplied with "predator guards" – mostly tin-plate (iron or aluminium) tubes or narrow cones, about 60 cm high which should prevent mink climbing. Nest sites were erected usually on open water, most often 5 – 10 m from the edge of emergent vegetation (usually common reed *Phragmites australis* or cattail *Typha* sp.). Area covered with continuous emergent plants which lacked a hard stem above water (e.g. floating sweet-grass *Gliceria fluitans*), were also used for artificial nest sites.

As a rule we avoided disturbing incubating ducks and checked nest sites when nesting season was over. Used nests (hay with down and egg shells) were removed and replaced with fresh hay or last years grass before every breeding season.

Due to the great number of inexperienced persons involved in making and erecting nest sites, the quality of nest sites varied widely. This raises difficulty in evaluating the occupancy rate of the nest sites. Therefore, we attempted to exclude from analysis nest sites which obviously were not suitable for nesting due to technical defects (usually insufficiently stable attachment to pole, insufficient inside space or a low amount of hay for nest).

The chi-square test with Yates' Correction was used to evaluate the significance in occupancy and nesting success.

Preliminary results obtained up till now have been published in Latvian, to maintain feedback with the network of nest site enthusiasts (Vīksne 2002; Vīksne, Laubergs 2004).

Results

Species composition of nesting ducks

Only Mallard was found nesting in the artificial nest sites. The only exception was a case when both Goldeneye *Bucephala clangula* and Goosander *Mergus merganser* attempted nesting in one wigwam on a pond near Saldus in 2002, but the nest was abandoned during egg laying. Some feathers of Shelduck *Tadorna tadorna* were found in a hay cylinder on Lake Engure in 1999, suggesting some interest of this species in artificial nest structures. Also, White Wagtail *Motacilla alba* quite often nested in artificial nest constructions but it could not be considered as competitor for Mallard. This paper deals only with Mallard nesting.

Differences in occupancy of nest sites among wetlands

There was a great difference in occupancy of nest sites on different wetlands (Table 2). Nest sites on ponds were favoured (46.8 %) over lakes rich in emergent vegetation mats and other potentially suitable substrates for nesting (3.3 %). Nest site occupancy by Mallard in single years did not exceed 10 % on Engure and 18.2 % on Kanieris. The very low occupancy on Lake Sarnate (1.5 %) might be explained by late erection of nest

Table 2. Occupancy of elevated artificial nest sites by Mallard *Anas platyrhynchos* on lakes and ponds in Latvia, 1999 - 2003

Type and name of wetland	Available	Occupied	
		number	%
L. Engure	44	3	6.8
L. Kanieris	52	4	7.6
L. Sarnate	206	3	1.5
Lakes, together	302	10	3.3
Ponds, together	421	197	46.8

sites in 2002 and unfavourable vegetation structure (many large continuous reed swards with very few interspersions of open water), compared with Engure and Kanieris. The difference in occupancy between ponds and lakes remains highly significant even if L. Sarnate is excluded (χ^2=36.04, p<0.0001).

Occupancy of different nest site types

To evaluate the occupancy of different nest site types we used data only from ponds. We also divided the records by periods: 1999 - 2002 when on many ponds experiments were started and both people and ducks had no or little experience with nest sites, and 2003 when no new nest site plots were set up by inexperienced persons (Table 3).

Nearly all types of nest sites were more occupied in 2003 than in 1999 - 2002 (hay cylinders: χ^2 = 4.12, p = 0.04; hay cylinders and wooden boxes, combined: χ^2 = 4.72, p = 0.03; two-entrance nest sites, combined: χ^2 = 6.1, p = 0.01), only stump nests were

Table 3. Occupancy of different types of artificial nest sites on ponds by Mallard *Anas platyrhynchos* in Latvia, 1999 - 2003

Type of nest site	1999-2002			2003			1999-2003		
	available	occupied		available	occupied		available	occupied	
		no.	%		no.	%		no.	%
Two-entrance nest sites on poles									
1. Hay cylinders	131	51	38.9	65	36	55.4	196	87	44.4
2. Wooden boxes	14	4	28.6	23	11	47.8	37	15	40.5
Total 1 + 2	145	55	37.9	88	47	53.4	233	102	43.8
3. Hollowed round timbers	8	6	75.0	4	4	100.0	12	10	83.3
4. Wigwams	3	2	66.7	8	6	75.0	11	8	72.7
Total 1 + 2 + 3 + 4	156	63	40.4	100	57	57.0	256	120	46.9
5. One-entrance nest sites on poles	76	24	31.6	32	16	50.0	108	40	37.0
Total 1 + 2 + 3 + 4 + 5	232	87	37.5	132	73	55.3	364	160	44.0
6. Stump nests	46	30	65.2	11	7	63.6	57	37	64.9

occupied equally in both periods.There was no difference between hay cylinder and wooden box occupancy ($\chi^2 = 0.15$, $p = 0.703$) in 2003, nor between these two types of nest sites combined and one-entrance nest sites ($\chi^2 = 0.02$, $p = 0.901$).

A very high occupancy of hollowed round timber nest sites and wigwams was observed. Combining all records for the whole period (1999 - 2003), occupancy of these nest sites was 83.3 % and 72.7 %, respectively, i.e. significantly higher than that of hay cylinders (44.4 %), wooden boxes (40.5 %) and one-entrance nest sites (37.0 %). The only explanation is that all the hollowed timber and wigwam nest sites were of ideal quality and mounted, from the ducks' point of view, in very suitable places. However, it is not excluded that wigwams were really more attractive and preferred by ducks due to their more natural appearance.

The idea of preference of nest sites which look as natural as possible was supported by much better occupancy of stump nests compared with all other nest sites mounted on poles in 1999 - 2003 (64.9 % versus 44.0 %, $\chi^2 = 7.87$, $p = 0.005$).

Occupancy and nest site density

Nest sites were set up on ponds of different size ranging from 4 to 30 ha (average 12.2 ha), with an average density of 1.7 nest sites per ha. Usually the number of nest sites was gradually increased when duck nesting was observed. The occupancy of nest sites increased with time which was evidently caused by two factors, i.e. increasing of skills of nest site managers and learning by ducks. Therefore, it is difficult to find a relationship between the density of nest sites and their occupancy by ducks.

The upper limit of Mallard occupied nests per ha that is possible by erecting nest sites is still unknown. Fig. 2 illustrates the number of artificial nest sites available, number of occupied nest sites and successfully hatched clutches on the pond Elkskene, 10 ha. The

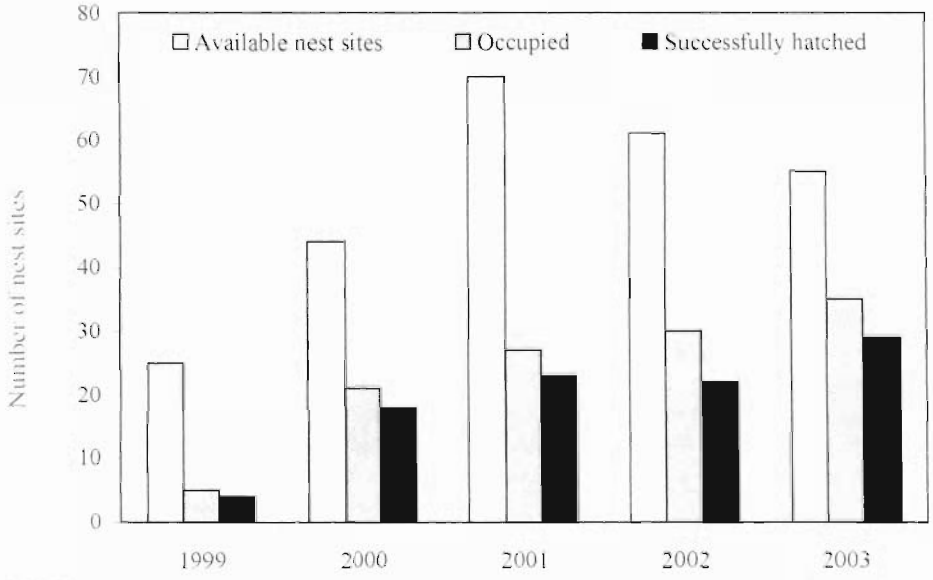

Fig. 2. Numbers of available and occupied by Mallard *Anas platyrhynchos* nest sites, and the number of successfully hatched broods on the pond Elkskene (10 ha), Latvia, 1999 - 2003.

number of nest sites here increased from 25 in 1999 to 70 in 2002, then declined to 61 in 2001 and 55 in 2003; the percent occupancy between 1999 and 2003 was 20, 48, 39, 49 and 64. During this time the number of nesting ducks increased every year from 5 in 1999 to 35 in 2003. The above indicates that it is possible to attract at least 3.5 pairs of Mallard per ha given a density of 5.5 available nest sites per ha. Probably even more can be attracted by increasing the number of nest sites and improving their quality.

Nesting success

No significant difference was found between nesting success of Mallards nesting in different nest sites mounted on poles with predator guards. The average nesting success in all these nest sites was 89.4 % (range 80 % to 100 % for different nest site types). In contrast, the nesting success of Mallards in stump nests accessible to climbing mammals was much lower – 67.6 % ($\chi^2 = 10.00$, $p = 0.002$). The difference between nesting success on Elkskene pond in predator guard supplemented nests and stump nests (the only location where we had this type of nests) was similar for the two types (87.7 % versus 67.6 %).

Reasons of nesting failure

In total, nesting was unsuccessful in 30 nests (Table 4) for two reasons – nest abandonment (14 nests) and predation (16 nests). The number of failed nests was too small to compare their frequency by different types of nest sites.

All abandoned nests contained incomplete clutches or, in some cases, probably complete clutches with fresh eggs. The widely known sensitivity of Mallard to disturbances during laying and early incubation can likely explain the nest abandonment in most cases.

The only nest predator recorded in elevated nest sites was American mink. Its predation rate differed between nest sites on poles with predator guard (2.9 %) and the easily accessible stump nests (29.7 %), thus proving the effectiveness of the predator

Table 4. Nesting success and failure of Mallard *Anas platyrhynchos* in artificial nest sites in Latvia, 1999 - 2003

Type of nest site	Nested	Hatched		Failed			
		no.	%	abandoned	predated	total	%
Hay cylinders	96	86	89.6	8	2	10	10.4
Wooden boxes	16	13	81.3	0	3	3	18.7
Hollowed round timbers	10	8	80.0	2	0	2	20.0
Wigwams	8	8	100.0	0	0	0	0
Two-entrance nest sites on poles, total	130	115	88.5	10	5	15	11.5
One-entrance nest sites on poles	40	37	92.5	3	0	3	7.5
Nest sites on poles, total	170	152	89.4	13	5	18	10.6
Stump nests	37	25	67.6	1	11	12	32.4
Total	207	177	85.5	14	16	30	14.5

guards. All five cases when American mink managed to predate clutches in nest sites on poles were recorded in 2003, four of them on the same pond where no mink predation was observed in artificial nest sites in 1999 - 2002 but was recorded in natural ground level nests. In all these cases, deviations from the recommended construction or placement (e.g. a supporting wire stretched between the landing platform and the pole below the predator guard, lack of or too narrow predator guard, cattail or shrubs growing very close to nest pole) were found.

Predation by avian predators such as Marsh Harrier, Hooded Crow and Raven was not observed. In a few cases, Magpie *Pica pica* was suspected at taking eggs from already abandoned nests.

Discussion

Mallard is adapted to an extremely wide range of habitats and nest sites. Although usually it nests on ground level, nesting on trees and different artificial constructions is known as well, even up to 32.5 m high. These elevated nests are not randomly distributed, as there are areas where they are quite common, separated by areas where they are very rare (Bauer, Glutz von Blotzheim 1968). One reason for the geographical patterns is probably the lack of such nesting experience in some populations. A switch to a new nesting habit could be prevented by abundant suitable natural (traditional) nesting sites, even though predation on these ground sites may be high. The site tenacity of Mallards which have successfully hatched ducklings in artificial nest sites (Doty, Lee 1974; Bishop et al. 1978; Majewski, Beszterda 1990; Yerkes 1999) allows to believe that a population will gradually switch from natural on-ground nests to artificial nests even in places where occupancy of newly mounted artificial nest sites is very low, as on natural lakes in our study and in some areas in the USA (Doty et al. 1975). In our conditions artificial nest sites could be very important as they prevent predation also by Marsh Harrier, probably also by Hooded Crow and Raven, which are the main Mallard nest predators on big coastal lakes.

It seems likely that American mink, the only nest predator recorded in artificial nest sites during this study, prefers searching for nests located at the ground level (on islets, along the shore line, on mats of emergent vegetation). Mink predation on natural nests placed on the ground level on ponds was higher (about 57 %) than on accessible nests placed on black alder stumps about 1 m above the water level (30 %). There also were cases when ducklings were successfully hatched in artificial nest sites that were accessible to mink due to some technical defects and placed very close to small grassy islets where mink predated natural ground nests. However, if mink succeeds in overcoming the predator guard, it is likely that it will show more interest in all elevated nest sites and that this experience could be spread among conspecifics.

The very high density of nesting Mallards achieved on the pond Elkskene during this study raises a special interest. The number of nesting ducks increased on the pond during five years, reaching 3.5 pairs per ha, and it is not excluded that a further increase could be expected. The density of nest sites available at Elkskene (5.5 nest sites per ha) exceeds that used and recommended in USA (Doty et al. 1975 – 2.5; Bishop, Barrat 1970 – 1.3 - 1.65; The Manitoba Habitat Heritage corporation – 0.6 - 1.2). However, in 2001 when the number of hatched broods was 23 (i.e. 2.3 broods per ha) the number of ducks on the pond declined dramatically at the opening of hunting season (August, 18). In 2002 nearly all

ducks had left the pond by hunting season (2.2 broods per ha, opening at August, 17). In 2003, when additional feeding of ducks with wheat was started already in July, a decline of duck numbers in mid- August was not observed. It seems that the lack of food was the reason for the unusually early departure of Mallards from the pond. Unfortunately, it is not clear whether and how increasing duck brood density influenced the amount and composition of zoobenthos organisms (the only information on benthos in Elkskene is from mid-October 2000, when 5040 and 5560 ind. m^2, correspondingly 122 and 73 g m^2, were found at the best sampling points – unpublished data by E. Parele).

It can be concluded that elevated artificial covered nest sites supplied with predator guards can be an effective management tool to increase nesting success and the breeding population size of Mallard. Considering the availability of different materials, ease of production, and longevity of nest sites, wooden boxes seem to be the most suitable type of nest site under the present Latvian conditions.

Acknowledgements

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Virs ūdens paceltas mākslīgās ligzdvietas meža pīlei *Anas platyrhynchos* Latvijā

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Kopsavilkums

Virs ūdens paceltas mākslīgās ligzdvietas meža pīlei *Anas platyrhynchos* izmēģinātas 1999. - 2003. gados piejūras ezeros un dažādos dīķos. Kopā analizētas 723 ligzdvietu kontroles. Meža pīle mākslīgās ligzdvietas daudz labāk aizņem dīķos (46,8 %) nekā lielos ezeros (3,3 %). Ligzdvietu aizņemtība ar gadiem pieaug, domājams, pateicoties gan to gatavotāju, gan pīļu lielākai pieredzei. Ligzdvietām, kuras izskatās dabiskākas, pīles dod priekšroku. Nav konstatētas būtiskas aizņemtības atšķirības divieeju ligzdvietām (siena cilindri, dēļu koridori) un vienieejas ligzdvietām (attiecīgi 53,4 % un 50,0 %) 2003. gadā. Vienā un tai pašā dīķī ligzdošanas sekmes bija augstākas ligzdvietās ar pretplēsēju aizsargiem (87,7 %), nekā bez tiem (67,6 %). Ligzdvietās ar pretplēsēju aizsargiem (visas vietas kopā) ligzdošanas sekmes vidēji bija 89,4 %, resp. ievērojami augstākas nekā dabiskajās ligzdās (Engurē – 23,3 %, Kaņierī – 54,3 %, dīķos – 25,0 %) tanī pat laikā. Ligzdvietās ar pretplēsēju aizsargiem 7,6 % dējumu tika pamesti, plēsēji (tikai Amerikas ūdele *Mustela vison*) izpostīja 2,9 %. Līdz šim virs ūdens paceltajās ligzdvietās nav konstatēti niedru lijas *Circus aeruginosus*, pelēkās vārnas *Corvus corone cornix* un kraukļa *Corvus corax* postījumi. Labākie rezultāti sasniegti kādā 10 ha lielā dīķī piektajā gadā (55 ligzdvietas, 35 meža pīles aizņemtas, 27 sekmīgi izvestas).

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Use of biometrical data to study Corncrake *Crex crex* population in Latvia

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Abstract

The Corncrake *Crex crex* population in Latvia was monitored by capturing 509 individuals in the time period between 1995 and 2003. During the breeding season Corncrakes attracted by playback of the territorial call of the male were captured at night, and during the migration birds were captured by cage traps. Only birds, which were identified as males (n = 498) and captured during the breeding season (May - July) were analyzed, except for variation in size by age, when all birds (including those captured on migration) with known age were used. Wing length of Corncrake males in Latvia varied between 130 mm and 152 mm (mean = 142.1; SD = 4.22; n = 455), tarsometatarsus length between 35.6 mm and 50.0 mm (mean = 40.5; SD = 2.94; n = 181), and weight between 134 g and 182 g (mean = 162.8; SD = 11.1; n = 120). Significant differences ($p < 0.01$) in wing length were observed in males from Latvia and other European countries, and comparing other countries with each other, but the expected tendency for northern animals of the species being larger than their con-specifics in the south was not clearly observed. The mean value of the wing maximum length in Corncrakes captured in different habitats, increased as follows: crops < pastures < cultivated meadows < uncultivated meadows < abandoned arable land < abandoned grasslands. The differences were statistically significant ($p < 0.05$), and might reflect the hierarchy of Corncrake males in habitat selection: larger males living in optimal habitats (e.g. abandoned grasslands), smaller – in suboptimal habitats (e.g. crops). Corncrake males captured in different months (May, June, July) had significantly different wing lengths ($p < 0.05$). This might be explained by immigration of birds from other populations later in season (June and July), when massive hay harvest begins to the south from Latvia (e.g. Poland), causing destruction of Corncrake nests and prohibiting successful re-nesting in the affected territories there.

Key words: body weight, *Crex crex*, morphometrical studies, tarsometatarsus length, wing length.

Introduction

Corncrake *Crex crex* is an open landscape species, today almost exclusively living in extensively managed agricultural lands, especially grasslands. Due to a cryptic lifestyle, the biology and ecology of the species have been poorly understood until the 1990s, when scientific research of Corncrake began (e.g. Schäffer 1999). The studies were stimulated by the need for effective conservation measures, since the introduction of intensive methods of agriculture in the 20th century caused dramatic population declines and fragmentation in most of its world range (Green et al. 1997). Species is included in the

IUCN Red List of Threatened Animals (Hilton-Taylor 2000). Corncrake population has declined also in Latvia (von Transehe 1965), but it is still numerous and the population increased in size since the major abandonment of agricultural lands in the 1990s (Keišs, Ķemlers 2000). An increase and recovery of the Corncrake population since 1998 can be observed also in the western Europe (e.g. The Netherlands – Koffijberg, van Dijk 2001). Since the individuals re-colonizing western Europe mostly derive from the East-European populations (Koffijberg, van Dijk 2001), it is important to understand Corncrake population processes at the local (e.g. Latvian) and global scales.

Individuals in a population might differ in various morphological traits. One of the important characteristics of a bird is its body size, which may indicate sex (Stresemann 1934), age (Stewart 1963), geographical origin (Stresemann 1934), and also its hierarchical level and mating success (Ligon 1999). Thus size may characterize a certain group of individuals of a species or population. As it is easy to take different measurements of a captured bird, there is an advantage of using body size in population studies, however overlap in size distributions among different groups may prevent use of size as an indicator of the group (e.g. age class: Jenni, Winkler 1994).

In the present study we use biometrical measurements (size of a bird) to examine the structure of the Corncrake population in Latvia, and provide possible explanations of the observed patterns.

Materials and methods

Systematic capture of Corncrakes (*Crex crex*) was initiated in Latvia in 1996, using the method of attracting birds at night by playback of its territorial call, then illuminating with a hand-light and capturing them by hand or spoon-net (the first attracted bird was captured during the day by mist-net in Jelgava in 1995). The majority of birds captured by the attracting method are males, but only those birds which are observed to call during

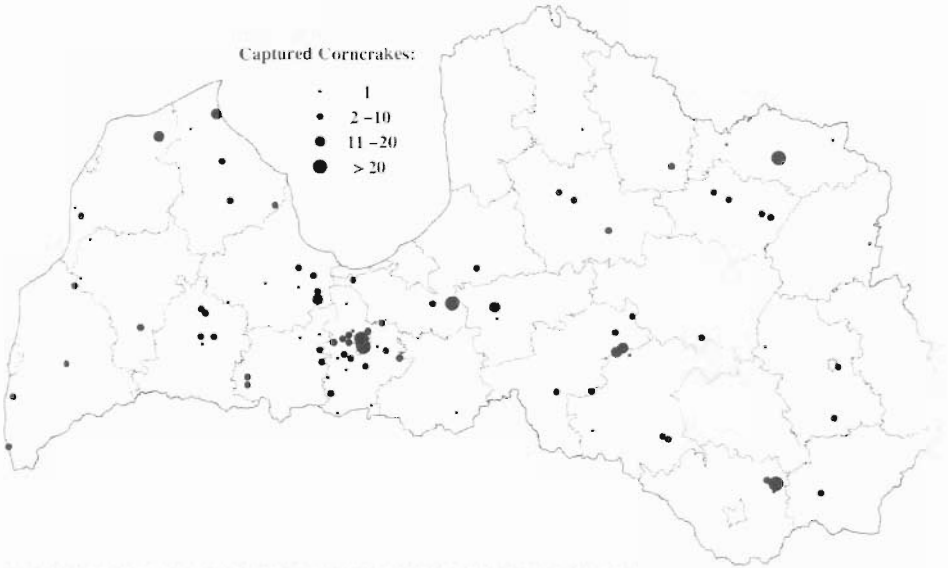

Fig. 1. Captured Corncrakes *C. crex* in Latvia between 1995 and 2003 (n = 509).

the process can be identified as males without doubt (Tyler et al. 1996). During autumn migration at the Pape Ornithological station Corncrakes were captured by cage traps. The authors captured and ringed 44 % of the birds, remaining data were collected by volunteer bird ringers from the Latvian Ringing Centre (Institute of Biology). Bird ringers were instructed how to capture and take measurements of Corncrakes in the seminars organized by the authors and the experts of the Latvian Ringing Centre in 1997, 1998 and 2001. The rings used for marking the Corncrakes were 5.5 mm diameter (LATVIA RIGA P). The geographic location for each capture was recorded (Fig. 1). The maximum wing lengths (length of flattened and straightened wing) of live birds were measured as described by Svensson (1992), using wooden rulers. A wooden rib at the zero mark of the ruler was constructed to facilitate bird wing measurement. All rulers were modified by the same person. The tarsometatarsus length was measured with standard technique as described by Svensson (1992). Birds were weighed in the field using spring balances (300 g spring-balance with 2 g precision: Pesola AG, Switzerland). Age according to iris colouration (Salzer, Schäffer 1997), morphological traits, wear of the primaries, behaviour and habitat category were recorded additionally. We recommended observers to use the following habitat categories: (i) cultivated (grass sown and fertilized) meadows, (ii) uncultivated (natural) meadows, (iii) cultivated (grass sown and fertilized) pastures, (iv) uncultivated (natural) pastures, (v) winter crops (rye, winter wheat), (vi) spring crops (barley, oats and mixed cereals), (vii) other arable land (potatoes, beats etc.), (viii) abandoned grasslands (previously used for moving or grazing), (ix) abandoned arable land (previously used for arable land), (x) abandoned agricultural land (unknown whether it was previously grassland or arable land), (xi) clear cuts in forests and (xii) other habitat. Not all of the habitat categories were represented in our sample (see Results).

Published data on Corncrake measurements (Table 2) were used to investigate the geographical variance in size of Corncrakes. Standard statistical procedures (two tailed *t*-test) were used to test for differences between different groups of birds (Zar 1996).

Results

A total of 509 Corncrakes were captured from 1995 to 2003 (Table 1), two of the birds were recaptured at the ringing site one year after the first capture. We divided the Corncrakes into three age groups – first calendar year (juveniles), second calendar year (one year old birds) and older birds (two years or older).

Juvenile birds captured at Pape were significantly smaller in size ($p = 0.05$) to

Table 1. Number of Corncrakes *C. crex* captured and measured in Latvia between 1995 and 2003 by months

	Males	Other (sex undetermined)		Total
	May - July	May - July	August - September	
Captured, of them:	498	6	5	509
– ringed	496	6	5	507
– wing length measured	455	6	5	466
– tarsometatarsus length measured	181	1	3	185
– weighted	120	2	1	123

Table 2. Biometrical measurements [range; mean; standard deviation (SD); sample size (n)] of Corncrake *C. crex* males in Europe

Country or region	Wing length (mm)				Weight (g)				Tarsometatarsus length (mm)				Source
	Range	Mean	SD	n	Range	Mean	SD	n	Range	Mean	SD	n	
Bavaria					139 - 190	164.0	12.1	25					Schäffer 1999
Belarus					145 - 180	163.6	9.7	9					Schäffer 1999
Estonia					155 - 170	162.0	5.6	6					Schäffer 1999
Latvia	130 - 152	142.1	4.2	455	134 - 182	162.8	11.1	120	35.6 - 50.0	40.5	2.9	181	this study
Nordrhein-Westfalen	132 - 148	140.4	3.7	49	138 - 176	161.9	8.8	40					Prünte 1972
Poland	136 - 154	143.4	3.3	258	133 - 190	161.7	10.8	272					Schäffer 1999
Scotland	141 - 158	149.9	3.6	60									Tyler et al. 1996
The Netherlands	139 - 150	144.0	4.2	15	135 - 202	169.0	18.7	28	37.0 - 43.0	40.0	1.8	36	L.M.J. van den Bergh (after Cramp, Simmons 1980)

compare with birds of the other two age categories. Significant differences between other age categories were not found.

Geographical differences in size

Wing length of Corncrake males captured in Latvia and males from other countries (Table 2) significantly differed ($p < 0.008$), except for The Netherlands ($p = 0.08$). Also, pair-wise comparisons of all but one pair of countries (Poland and The Netherlands) were highly significant ($p < 0.005$). Tarsometatarsus length was measured only in The Netherlands, where it was slightly shorter than in Latvia, but the difference was not statistically significant ($p = 0.3$). Corncrake males weighed (Table 2) during the breeding season (May - July) did not differ significantly between several European countries excepting that Dutch birds were significantly heavier than birds from Poland ($p < 0.002$), Latvia ($p < 0.03$) and Nordrhein-Westfalen ($p < 0.04$).

Size differences among habitats

Only the wing length was compared between birds captured in different habitats, due to small sample size of other measurements,. To enlarge the sample size, we combined cultivated and uncultivated pastures into the category "pastures" and winter and spring crops into the category "crops". The mean value of the wing maximum length increased as follows: crops < pastures < cultivated meadows < uncultivated meadows < abandoned arable land < abandoned grasslands (Fig. 2). Wings of Corncrake males captured in crops (140.3; SD = 4.8; n = 15) and pastures (140.6; SD = 3.0; n = 16) had a similar length ($p = 0.84$), and males in cultivated meadows (141.2; SD = 4.1; n = 80) were non significantly ($p = 0.5$) larger comparing with those in crops and pastures. Corncrake males inhabiting uncultivated meadows (142.4; SD = 4.3; n = 163) had larger wings in comparison with inhabitants of crops ($p = 0.08$), pastures ($p = 0.11$) and, significantly, cultivated meadows ($p < 0.05$). Birds in abandoned grasslands (143.3; SD = 4.2; n = 35) were significantly larger than birds captured in crops ($p < 0.04$), pastures ($p < 0.04$) and cultivated

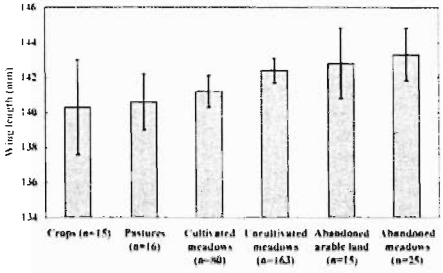

Fig. 2. Wing length of male Corncrakes *C. crex* captured in different habitat categories in Latvia between 1995 and 2003.

meadows ($p < 0.02$). This is also true for birds in abandoned arable land (142.8; SD = 3.7; n = 15) but the differences were not significant. Inhabitants of abandoned arable lands and grasslands had similar size ($p = 0.72$).

Size differences during the season

Different individuals captured at different times of the breeding season showed differences in size (Fig. 3). Males captured early in the season in May were larger than those caught later in June and July. This pattern was observed for all size measurements – tarsometatarsus length, wing length and weight, and the differences in the latter two were statistically significant between May and June, May and July ($p < 0.003$). June and July sizes were significantly ($p < 0.04$) different only in wing length: males, captured in July had longer wings.

Discussion

The small sample size (n = 30) of old birds (at least 2 years old) might explain why significant differences between yearlings and older birds were not found. Salzer and Schäffer (1997) suggest to use colour of the iris to determine age of Corncrake, but artificial light conditions at night inconvenience its use and might be potential source of error.

The results showed significant geographical differences in wing length of Corncrakes. It is not known, whether these differences are determined by a specific factor. Expected tendency for northern animals of the species being larger than their con-specifics in the south (Stresemann 1934; Stevens 1989) was not clearly observed. All birds used to test for geographical differences were captured during the breeding season. Moult can be excluded as a possible cause of errors, since complete moult in Corncrake occurs after the breeding season in August (Cramp, Simmons 1980). It is possible, however, that using our capture method, aggressive males were selectively caught. These males are expected to be larger than the rest of population; i. e. the population was not sampled randomly. We also lack data on Corncrake measurements in other countries with large populations (e.g. Lithuania, Belarus, Ukraine). Comparing the size measurements of birds captured in May – "true" Latvian population, the pattern might be more pronounced – Latvian birds has longer tarsometatarsus and they are heavier (Fig. 4).

We assume that Corncrakes with longer wings are higher in hierarchy and therefore

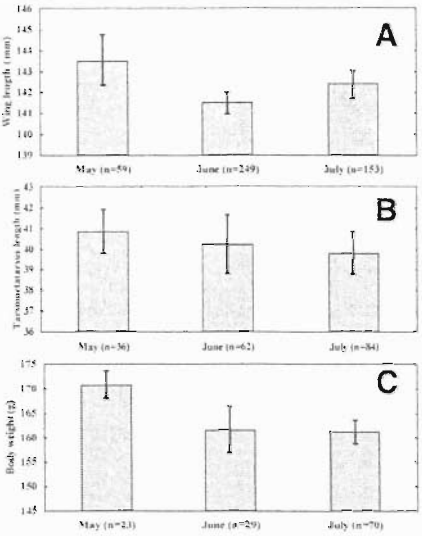

Fig. 3. Morphometrical data of male Corncrakes *C. crex* captured at different time of season in Latvia between 1995 and 2003: A, maximal wing length; B, tarsometatarsus length; C, body weight.

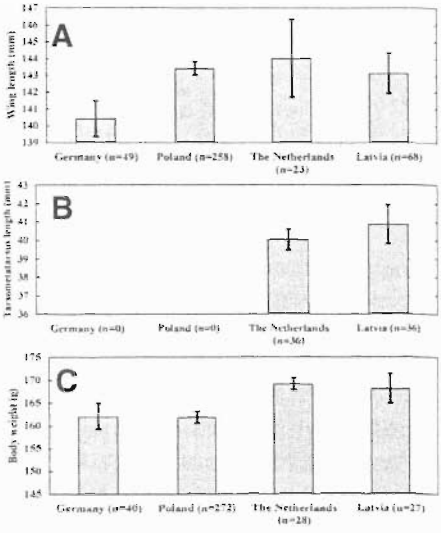

Fig. 4. Morphometrical data of male Corncrakes *C. crex* captured in Latvia in May and in Germany, Poland and The Netherlands: A, maximal wing length; B, tarsometatarsus length; C, body weight.

occupy better habitats. Our observed gradient of wing length in different habitats perfectly coincides with the preferred habitat gradient of Corncrakes in Latvia estimated by the density of calling males (Keišs 1997). Corncrakes avoid crops and pastures (we also found the shortest wings of males captured in these habitats), cultivated meadows are a neutral habitat (wing length have a medium value between crops/pastures and uncultivated meadows/abandoned lands), and uncultivated meadows and abandoned lands (the longest wings) are preferred by Corncrake males (Keišs 1997). Nevertheless a behavioral study of Corncrake males is required to confirm this observation.

Changes in size of Corncrakes during the season might have several explanations. It is clear that early in the season in May captured birds were larger. Assuming that also Corncrakes are lager in northern populations, these might have been birds from the "true" Latvian population, which later in the season mix with short-winged birds displaced by grass mowing in south. Recovery of a Corncrake ringed in May 23, 1972 in The Netherlands and found in Latvia in August 1, 1972, supports this hypothesis (Latvian Ringing Center, unpublished data). Also ringing results of Brüger and Pykal (2000) in the Czech Republic showed one Corncrake male calling in the same breeding season at two places 600 km apart. Another explanation is that the large birds were dominant males, arriving and starting to call early. They also might have been individuals still on migration to North, but this explanation is unlikely, since Corncrakes first start to call only a few days after arrival at their breeding grounds (Beme et al. 1987). According to Tyler et al. (1996), males are silent when accompanied by female, which be the case of "true" Latvian males in June, when immigrants are the most active at calling and therefore are captured. The calling activity (and capture rate) of local males increases again in July before the second brood.

The obtained results support observations made in previous studies on Corncrake habitat selection in Latvia. The data suggest that seasonal variation in size can be explained by movements of Corncrake males during the breeding season, but behavioral study and simultaneous ringing efforts in European countries during the breeding season are needed for conformation.

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Biometrisko mērījumu izmantošana, pētot griezes *Crex crex* populāciju Latvijā

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Kopsavilkums

No 1995. līdz 2003. gadam Latvijā tika noķertas un apgredzenotas 509 griezes *Crex crex*. Ligzdošanas laikā griezes tika ķertas naktī, pievilinot tās ar tēviņa balss ierakstu, bet migrāciju laikā – ar ķeramkastēm. Biometrisko mērījumu analīzē izmantoti dati tikai par putniem (n=498), kuri noķerti ligzdošanas sezonas laikā (maijā - jūlijā) un kuru dzimums (tēviņš) tika nešaubīgi noteikts, izņēmums ir lieluma variāciju analīze pēc vecuma, kurā izmantoti visi putni ar zināmu vecumu (t.sk. migrāciju laikā noķertie). Latvijas griežu tēviņu spārna garums variēja no 130 līdz 152 mm (vidējais=142,1; SD=4,22; n=455), stulma garums 35,6–50,0 mm (atbilstoši: 40,5; 2,94; 181), svars 134–182 g (atbilstoši: 162,8; 11,1; 120). Salīdzinot spārna garumu, Latvijas griežu tēviņi būtiski ($p<0.01$) atšķīrās no citās Eiropas valstīs ligzdošanas laikā mērītajiem griežu tēviņiem. Savstarpēji atšķīrās arī šo valstu (Polijas, Skotijas, Vācijas) tēviņu mērījumu rezultāti, taču sagaidāmā tendence, ka ziemeļu populāciju īpatņi ir lielāki, netika skaidri konstatēta. Dažādus biotopus pēc noķerto griežu vidējā spārna garuma no īsākā uz garāko varēja sakārtot šādā secībā: 1) labība; 2) ganības; 3) kultivētas pļavas; 4) nekultivētas pļavas; 5) aramzeme atmatā; 6) pļavas atmatā. Atšķirības bija statistiski būtiskas ($p<0.05$) un, iespējams, atspoguļo to, ka hierarhijā augstāk stāvoši (lielāki) tēviņi ieņem teritorijas optimālos biotopos (piemēram, atmatās), bet zemāk stāvoši – suboptimālos biotopos (piemēram, labībā un ganībās). Sezonas gaitā (maijā, jūnijā, jūlijā) noķerto tēviņu spārna garums būtiski atšķīrās ($p<0.05$). Iespējams, to var izskaidrot ar griežu imigrāciju no citām populācijām jūnijā un jūlijā, kad lielās platībās sākas siena pļauja uz dienvidiem no Latvijas un iznīcina iesāktās griežu ligzdas, kā arī padara neiespējamu atkārtotu ligzdošanu šajās teritorijās. Par griežu pārvietošanos ligzdošanas laikā lielā attālumā (>600 km) liecina divu gredzenoto griežu atradumi (gredzenotas un atrastas vienā un tajā pašā ligzdošanas sezonā).

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Vertigo moulinsiana (Dupuy, 1849) (Gastropoda: Pulmonata) in Latvia

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Abstract

Vertigo moulinsiana (Dupuy, 1849) was recorded in a wet black alder forest (Cl. *Alnetea glutinosae*) in 1997 in the Forest Management District Aizkraukle. The locality is situated on a rised island of mineral soil in a raised bog. This is the first record of the species in Latvia.

Key words: Gastropoda, Latvia, Pulmonata, *Vertigo moulinsiana*.

Introduction

Vertigo moulinsiana belongs to family *Vertiginidae*. The shell height is 2.2 - 2.7 mm and average width is 1.5 mm. The species prefers open habitats, and is characteristic in wet forests and marshlands, and calcareous fens. Most frequently it has been found on lake banks and in reed, sedge or *Glyceria* debris on the banks of rivers (Kerney et al. 1983; Pokryszko 1990). In Sweden, England and Scotland, the species occurs in wet forests including black alder swamps (Ehnström, Walden 1986; Killeen 2003). During the summer, the snails live on plant stems and leaves, preferably on *Typha*, *Iris*, *Glyceria*, *Carex* and *Phragmites* leaves at a height of 30 - 50 cm. Snails hibernate in plant litter where they can be found also in spring and autumn. Until now, the species has not been recorded in habitats managed by regular haymaking or grazing. The snails feed upon fungi covering the plants growing in wet habitats (Pokryszko 1990).

The distribution of the species is interpreted differently. One opinion is that it has a European and probably Holarctic distribution (Kerney et al. 1983). Other consider an Atlantic-Mediterranean distribution (Pokryszko 1990) or Northern European and Middle European distribution (Liharev, Rammelmeier 1952). The southern border of its distribution is not clear (Pokryszko 1990). To the North *V. moulinsiana* has been found in Lithuania and in South Sweden (Liharev, Rammelmeier 1952; Ehnström, Walden 1986; Pokryszko 1990). The species has not been recorded in Finland and Russian Karelia (Valovirta 2003). There are no data records from Estonia. However, in the collection of the Latvian Museum of Natural History, there are specimens of *V. moulinsiana* (LDM Z-5) collected in the Zaonezhje Region of Karelia at the end of July in 2003, where the author collected 21 specimens in a wet black alder forest on B. Lelikovskij Island of the Kizhi archipelago.

Species is protected in the European Union (EU Directive 1992). Particularly protected territories must be established in the sites with populations.

Methods

The influence of forest management on terrestrial snails was studied in 1995 and 1997 on mineral soil islands in the Aizkraukle bog and also in three other sites in Latvia: Slītere, Līvbērze and Mežole. The studies covered five forest habitat groups – pioneer phase of deciduous forests, pine forests, spruce forests, wet black alder forests and broadleaved forests.

Vertigo moulinsiana (Dupuy, 1849) is mentioned as a possible species of the fauna of snails in Latvia (Schlesch 1942; Sloka, Sloka 1957; Rudzīte 1999), but no records previously existed.

The species was collected in wet black alder forests (Cl. *Alnetea glutinosae*) in Aizkraukle bog at the end of July 1997. At first, the species was identified as *V. geyeri* and was published under this name (Pilāte 2000; Pilāte 2003). After rechecking the collected snails, it was recognised that the species was misidentified and that it actually was *V. moulinsiana*.

Results and discussion

The two localities in black alder forests are situated on a bog island on an ecotone between raised bog and broadleaved-spruce forests. Groundwater flow with a high groundwater table is found in both habitats. The forests restored naturally after a clear-cut harvest around 1950th in one locality and in 1934 in another locality (blocks 175 and 174 in the Aizkraukle Forest Management District; Suško 1998). 21 species of vascular plants were found in the forest blocks, including *Typha*, *Iris*, *Glyceria*, *Carex* un *Phragmites* with a high diversity of *Carex* – 17 species (Suško 1998). These plants are preferred by *V. moulinsiana*.

V. moulinsiana was recorded in material collected by area sampling method (Dunger, Fiedler 1997). 22 land snail species were recorded in one locality where *V. moulinsiana* was dominant, 17 species – in another locality where *V. moulinsiana* was subdominant. The frequency of specimens was high in both localities. Two recorded species, *Clausilia dubia* (Draparnaud, 1805) and *Ruthenica filograna* (Rossmässler, 1836), are characteristic to calcareous habitats.

V. moulinsiana, probably, is really very rare, as it has not been found in other similar black alder forests in the Aizkraukle Forest Management District. The species has not been found also in other investigated sites (Slītere, Līvbērze and Mežole) with black alder forests. Sampling time could be another reason for not finding the species. There is a low probability of finding *V. moulinsiana* if samples are collected in late spring or early summer. During this period the snails live on plants. Black alder forests are flooded in the early spring and it is difficult to sample plant litter. Probably, searches for the species should be started at the end of July.

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Vertigo moulinsiana (Dupuy, 1849) (Gastropoda: Pulmonata) Latvijā

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Kopsavilkums

Vertigo moulinsiana (Dupuy, 1849) atrasta 1997 gadā slapjā melnalkšņu mežā (Cl. *Alnetea glutinosae*) Aizkraukles mežniecībā. Atradne lokalizēta augstā purva salā uz minerālās augsnes. Šis ir pirmais sugas dokumentējums Latvijā.

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Relationships between the nest predation rates caused by different waterfowl nest predators: an artificial nest experiment

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Abstract

Nest predation is crucial process affecting the breeding success of birds and their fitness. Numerous studies have been carried out using natural and artificial nests to test nest failures caused by different predator species. The aim of this study was to investigate the interaction among nest predation rates due to different predators, i.e. how changes in one predator's predation rate and consumption of the nests are related to the predation rate of other predators? Depredation of artificial nests was studied in 5.68 ha and 10.17 ha plot on Lielrova island, in Lake Engure in Latvia, in 2002 and 2003 respectively. This study shows that food availability (e.g. number of nests) and activity of other predators was significantly related to the nest predation rate by interspecific predators in a waterfowl nest predator community. Nest daily mortality rates caused by predators were related to changes in the number of exposed nests. The experimental study showed that predation on artificial nests by competitive nest predators was a significant predicator of the possibility of nest predation by other members of the local predator community.

Key words: artificial nest, corvids, mammals, marsh harrier, nest predation.

Introduction

Nest predation is a major factor affecting nesting success and is a driving force in the evolution of birds (Martin 1988; Ricklefs 2000). As artificial nests are easy to construct they are frequently used to measure the variation in predation rate associated with characteristics of eggs, nests, habitat, location of nests, nest density and season (Major, Kendal 1996). These factors allow the investigators to control variables of interest and to work with adequate samples.

In most studies of nest predation, the nest fate is measured in general (successful vs. depredated) or the study is focused on a single target nest predator species or on a group of species (e.g. avian vs. nonavian; Yahner, Cypher 1987; Yahner, Scott 1988) and the nest predation rates of different nest predator species are overlooked. In the majority of ecosystems there are several nest predator species. Knowledge about interactions among predators is required in order to determine their effect on the prey population. The influence of predators on the risk of nest predation has been investigated in relation to habitat quality (Yahner, Cypher 1987; Eriksson 2001) or predator and nest characteristics (Crabtree et al. 1989; Cresswell 1997; Opermanis et al. 2001), interactions between competitors in waterfowl nest predator assemblages are poorly understood.

The aim of this study was to investigate the interactions between nest predation rates due to interspecific predators depending on nest availability, i.e. how the changes in the predation rate of one predator and the consumption of the nests are related to predation rate of other predators.

Materials and methods

Study area

This study was conducted on Lake Engure, Latvia (57° 15' N, 23° 07' E), an eutrophic wetland encompassing 3,500 ha. About 40 % of the lake is covered by emergent vegetation, mainly common reed *Phragmites australis*, narrow-leaved cattail *Typha angustifolia* and bulrush *Scirpus* spp. The data on artificial nests were collected on Lielrova island and the most accessible small artificial islands. The islands were open, flat and the vegetation was dominated by grasses *Poaceae*, occasionally interspersed with reeds, sallow *Salix* spp. (but mostly *Salix repens*) and sedges *Carex* spp. The study area was 5.68 ha and 10.17 ha, in 2002 and 2003 respectively. The size of the experimental plots was selected according to the number of nests necessary to statistically process the data. The area of the study plots was estimated using an aerial photo (1 : 10000 scale) and ArcView version 8 software package. The location and size of the experimental plots were selected so that the plots had sufficient numbers of natural duck nests in the particular year. In the study plots Mallard *Anas platyrynchos* were common breeders and Gadwall *Anas strepera*, Northern Shoveler *A. clypeata*, Garganey *A. querquedula*, Tufted Duck *Aythya fuligula* and Common Pockard *A. ferrina* nests were rare during the breeding season.

Experimental design and field procedures

The artificial nest experiment was carried out from July 11 till October 8 in 2002 and from August 30 till October 17 in 2003. To minimize interference to the natural predator-prey ecosystem and to exclude an increase of predation rate in real duck nests stemming from the attraction of predators, the artificial nests experiment was carried out after the duck breeding season (Association for the Study of Animal Behaviour, Animal Behaviour Society 2001). The lengths of trials were five and seven days. During each trial, 50 and 32 atrificial nests were exposed, in 2002 and 2003 respectively. The number of artificial nests in each trial was equal to the number of duck nests in the study area in the same year. Each artificial nest was randomly placed where suitable substrate for duck nesting was present. The minimal distance between nearest neighbouring nests was 10 m. To relocate the nests they were marked using 1.5-m-long willow canes. To decrease the probability that potential nest predators use this mark to locate the nest, the canes were placed 2 m away from the nests (Hein, Hein 1996). The artificial nests were constructed to resemble duck nests. Nest bowls were made by creating a shallow depression in the ground approximately 15 cm in diameter. Six fresh brown hen eggs were placed in each bowl. Bowls were concealed by vegetation so that two or three eggs were visible from above. Since the nests were partly covered by vegetation, they appeared like nests abandoned or from which the female duck was flushed (Opermanis 2004). Since non-cryptic hen eggs were used, their colour was similar to that of duck eggs and they did not affect the detectability of the nests by visually oriented predators (Weidinger 2001).

Nest-site characteristics were recorded in each experimental trial: species of plants in

a 1-m radius around the nest, height of vegetation around the nest estimated using five randomly placed measurements in a 1-m radius around the nest, and location (natural vs. artificial island). The date of the placement of the nest and subsequent relocations were recorded. If the nest was depredated, the number of damage or missing eggs was recorded.

All nests were exposed for 24 or 25 days, which was close to the mean duration of the egg stage of real duck nests. If the nest was successful after this time, it was removed and reset in a different location, keeping total number of the nests in each trial constant.

The fate of the nests was measured at the end of each trial. A nest was considered depredated when at least one egg was damaged or missing. Depredated nests were removed and reset in different locations, maintaining the total number of nests in each trial constant. The nest predators were identified using careful examination of the remaining eggshells and additional signs in and around the nest (Opermanis et al. 2001). The nest predators were divided in four groups using the as precise as possible taxonomical level: (i) 'Harriers' – including Marsh Harrier *Circus aeruginosus*; (ii) 'Corvids' – including Raven *Corvus corax*, Hooded Crow *C. corone cornix* and Magpie *Pica pica*; (iii) 'Mammals' – including American Mink *Mustela vision*, Raccoon Dog *Nyctereutes procyonioides* and Red Fox *Vulpes vulpes*; (iv) 'Unknown' – including all cases where the predator was not possible to identify sufficiently precisely or where multiple predators were present. As mammals as predators were detected only in one trial in 2003, this data was pooled with the 'Unknown' predator group and designated in further as 'Other' predators.

Data analysis

Data from both years were analysed separately, as recommended by Butler and Rotella (1998). The daily mortality rates (hereafter DMR) of nests were estimated by the Mayfield method (Mayfield 1961; Mayfield 1975). DMR of each trial was divided in separate days. The nest-day was used as the unit of exposure because the number of nest exposure days in trials differed – 5 days in 2002 and 7 days in 2003.

To identify interactions between the nest predation rates due to interspecific predators, multiple linear regression analysis was used as follows – one by one the DMR by each predator was used as a dependent variable and the DMR's by the remaining predators were used as predictor variables. In multiple linear regression analyses, the daily mortality rates were arcsine transformed. The significance of the regression models was estimated using ANOVA. The significance of coefficients of multiple linear regression equiations was estimated using *t* statistics. The nests availability rate for each predator was calculated as the daily survival probability after depredation by the remaining predators. Spearman rank correlation was used to detect association between the DMR by each predator type and the nest availability rate for a particular predator.

All statistical tests were conducted using SPSS statistical software package. All tests (where were preference) were two tailed.

Results

All multiple linear regression models in both years demonstrate significant relationships between the DMR's by different predators (Table 1). Significant negative relations in year 2002 were detected (i.e. significant unstandardized coefficient in multiple linear model

Table 1. Multiple regression analysis of the effects of the predation rates of different predator species (predictor variable) on the nest daily mortality rates by specific predator groups (dependent variable) in an artificial nests experiment at the Lake Engure, Latvia in 2002 and 2003

Year	Regression model estimation					Regression equation coefficient estimation			
	Dependent variable r^2	Coefficient of determination d.f.	Degree of freedom	F-value	Significance P	Predictor variable	Unstandardized coefficient	Student's t test	Significance P
2002	Harriers	0.666	89	57.091	< 0.001	Constant	0.099	20.260	< 0.001
						Corvids	-0.615	-9.461	< 0.001
						Mammals	0.209	1.079	0.284
						Unknown	-0.609	-4.042	< 0.001
	Corvids	0.623	89	47.337	< 0.001	Constant	0.097	11.179	< 0.001
						Mammals	-0.444	-2.002	0.048
						Unknown	-0.026	-0.136	0.892
						Harriers	-0.829	-9.461	< 0.001
	Mammals	0.352	89	15.577	< 0.001	Constant	0.0002	0.043	0.966
						Unknown	0.458	6.008	< 0.001
						Harriers	0.064	1.079	0.284
						Corvids	-0.100	-2.002	0.048
	Unknown	0.448	89	23.252	< 0.001	Constant	0.037	5.556	< 0.001
						Harriers	-0.262	-4.042	< 0.001
						Corvids	-0.008	-0.136	0.892
						Mammals	0.645	6.008	< 0.001
2003	Harriers	0.200	48	5.756	0.006	Constant	0.044	3.240	0.002
						Corvids	-0.745	-0.870	0.389
						Other	-0.204	-0.565	0.575
	Corvids	0.832	48	113.603	< 0.001	Constant	0.015	11.877	< 0.001
						Other	0.373	13.192	< 0.001
						Harriers	-0.022	-0.870	0.389
	Other	0.830	48	112.326	< 0.001	Constant	-0.025	-5.078	< 0.001
						Harriers	-0.034	-0.565	0.575
						Corvids	2.118	13.192	< 0.001

Table 2. Spearman rank correlations between daily mortality rate by each nests predator and the availability of remaining nests after depredation by the other predators, in an artificial nest experiment during 2002 and 2003 at Lake Engure, Latvia

Nest predator	Year	Spearman rank correlation coefficient r_s	Number of observations (days)	Significance P (two tailed)
Harriers	2002	0.763	90	< 0.001
Corvids	2002	0.549	90	< 0.001
Mammals	2002	-0.055	90	0.609
Unknown	2002	0.012	90	0.907
Harriers	2003	0.555	49	< 0.001
Corvids	2003	-0.430	49	0.002
Other	2003	-0.382	49	0.007

equitation) between Harriers and Corvids, and between Harriers and Unknown predators: an increasing daily mortality rate due to Corvids and Unknown predators was related with a decrease in the Harrier-caused nest depredation rate (or vice versa). An increasing daily mortality rate by Corvids occurred with a decrease in Mammal nest predation rate (or vice versa). A significant positive relation between Mammals and Unknown predators was identified in 2002. In 2003 a significant positive relation was found only between Corvids and Other (Table 1).

The total nest exposure to possible depredation significantly increased the likelihood of nest mortality by Harriers and Corvids in both years and by Other only in 2003 (Table 2).

Discussion

The artificial nest experiment demosntrates that nest predators can affect predation rates of each other. Although the nest predators were not identified to species, probably most of the Unknown predators in 2002 were mammals, as the late summer was dry and the water level was low. These conditions enable Racoon Dogs and Foxes to use small ridges to reach the island from the lake share, which was confirmed by Mammalian faeces found on the island. In 2003 Ravens were believed to be responsible for majority of depredated nests by Other predators, since all egs were absent in some nests and there were no additional signs. The above explain the positive relationships between Mammals and Unknown in 2002 and between Corvids and Other in 2003.

Since the artificial nest experiment was carried out beyond the breeding season (i.e. no real duck nests) the study area had a higher density of nests. Aggregation of predators (mainly magpies) was observed during the experiment. Bendekoff et al. (1997) demonstrated that *Corvidae* successfully apply 'Spatial information processing' to make their own foraging more effective. This has also been confirmed in studies where corvids were natural (Erikstad et al. 1982) or artificial (Picozzi 1975) nest predators. Therefore, it is possible that corvids changed their feeding behaviour, thereby significantly affecting other predators. Predators use prey selection according to prey species, age or sex and

interindividual variations in hunting behavior, and predatory behavior may differ not only between habitats, but also within similar environments (Mitani et al. 2001). Sparrowhawk *Accipiter nisus* consistently selectively decides to hunt the weakest and most vulnerable individuals in a population (Götmark 2002; Quinn, Cresswell 2004), but nests with openly visible eggs are vulnerable to visually oriented nest predators (Opermanis 2004). Thus, openly placed artificial nests, as immobile and vulnerable pray, are more susceptible to successful attack of a predator. The Harrier is a generalist predator, feeding on a wide variety of prey (Cramp, Simmons 1980) and can change its foraging behaviour and composition of the diet according to changes in the availability of different food items (Underhill-Day 1985), which is also true for other bird species (Bryant et al. 1999; Murakami 2002). Non-specific predation on nests of some duck species with different appearances, location and temporality showed that Harriers were generalist predators on waterfowl nests (Opermanis et al. 2001). Quartering hunting flights have been described earlier (Schipper 1977) and if predators find nests by random or systematic search, nests should be equally vulnerable. In our experiment similar hunting techniques were most frequently observed, but Harriers usually used transect searching flights to follow habitat edges (open water/reed stand) in late summer. Since waterfowl eggs contribute a small part of the Harrier diet (Kasparsons 1960; Schipper 1973; Underhill-Day 1985), there is a weak response to artificial nests as supplementary food items.

Norrdahl and Korpimäki (2002) demonstrated a response of diurnal raptors to a change of prey availability following cyclic fluctuations in rodent populations. They found a short time lag response in late summer, when non- and post-breeding individuals were free to search for patches with a high pray density. In this context, it was possible that Harriers prefer young waterfowl and reed breeding passerines, as they were abundant in the study area in late summer. Therefore, artificial nests in our experiment were not an important part of the Harrier diet and Harriers were likely pronounced opportunistic nests predators. This confirms an earlier observation by Opermanis (2001) that Harriers never consume entire egg contents. Thus, Harriers are generally weak competitors for artificial nests as a food item.

Mammal predation on artificial nests on islands have a chance character because of the difficulty in arriving on the island. Depredation by American Mink was low in both years because they depredate nests more frequently near hatching eggs or ducklings (A. Mednis, personal communication). DMR due to Mammals were analysed separately only in 2002, when foxes were observed on the island. The data are still insufficient, but it is clear that there is no relation between DMR by Mammals and DMR by specific avian predators – Harriers and Corvids (Table 1). Avian nest predators use visual cues to locate nests (Dwernychuk, Boag 1972) while mammals are olfactory oriented predators, resulting in a low possibility for an interaction between the predation rates. However further study is still required in this context.

Since the nest availability had a significant positive correlation with the daily mortality rates by Harriers and Corvids in both years (Table 2), the daily mortality rates by these predators influenced food availability and the success of nest searching by other predators.

Our experiment and other studies (Sutherland 1996; Krebs, Davies 1997) provide evidence that the interactions between nest predation rates by interspecific predators in the waterfowl nest predator community result from competition between different

predators for food, depending on the importance of eggs in the diet of the predator species. Predation on artificial nest by competitive nest predators was a significant predictor of the possibility of nest predation by other predators. The nest failure may be affected by area-dependent changes in predator assemblages on the landscape-level, and other factors also may be important. As the nests were only partly covered, the daily mortality rates more represented the activity of predators than successful searching for the nests. The experiment showed that the effects of food availability (e.g. numbers of nests) and the activity of one predator may influence nest predation rate by another predator in a waterfowl nest predator community. This study suggests that the traditional measure of nest success (failed vs. fledged at least one young) may not be appropriate in investigating predation, without careful validation of the experiment design and results, because changes in the predation rate by one predator may influence predation rate of another predator.

As this study presented the response of predators on artificial nests beyond real duck breeding season, future experiments should be carried out to examine whether the prey-breeding season determines the nest search behaviour of different predators.

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Sakarības starp dažādu ligzdu postītāju veikto ūdensputnu ligzdu postījumu apjomu: mākslīgo ligzdu eksperiments

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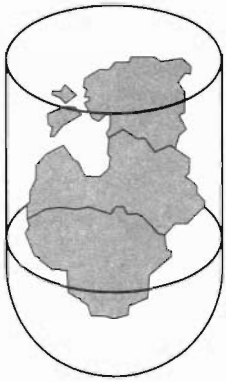
Kopsavilkums

Ligzdu postījumiem ir nozīmīga ietekme uz putnu ligzdošanas sekmēm un to izdzīvošanu. Lai izpētītu dažādu plēsēju sugu veiktos ligzdu postījumus, ir veikti daudzi pētījumi, izmantojot gan dabīgās, gan mākslīgās ligzdas. Šī darba mērķis bija noskaidrot atsevišķu ligzdu postītāju veikto ligzdu postījumu mijiedarbību, t.i., vai izmaiņas viena plēsēja veikto ligzdu postījumu biežumā un ligzdu patēriņā izmaina citu plēsēju veikto ligzdu postījumu biežumu. Mākslīgo ligzdu postījumu eksperiments tika veikts Engures ezera Lielrovas salā 2002. un 2003. gadā, attiecīgi 5,68 ha un 10,17 ha lielos parauglaukumos. Eksperiments parādīja, ka barības pieejamība (t.i., ligzdu skaits) un citu plēsēju darbība būtiski ietekmē pārējo ūdensputnu ligzdu postītāju sabiedrības sugu veikto ligzdu postījumu apjomu. Plēsēju veikto ligzdu postījumu biežums bija atkarīgs no tiem pieejamo ligzdu skaita. Iegūtie eksperimenta rezultāti parādīja, ka izmaiņas savstarpēji konkurējošu mākslīgo ligzdu postītāju veikto ligzdu postījumu biežumā būtiski ietekmē citu šajā teritorijā sastopamo plēsēju veikto ligzdu postījumu biežumu.



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Regulation of cytokinin response-competence by cold treatment of mature *Pinus sylvestris* tissues *in vitro*

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Abstract

One of the possible reasons for lack of morphogenesis during tissue culture of mature tissues is the inability to respond to cytokinin. In the present experiments, cold incubation of pine (*Pinus sylvestris* L.) bud explants was used as a means to affect cytokinin response competence. Pulse treatment with cytokinins of pine bud explants cultivated at 22 °C did not result in increased morphogenesis. New bud and shoot formation was achieved on pine bud explants which were pulse-treated with BAP in combination with cold incubation. It is concluded that, due to the temporary nature of juvenilization of mature pine bud explants, it is necessary to combine both physical and biochemical means to promote morphogenic competence and to achieve further development in tissue culture.

Key words: cold incubation, cytokinin response competence, morphogenesis *in vitro*, *Pinus sylvestris*.

Introduction

Micropropagation is the most effective vegetative reproduction technique permitting rapid propagation and distribution of elite genotypes. Micropropagation of woody species with mature tissues could be very valuable, but it is often difficult, especially, with conifers. Mature tissues of *Pinus sylvestris* L. have a very low morphogenic potential *in vitro* (Bonga 1987; Hohtola 1988).

Several methods of pretreatment for the rejuvenation of mature conifer tissues or the increase of their morphogenic potential have been described – serial grafting (Huang et al. 1992; Ewald, Kretzschmar 1996; Centeno et al. 1998), cold or frozen storage of detached resting buds (Andersone, Ievinsh 2002), spraying of donor plants with cytokinin (Salonen 1991). The timing of collections is also a critical factor (Bonga 1987; Andersone, Ievinsh 2002). However, only brachioblast and needle formation is achieved on mature Scots pine bud explants (Andersone, Ievinsh 2002). Cultivation of the explants on media containing cytokinin did not result in new bud or shoot formation. One of the reasons for that could be very low cytokinin response-competence of the mature *P. sylvestris* tissue.

It is well known that bud production or adventitious branching is usually achieved by increasing the cytokinin level in the tissues. However, extremely rapid multiplication

in tissue culture due to high levels of cytokinin may lead to genetic changes (Peschke, Phillips 1992). It is also known that a high concentration of cytokinin, while stimulating organ induction, inhits their elongation and development (Salonen 1991). Therefore, pulse treatment with cytokinin between subcultures can be used to achieve a high level of multiplication without undesirable genetic effects. In experiments with mature intact conifer trees, repetitive cytokinin treatment has been used for juvenilization leading to development of new shoots (Krikorian 1982).

The aim of the present study was to investigate the possibility of using a pulse treatment with cytokinin as well as to affect cytokinin response competence in mature P. *sylvestris* tissues by means of cold treatment of established *in vitro* culture of bud explants.

Materials and methods

Plant material was collected from mature pine (*Pinus sylvestris* L.) trees in a seed orchard near Salaspils (Riga Region, Latvia). Buds were taken randomly from different trees from the lower part of the crown. Collection was carried out from the second half of July till the first half of August. Newly formed buds were used. They were surface sterilized with a half-diluted commercial bleach ACE (Procture and Gamble; containing 5 - 15 % sodium hypochlorite) for 20 min, rinsed for 10 min in sterile distilled water, sterilized again in 15 % hydrogen peroxide and rinsed three times for 10 min in sterile distilled water. The buds were peeled and dissected aseptically. Explants were cultivated in 20 × 200 mm glass test-tubes containing 10 ml agarized nutrient medium. Tubes were closed with cotton-wool

Table 1. Media used for cultivation of *Pinus sylvestris* bud explants

	Basal medium (BM)	Medium No. 1 (M1)	Medium No. 2 (M2)	Medium No. 3 (M3)
Woody Plant Medium mineral salts	+	+	+	+
Myo-inositol	100 mg l^{-1}	100 mg l^{-1}	100 mg l^{-1}	100 mg l^{-1}
Thiamine hydrochloride	30 mg l^{-1}	30 mg l^{-1}	30 mg l^{-1}	30 mg l^{-1}
Pyridoxine hydrochloride	10 mg l^{-1}	10 mg l^{-1}	10 mg l^{-1}	10 mg l^{-1}
Nicotinic acid	10 mg l^{-1}	10 mg l^{-1}	10 mg l^{-1}	10 mg l^{-1}
Glycine	1 mg l^{-1}	1 mg l^{-1}	1 mg l^{-1}	1 mg l^{-1}
Benzylaminopurine	–	–	100 mg l^{-1}	–
Naphthylacetic acid	–	0.1 mg l^{-1}	0.2 mg l^{-1}	0.2 mg l^{-1}
Adenin	–	10 mg l^{-1}	–	250 mg l^{-1}
Kinetin	–	1 mg l^{-1}	–	25 mg l^{-1}
Sucrose	45 g l^{-1}	45 g l^{-1}	45 g l^{-1}	45 g l^{-1}
Agar	7 g l^{-1}	7 g l^{-1}	7 g l^{-1}	7 g l^{-1}
pH	5.6 - 5.7	5.6 - 5.7	5.6 - 5.7	5.6 - 5.7

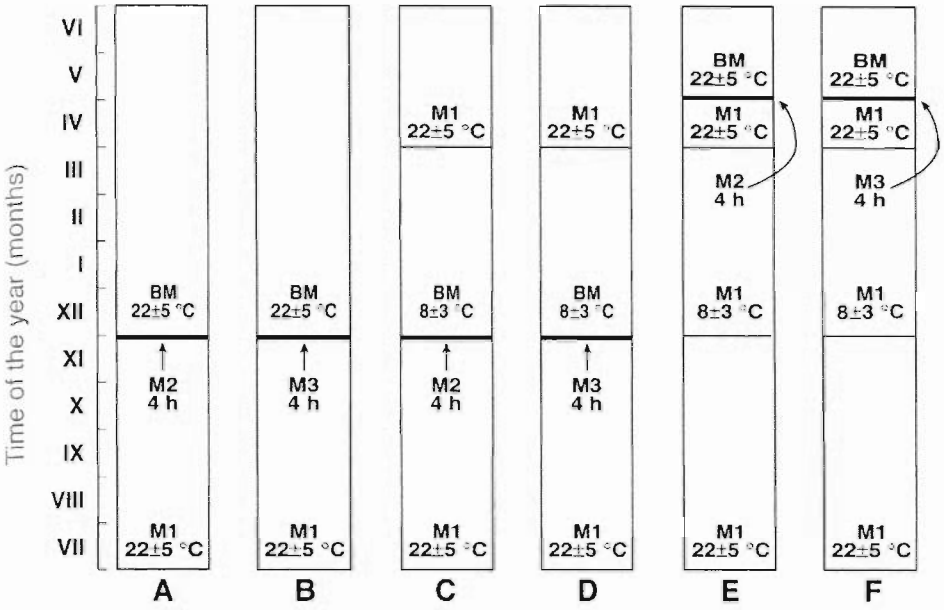

Fig. 1. A diagram outlining the experimental procedure. Time sequence, cultivation media used, as well as temperature regimes are shown for experimental variants A to F.

plugs, and covered also with polythene film fixed with a rubber band. Each tube contained one explant. During the entire time they were cultivated in natural lighting.

For culture establishment medium M1 was used (Table 1). Pulse treatment with high concentrations of cytokinins was carried out on media M2 (containing benzylaminopurine, BAP) or M3 (containing kinetin and adenin) for 4 h. After the cytokinin treatment, basal medium (BM) without growth regulators was used. During cold storage, explants were cultivated at 8 ± 3 °C; control explants were maintained at 22 ± 5 °C.

Six different variants of cytokinine treatment in combination with or without cold storage were tested (Fig. 1). For each variant three replicates of 10 bud explants per treatment were used.

Results

Pulse treatment with cytokinins of pine bud explants cultivated at 22 °C (variants A and B) did not result in increased morphogenesis (Table 2). New bud and shoot formation was achieved on pine bud explants (variants C and E) which were pulse-treated with BAP in combination with cold incubation (Table 2). The effect was more pronounced when the treatment was performed after the cold incubation (40 %) in comparison with the treatment performed before the cold incubation (20 %). Also, the average amount of microshoots per one shoot-forming explant was higher in variant E than in variant C (Table 2). Kinetin in combination with adenin did not result in bud and shoot formation (Table 2, variants D and F).

In both variants of BAP treatment in combination with cold incubation (variants C

Table 2. The effect of cold storage on cytokinin response-competence of mature *Pinus sylvestris* tissues *in vitro*. Data are means from 3 replicates (10 explants per treatment) ±SE

	Treatment					
	A BAP	B Kinetin + adenin	C BAP followed by cold	D Kinetin + adenin followed by cold	E Cold followed by BAP	F Cold followed by kinetin + adenin
Bud explants, forming microshoots (%)	0	0	20 ± 2	0	40 ± 3	0
Microshoots per explant	0	0	5.5 ± 0.8	0	6.8 ± 1.2	0

and E), buds and microshoots developed directly on the lower part of the explants (Fig. 2A, D). Elongation of these buds occurred only in variant E (Fig. 2D). In variants C and E, also brachioblasts with short needles were formed on upper and middle part of explants (Fig. 2A, D). In variant C, axillary shoots developed between these needles and subsequentially elongated (Fig. 2B). The longest of these elongated axillary microshoots were isolated and transferred to a fresh BM medium (Fig. 2C).

Discussion

In previous experiments with *P. sylvestris* it was found that about 15 % of mature pine buds introduced *in vitro* in the second half of summer form brachioblasts with needles (Andersone, Ievinsh 2002). However, further development of these explants was not obtained: buds with needles as well as buds without needles did not survive longer than eight months *in vitro*. No further multiplication was achieved even on media with high concentrations of cytokinins. This situation is similar to the observation that micropropagated plants of mature origin may retain their physiological maturity during tissue culture (Nas et al. 2003). Consequently, inability to respond to growth regulators, e.g. cytokinins, is one of the characteristics of the "out of hormonal response competence" state of mature tissues during *in vitro* cultivation.

It has been suggested that accumulation of competence to induction is important before a particular induction phase during shoot organogenesis (Christianson, Warnick 1988). Thus, to deal with a lack of induction competence in mature pine explants reflected in lack of cytokinin-response competence, certain conditions should be met. As a possible candidate for such conditions, cold treatment can be used, as it was shown for intact juvenile *P. sylvestris* that cold exposure is necessary for elongation of cytokinin treatment-induced buds (Salonen 1991). In order to improve cytokinin response-competence of pine bud tissues, cold treatment of established *in vitro* cultures at 8 °C was used in the present experiments.

Due to a relatively slow transport of exogenous cytokinins, the concentration of BAP in pine bud tissues obviously decreased in the direction towards the apical part of the bud, explaining why the largest amount of shoots formed at the lower part of the explants. In

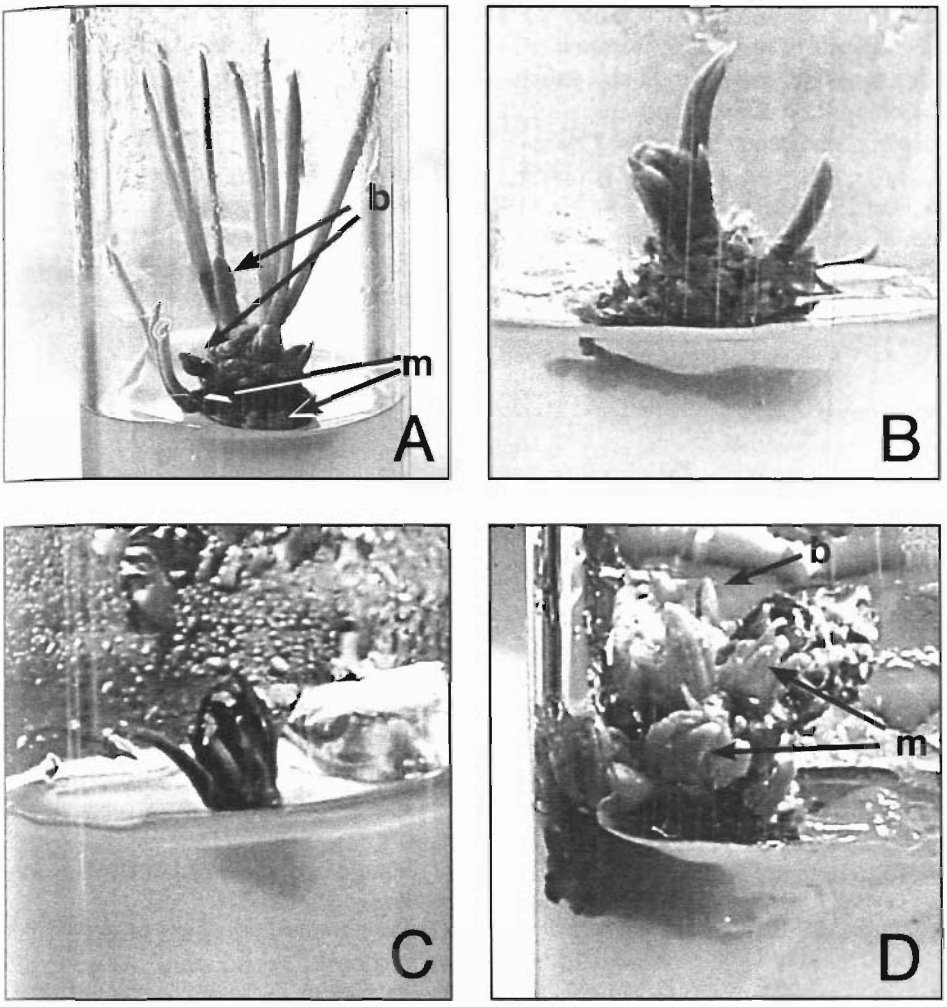

Fig. 2. Development of mature *Pinus sylvestris* bud explants *in vitro* under the effect of cold and cytokinin treatment. A, experimental variant C (according to Fig. 1, pulse treatment with BAP before cold incubation), 4 weeks after the end of the cold incubation; B, variant C, 6 weeks after the end of the cold treatment; C, variant C, isolated axillary shoot on BM medium, 2 weeks after isolation; D, variant E (pulse treatment with BAP after cold incubation), 6 weeks after the BAP treatment. m, microshoots; b, brachioblast with needles.

contrast, secondary needles were formed only in the central or upper part of the pine bud explants, which is in accordance to the observation that a high concentration of cytokinin prevents secondary needle formation (Zhang et al. 2003). Our results were similar to those obtained with *Actinidia deliciosa* explants, where gradient of exogenously applied BAP was responsible for different developmental patterns (Feito et al. 2001).

The effect of cytokinin was more pronounced when the pulse treatment with BAP was performed after the cold incubation (variant E), in comparison with the pulse treatment

before the cold incubation (variant C). Consequently, in the latter variant, during the development of competence in conditions of cold treatment, exogenously applied BAP was considerably decomposed in pine tissues leading to less pronounced cytokinin response. Decomposition of exogenously applied citokinin-like substances in plant tissues is a well known phenomenon (Harrison, Kaufman 1984).

In conclusion, due to the temporary nature of juvenilization of mature pine bud explants, it is necessary to combine both physical and biochemical means to promote morphogenic competence and to achieve further development in tissue culture.

Acknowledgements

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Factors affecting morphogenesis in tissue culture of linseed flax (*Linum usitatissimum* L.)

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Abstract

Factors influencing the morphogenic capability of linseed flax cultivars 'Lirina', 'Barbara' and 'Szaphir' in tissue culture were determined. Two different types of explants (stem segments and hypocotyls) of the three genotypes were cultivated in nutrient media differing in their macro and micro salts concentration and different levels of growth regulators. Our results showed, that callus induction, root formation and shoot regeneration was possible in all types of nutrient media. However, the intensity of morphogenesis was affected not only by the used exogenous growth regulators, but also by the type of explant and genotype. Hypocotyls from all tested genotypes were more responsive in shoot induction than stem segments on all tested media. Calli, shoots and roots were derived at the same time from tested explants in all media depending on the genotype. The best morphogenetic capabilities were demonstrated for cultivar 'Szaphir'. For all cultivars the largest number of shoots was produced on MSB_5 nutrient medium supplemented with cytokinin 6-(y-y-dimethylallylamine)purine (2.0 mg l^{-1}).

Key words: genotype, growth regulators, flax, media composition, morphogenesis, tissue culture.

Introduction

Flax (*Linum usitatissimum* L.) is a dicotyledonous plant of the family *Linaceae*. Commonly, flax breeders use pedigree selection or bulk breeding methods to create novel lines (Steiss et al.1996). The application of biotechnology have been helpful in accelerating breeding programmes or improving the efficiency of selection, as demonstrated in linseed flax and other oil crop species (Friedt 1990). Tissue culture techniques developed for flax mainly aim to obtain valuable and desirable new traits in flax cultivars (resistance to fungal diseases, oil quality improvement and herbicide tolerance) through somatic hybridization and somaclonal variation (Basiran et al. 1987). Cotyledons, hypocotyls, meristems, and stem segments have been used as explants for culture initiation in flax, but only plant regeneration from hypocotyl segments has proven to be highly efficient (Friedt 1990).

The aim of this research was to determine the effect of genotype, explant type and medium composition on linseed flax morphogenesis *in vitro*.

Materials and methods

The investigation was carried out with three linseed flax (*Linum usitatissimum* L.) cvs. 'Lirina', 'Barbara', 'Szaphir'. Stem segments and hypocotyls were used as explants to

study linseed flax morphogenesis. Tissue culture protocols were the same as described previously (Bretagne et. al. 1994; Blinstrubiene et. al. 2004). Explants were placed on combined medium MSB_5 [MS macro salts (Murashige, Skoog 1962) and B_5 micro salts with vitamins (Gamborg 1968)], $0.75MSB_5$ (75 % MS macro salts and B_5 micro salts with vitamins), $0.5MSB_5$ (50 % MS macro salts and B5 micro salts with vitamins) supplemented with sucrose 30 g l^{-1} and Difco-Bacto agar 6 g l^{-1}. The following growth regulators were used: 1.0 mg l^{-1} 6-furfurylaminopurine (kinetin) + 0.1 mg l^{-1} indole-3-acetic acid (IAA), 1.0 mg l^{-1} 6-benzylaminopurine (BA) + 0.05 mg l^{-1} α-naphthylacetic acid (NAA), 2.0 mg l^{-1} 6-(y-y-dimethylallylamine)purine (2iP), 1.0 mg l^{-1} kinetin. The media pH was 5.7 ± 0.1, illumination – 5000 lx, photoperiod – 16 h, temperature 25 ± 2 °C. Each variant consisted of 50 explants and four replications were used. Explants were transferred to 50 ml fresh medium every 4 weeks into 200 ml glassware.

The morphogenetic potential of tissues was evaluated by analyzing the morphological parameters of the structures formed in the explants. The evaluation was based on the relative frequency of explants forming callus, shoots, and roots.

Significant differences were determined using a computer programme (Tarakanovas 1996) for analysis of variance, grouped by Duncan's criteria $P \leq 0.05$.

Results

The tested genotypes and explant types had different intensities of callus formation (Table 1). The percentage of explants producing callus varied from 48 % on hypocotyls of 'Lirina' in $0.5MSB_5$ with 1.0 kinetin + 0.1 IAA) to 100 %. The affect of medium composition on callogenesis was genotype dependent. Cultivar 'Barbara' had the highest frequency of callus formation in all media. The medium MSB_5 with 1.0 mg l^{-1} kinetin

Table 1. Influence of genotype, explant type and medium composition on callogenesis (%) of linseed flax in tissue culture. Means are significantly different at $P \leq 0.05$ (Duncan's multiple range test)

Nutrient media	'Lirina'		'Barbara'		'Szaphir'	
	Stem segment	Hypocotyl	Stem segment	Hypocotyl	Stem segment	Hypocotyl
MSB_5+1.0 kinetin+0.1 IAA	93^b	80^b	100^a	90^b	98^a	85^b
MSB_5+1.0 kinetin	100^a	88^b	100^a	96^a	100^a	95^a
MSB_5+1.0 BA+ 0.05 NAA	95^a	82^b	100^a	90^b	100^a	90^b
MSB_5+2.0 2iP	97^a	85^b	100^a	93^b	100^a	92^b
$0.75MSB_5$+1.0 kinetin+0.1 IAA	60^c	57^d	80^b	70^c	75^c	68^c
$0.75MSB_5$+1.0 kinetin	70^c	68^c	78^c	70^c	75^c	70^c
$0.75MSB_5$+1.0 BA+ 0.05 NAA	100^a	85^b	100^a	87^b	100^a	78^c
$0.75MSB_5$+2.0 2iP	85^b	80^b	100^a	85^b	100^a	80^b
$0.5MSB_5$+1.0 kinetin+0.1 IAA	53^d	48^d	68^c	60^c	65^c	57^d
$0.5MSB_5$+1.0 kinetin	63^c	58^d	62^c	58^d	70^c	50^e
$0.5MSB_5$+1.0 BA+ 0.05 NAA	100^a	68^c	100^a	94^b	100^a	85^b
$0.5MSB_5$+2.0 2iP	80^b	92^b	95^a	85^b	93^b	80^b

Table 2. Influence of genotype, explant type and medium composition on shoot regeneration (%) of linseed flax in tissue culture. Means are significantly different at $P \leq 0.05$ (Duncan's multiple range test)

Nutrient media	'Lirina'		'Barbara'		'Szaphir'	
	Stem segment	Hypocotyl	Stem segment	Hypocotyl	Stem segment	Hypocotyl
MSB_5+1.0 kinetin+0.1 IAA	60^{c}	73^{c}	75^{c}	80^{b}	100^{a}	100^{a}
MSB_5+1.0 kinetin	60^{c}	65^{c}	98^{a}	98^{a}	97^{a}	100^{a}
MSB_5+1.0 BA+ 0.05 NAA	75^{c}	80^{b}	98^{a}	100^{a}	85^{b}	90^{b}
MSB_5+2.0 2iP	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
$0.75MSB_5$+1.0 kinetin+0.1 IAA	8^{e}	15^{e}	10^{e}	18^{e}	15^{d}	23^{d}
$0.75MSB_5$+1.0 kinetin	10^{d}	18^{d}	12^{d}	26^{d}	15^{d}	30^{d}
$0.75MSB_5$+1.0 BA+ 0.05 NAA	14^{d}	20^{d}	20^{c}	24^{d}	30^{d}	38^{d}
$0.75MSB_5$+2.0 2iP	30^{d}	44^{d}	30^{d}	50^{c}	50^{c}	62^{c}
$0.5MSB_5$+1.0 kinetin+0.1 IAA	2^{e}	8^{e}	3^{e}	10^{d}	15^{d}	20^{d}
$0.5MSB_5$+1.0 kinetin	5^{e}	10^{d}	8^{e}	12^{d}	10^{d}	15^{d}
$0.5MSB_5$+1.0 BA+ 0.05 NAA	8^{e}	12^{c}	10^{e}	14^{d}	10^{d}	16^{b}
$0.5MSB_5$+2.0 2iP	18^{d}	30^{d}	25^{d}	30^{d}	35^{d}	42^{b}

was the most suitable for callus induction for all tested genotypes. On this medium, stem segments and hypocotyls of all tested genotypes showed the highest potential of callogenesis. Hypocotyls from all tested genotypes had lower levels of callus induction than stem segments on all media types.

Morphogenesis of the tested linseed flax cultivars depended on the genotype, explant type and nutrient medium composition (Table 2). Shoot induction was higher on hypocotyls than on stem segments from all tested genotypes and media. The genotype 'Szaphir' demonstrated the highest frequency of morphogenesis on all used types of nutrient media, in comparison with 'Barbara' and 'Lirina'. However, the nutrient medium MSB_5 with cytokinin 2iP was mostly suitable for organogenesis in all genotypes, especially for shoot formation. Also, this medium and hormone combination showed the best rhizogenesis in all of the tested cultivars (data not show). Isolated explants of the cultivars 'Barbara' and 'Lirina' also demonstrated the most intensive morphogenesis in complex MSB_5 medium supplemented with cytokinin 2iP, in comparison with the another medium treatments.

Discussion

It is widely considered that morphogenesis is strongly affected by genetic and exogenous factors (Bhaskaran, Smith 1990; Bjowani, Razdan 1990). Our results illustrate that the genetic background is important both for callus induction and shoot regeneration of in linseed flax tissue culture. The linseed flax cv. 'Szaphir' showed the superior morphogenetic capability. The organogenesis capacity was higher in cv. 'Szaphir' by 1.5 and 1.1 times compared to cvs. 'Lirina' and 'Barbara', respectively.

Depending on the plant species, nutrient media are often modified by adding different compositions of vitamins and growth regulators. The most widely used growth regulators

are the cytokinins BAP, 2iP and kinetin (Bjowani, Razdan 1990), and the auxins IAA and NAA. The effect of medium composition on linseed flax callogenesis, shoot formation and rhizogenesis strongly depended on the genotype and the type of the explant, with the three tested linseed flax cultivars exhibiting different regeneration responses. Hypocotyl segments from the linseed flax cultivar 'Szaphir' gave the best results. It can be assumed that the differences in morphogenetic reaction of different linseed flax genotypes are determined by the balance of endogenous hormones. The combinations of growth regulators optimal for callus induction, root formation and shoot regeneration differed. Our results show that linseed morphogenesis capacity depends not only on growth regulators, but also on the other components (macro salts, micro salts, vitamins). The best medium (MSB_5 + 2.0 mg l^{-1} 2iP) differed from the others in raised quantities of added vitamins and amino acids, which allowed more intensive development of callusogenesis and organogenesis in the linseed flax tissue culture.

In conclusion, linseed flax morphogenesis in tissue culture is influenced by endogenous and exogenous factors: cv. 'Barbara' had the highest frequency of callus formation in all media, but the best morphogenetic capability was demonstrated by the cv. 'Szaphir'; hypocotyls showed better morphogenetical ability in comporison with stem segments. MSB_5 nutrient medium supplemented with cytokinin 2iP (2.0 mg l^{-1}) was the mostly suitable medium for linseed flax organogenesis *in vitro*.

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Embryogenesis, callogenesis and plant regeneration from anther cultures of spring rape (*Brassica napus* L.)

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Abstract

Anther culture is a very important and useful tool in plant breeding for haploid production. The investigation was carried out with three spring rape cultivars, 'Trend', 'Landmark' and 'Auksiai'. Rape anthers were cultivated in modified Nitch and Nitch induction medium supplemented with 13 % sucrose and 0.4 % agarose. Embryo regeneration took place in a modified B_5 Gamborg nutrient medium supplemented with 0.1 mg l^{-1} GA_3 and 0.8 % agar. Temperature pretreatment of flower buds was at 35 °C for 72 h. After 72 h thermal shock pretreatment, the callus formation frequency differed for the tested genotypes. Thermal shock pretreatment on the callogenesis process appeared to be genotype dependent, since this treatment failed to significantly increase for 'Auksiai' and significantly decrease callus formation for the 'Landmark' and 'Trend' cultivars. Further embryo development was noted only in genotype 'Trend'. The frequency of embryo formation from anthers treated with high temperature was higher than the control. It was observed that in haploids the stomata length was reduced by 1.4 and their number in the vision field increased by approximately 1.1 in comparison with the diploid parent plants. The height of the haploid plants was 1.4 times less than parent plants. Plant regenerants were 100 % haploids.

Key words: anther culture; *Brassica napus* L; callogenesis; embryogenesis; nutrient media; regeneration; temperature pretreatment.

Introduction

The oilseed rapes (*Brassica napus*, *B. rapa* and *B. juncea*) are now the third most important source of edible vegetable oil in the world. Over the last decade, researchers have made great efforts into developing biotechnological methods to facilitate rape breeding (Kott 1998). Anther culture is an important technique for immediate fixation of homozygosity and shortening the breeding cycle in varietal improvement. The selection efficiency with the doubled haploid lines is higher, especially when dominance variation is significant. Through anther culture, considerable progress and success have been achieved for a large number of economically important crop species, such as barley, wheat, rapeseed, rice, and maize (Lelu et al. 1990). Application of heat stress pretreatment has been an essential factor to increase the efficiency of androgenesis in different species (Kumar et al. 2003). Heat pretreatment has been used to induce embryogenesis from isolated anthers, as it disrupts the cytoskeleton in microspores in the initial phase (Ferrie et al. 1995). Optimal temperature and duration of the pretreatment varies from species to species (Ferrie et al.

1995).

High temperature pretreatment disrupts the normal integrated development of somatic anther tissue and subsequently may synchronise the physiological states of the two tissues, thereby stimulating the induction process (Dunwell et al. 1983).

There are many factors that influence the response frequencies from *in vitro* androgenesis, and these factors may also interact. Some of major factors are genotype, donor plant growth conditions, anther pretreatment, cultivation media composition and environment conditions.

The aim of this research was to investigate the morphogenesis *in vitro* in anther culture of spring rape.

Materials and methods

The investigation was carried out with three spring rape cultivars, 'Trend', 'Landmark' and 'Auksiai'. The donor plants were grown in the growth room with a light intensity of 5000 lx and a 16-h photoperiod. Buds (3 - 4 mm in length) were collected when the microspores were at the mid uninucleate stage. Temperature pretreatment of flower buds was performed at 35 °C for 72 h. Buds were surface sterilized in 70 % ethanol for 2 min and rinsed three times with sterile distilled water. Rape anthers were cultivated in modified Nitsch and Nitsch (Fletcher et al. 1998) induction medium supplemented with 13 % sucrose and solidified 0.4 % agarose. The macro, micro salts and agarose were autoclaved at 115 °C for 30 min whereas the other components of the medium were 0.2 mm filter sterilized. The ready medium was poured into 90 mm diamter sterile plastic Petri dishes (15 - 20 ml medium per dish). Anthers from each bud were removed under a dissecting and inoculated onto the induction medium (20 anthers per Petri dish). The dishes were wrapped with double layers of parafilm and cultivated in the dark. For each treatment, 100 anthers were cultured and the experiment was repeated three times. The maturity morphologic embryos in 28 days after anther isolation were transferred to B_5 Gamborg (Fletcher et al. 1998) regeneration medium supplemented with 0.1 mg l^{-1} GA_3 and 0.8 % agar and cultivated at 25 °C temperature, a 16-h photoperiod, and 5000 lx light intensity. After 30 days, the plantlets with roots, stems and at least three true leaves were transferred to soil. The ploidy level of regenerated plants was determined according to the stomata length at the seventh leaf unfolded growth stage. At flowering, the regenerated plants were evaluated by inspection of the morphological characteristics of flower buds, anthers and pistils, and production of mature pollen grains (Chen et al. 1994).

Data were analyzed using analysis of variance and mean comparisons were made by protected least significant difference at 1 % level of probability.

Results

The whole androgenetic process of anther culture in this study was similar as described previously (Kupriene et al. 2004). Microscopic calli were visible on the surface of the anther following 12 to 14 days culture in induction medium. When thermal shock was not applied the callusgenesis process in induction medium was most intensive for the genotypes 'Trend' and 'Landmark' in comparison with 'Auksiai' (Fig. 1). After 72-h thermal shock pretreatment the callus formation frequency differed for the tested genotypes.

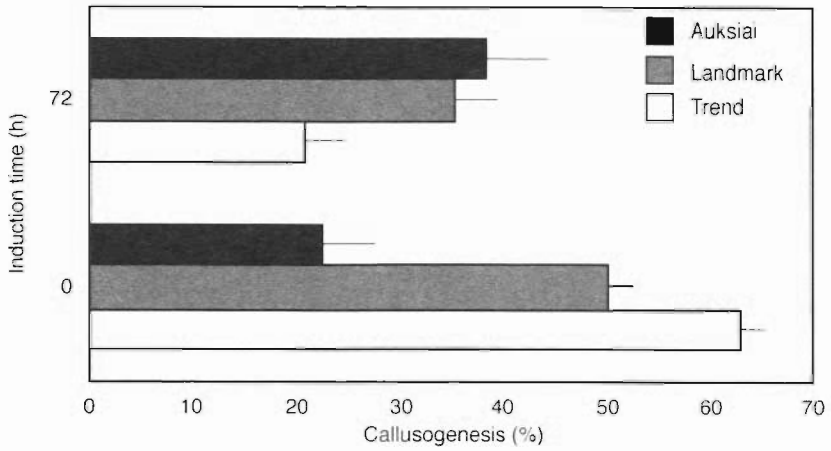

Fig. 1. Callogenesis in spring rape anther culture after 28 days of cultivation

Thermal shock significantly improved the percentage of anthers producing calli for 'Auksiai' from 22.5 % to 38.3 %. However, this same treatment decreased the overall efficiency of callusogenesis for 'Trend' and 'Landmark', in comparison with control, with decreases from 62.9 % to 21.0 % and from 50 % to 35.4 % respectively. After 28 days of cultivation of rape anthers, the callus was yellow white and had watery structure (Fig. 2A). It was transferred to regeneration medium where it did not develop further and did not induce shoot formation.

Direct embryogenesis of anther culture was obtained on induction medium. Embryo development for the genotypes 'Auksiai' and 'Landmark' often stopped after several divisions or during the globular embryo to the heart-shaped embryo stage, followed by embryo death. Further embryo development was noted only in anthers of the genotype Trend when they were cultivated after 72 h of thermal shock. The developmental stages (globular, torpedo and cotyledonary embryos) were observed in embryos developed from generative cells by direct embryogenesis (Fig. 2B, C, D). The frequency of embryo formation from anthers treated with high temperature was higher than for the control (data not show). After 19 days the first morphologically mature embryos were noted. In regeneration medium, embryos first developed a strong primary root with masses of root hairs and in the following week the shoot apex begun to produce young leaves. However not all the embryos were physiologically ready for morphogenesis after 28 days of cultivation. When transferred onto regeneration medium, the morphologically mature embryos formed 62.5 % plants with roots and 37.5 % without roots. Regenerants with roots, stems and at least three true leaves were transferred to soil. Plant survival following transfer to soil was 100 % (Fig. 2F). At the seventh leaf unfolded growth stage, the haploids plants were evaluated for leaf morphometric parameters and stomata length. It was observed that for the haploids, the stomata length was reduced by 1.4 and their number in the vision field increased by approximately by 1.1 in comparison with the diploid parent plants (data not shown). At flowering, the haploids had smaller flowers and did not produce pollens (Fig. 3). The height of the haploids was 1.4 times less than that of parent plants. The plant regenerants were 100 % haploids.

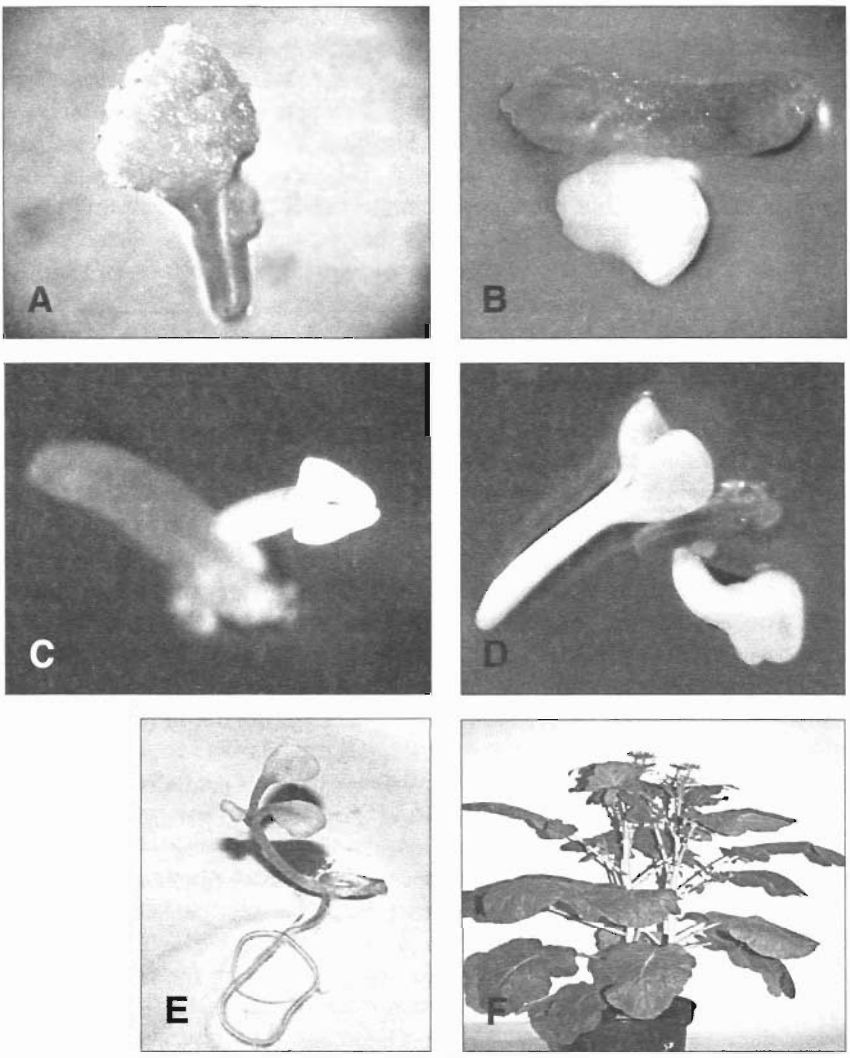

Fig. 2. Embryogenesis, callogenesis and plant regeneration in cultured anthers of rapeseed. A, callus emerging from anthers after 14 days of anther isolation; B, embryos after 15 days of anther isolation; C, direct embryo formation from cultured anthers after 19 days; D, microspore-derived embryo after 28 days of anther isolation; E, regenerated plantlet on B_5 medium; F, haploid plant.

Discussion

High temperature stress is one of the most important but least studied abiotic stresses affecting plant development in tissue culture. The reproductive stage is the most susceptible stage for temperature stress in most crops in which temperature response has been studied (Paulsen 1994; Angadi et al. 2000). Our previous results showed that 35 °C temperature pretreatment for 72 h had a positive effect on morphogenesis process of

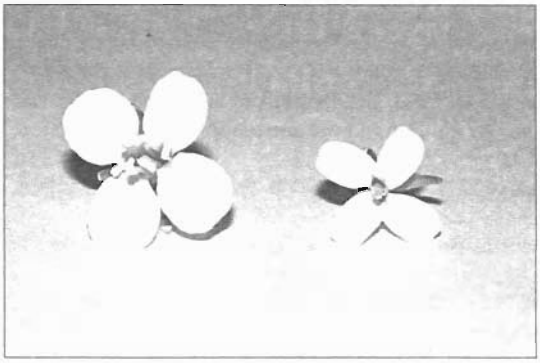

Fig. 3. Flowers of rape regenerants (left diploids, right haploids).

spring rape in isolated anther culture (Kuprienė et al. 2004). However, the effect of anther pretreatment on callogenesis appeared to be genotype dependent since this treatment failed to significantly increase for 'Auksiai' and significantly decreased callus formation for the 'Landmark' and 'Trend' cultivars. We found that the high temperature pretreatment was beneficial for embryogenesis only for the Trend genotype. Morphological variability of embryos among individual cultures is a result of the specific embryogenic competence of the original culture (Burbulis et al. 2000). Although incubation temperature and other cultivation conditions influenced embryo quality, the genetic factor appeared to play an important role in normal development (Chuong et al. 1987; Kott, Beversdorf 1990): the frequencies of normal embryos characterized by a root-shoot axis and cotyledons depended on the genotypes. Frequencies of normal embryos were generally constant in Trend cultures after 35 °C temperature pretreatment (data not shown) while embryos were not found in cultures of 'Landmark' and 'Auksiai'. Genotypic differences in anther culture responses have been previously reported in flax (Nichterlein et al. 1991) and rape (Dunwell et al. 1983).

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In vitro germination of seeds of some rare tropical orchids

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Abstract

The objective of the study was to develop an appropriate method for *in vitro* seed propagation of *Cattleya aclandiae* Lindl., *Cattleya bowringiana* Veitch, *Cattleya granulosa* Lindl., *Cattleya percivaliana* O'Brien, *Cattleyopsis lindenii* (Lindl.) Cogn., and *Dendrobium parishii* Rchb.f. species, members of the *Epidendroideae* subfamily of the *Orchidaceae* family. Special attention was paid to studying the morphology of seeds, different stages of protocorms, and seedlings produced *in vitro*. Two seed types were been recognized according to the classification of Dressler. The highest percent of seed germination (about 100 %) was obtained on the standard Knudson medium supplemented by 2 g l^{-1} peptone, 50 mg l^{-1} potassium hummate and 1 g l^{-1} activated charcoal. It was shown that *Dendrobium parishii* had the shortest (about 300 days) period of *in vitro* cultivation, and *Cattleyopsis lindenii* had the longest (about 600 days). The formation of *Cattleya* seedlings, on average, took about 500 days. By this time, the most advanced seedlings had been transferred to glasshouse. The use of immature seeds capsules as a seed source shortened the reproductive cycle by approximately 2 to 2.5 months. The results of these experiments showed that all of the studied species can be effectively propagated by *in vitro* seed culture with the aim of *ex situ* biodiversity conservation.

Key words: *in vitro* culture, orchids, propagation, seed germination.

Introduction

The application of *in vitro* seed propagation technique to rare tropical orchid species, which are suffering from over-collecting and continuous loss of their natural habitats, undoubtedly is a powerful tool for *ex situ* biodiversity conservation (Stenberg, Kane 1998; Gangaprasad et al. 1999). Many tropical native orchid species have been propagated in the National Botanic Garden of National Academy of Sciences of Ukraine through a range of asymbiotic seed germination techniques and tissue culture procedures aimed to preserve a number of individuals under artificial conditions in glasshouses in the temperate zone, with the aim to protect these species from complete extinction.

The objectives of this study were (i) to elaborate a method of seed propagation for four *Cattleya* species – *C. aclandiae* Lindl., *C. bowringiana* Veitch, *C. granulosa* Lindl., *C. percivaliana* O'Brien, as well as *Cattleyopsis lindenii* (Lindl.) Cogn. and *Dendrobium parishii* Rchb.f.; (ii) to describe the seed morphology; (iii) and to study the development of protocorms and seedlings *in vitro*.

Materials and methods

To obtain seeds, flowers of the studied species were self-pollinated by hand under glasshouse conditions in the National Botanic Garden. Seeds and young seedlings were grown on Knudson (1992) medium modified by addition of 2 g l^{-1} peptone, 50 mg l^{-1} potassium hummate, 1 g l^{-1} activated charcoal. For proliferation of protocorms, Murashige and Skoog (1962; MS) medium supplemented by 5 mg l^{-1} benzylaminopurine (BAP) and 2 mg l^{-1} naphthalenacetic acid (NAA) was used.

Seeds from dehisced capsules were sterilized in 10 % Clorox for 15 to 20 min, in 15 % H_2O_2 for 10 min, then rinsed two times with sterile distilled water. Undehisced immature capsules were surface-sterilized as follows: rinsed with tap water for few minutes, then flamed after spraying with 96 % ethanol. Capsules were cut open and seeds were transferred to cultivation media.

The cultures were incubated in 250-ml Erlenmeyer glass flasks in the laboratory at 25 - 26 °C, photoperiod 16 h and relative moisture of air 70 %. After sowing of seeds, flasks were inspected for seed germination every seven days. The examination of seeds under a SELMI REMMA-102 scanning electron microscope was carried out in the secondary electron emission regime. Prior to examination, dry seeds were mounted with double-sided adhesive tape on aluminium stubs. Then they were carbon-copper-coated using a rotation and tilting specimen stage. For uniform coating of carbon and copper a thermal vacuum evaporator was used. The size of the seeds were measured on the scanning electron micrographs. Micrography was undertaken at an accelerating voltage of 15 kV and working distance of 19 - 22 mm. General classification of seed types followed Dressler (1993).

Results and discussion

The seeds of all species studied were minute, dust-like, ranging from 280 μm (*Dendrobium parisii*) to 830 μm (*Cattleya bowringiana*) in length. The width of seeds did not differ significantly and ranged from 70 μm (*Cattleya percivaliana*) to 100 μm (*Cattleya granulosa*). The seeds of *Cattleya* spp. and *Cattleyopsis lindenii* usually were yellowish, elongate, oblong or slightly fusiform or narrow spindle-shaped. The general morphology of the seeds of all *Cattleya* spp. and *C. lindenii* were almost identical (Fig. 1), and belonged to the *Epidendrum*-type (Dressler 1993). Seeds of *D. parishii* were brightly-yellow, seed testa was intransparent, covered by very fine warts (Fig. 1F), corresponding to the *Dendrobium*-type (Dressler 1993). The seeds usually had the largest diameter near the middle, narrowing toward the polar ends. All seeds had an aperture, the micropylar pole, in one of the ends.

Seed germination of *Cattleya* species on average began after 2 or 3 weeks of culture. By this time embryos had enlarged by two times and occupied the whole seed coat. The seed coat split and the embryos developed into protocorms of 1 to 1.8 mm in length one month after sowing. Initially developing protocorms were elliptic or elongate, becoming clavate or pearshaped with a blunt apex. The colouration of protocorms varied from milk-white at the beginning of germination to brigth-green some time later. As a rule, three to four or more epidermal hairs were produced at this stage. It was observed that the shape of protocorms was species-specific. The protocorms were formed by undifferentiated highly

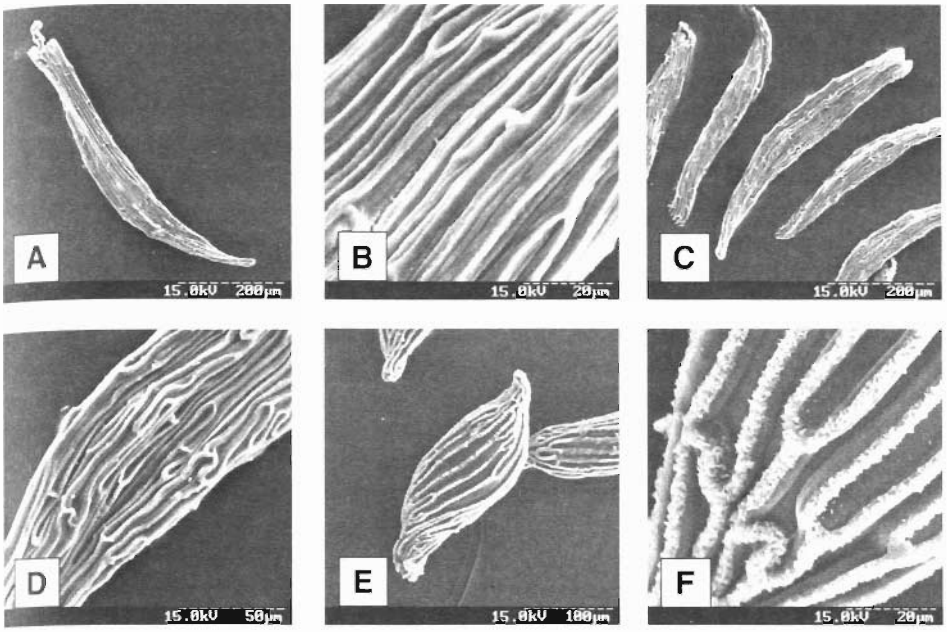

Fig. 1. Scanning electron micrographs of seed specimens studied. A. *Cattleyopsis lindenii*, general view of seed. B. *Cattleyopsis lindenii*, testa cells. C. *Cattleya percivaliana*, general view of seed. D. *Cattleya percivaliana*, testa cells. E. *Dendrobium parishii*, general view of seed. F. *Dendrobium parishii*, testa cells.

vacuolated parenchymal cells, which were surrounded by a single layer of epidermal cells. Later, in apical zone of protocorms the formation of apex and leaf primordia were observed. This was accompanied by the differentiation of procambial and vascular bundles. The formation of protocorms with many meristematic apices began.

In general, during the *in vitro* germination of seeds of *Cattleyopsis lindenii* and *D. parishii*, similar developmental stages of protocorms and seedling were observed. Newly flasked seed of *D. parishii* were noticeably swollen and began greening after 15 to 20 days on nutrient medium. By approximately 45 to 50 days the protocorm stage was reached. At that time the apical meristem with a single leaf primordium had formed on the upper part of the protocorm. One-year-old seedlings of *D. parishii* generally consisted of 3 or 4 leaves and 2 or 3 axillary buds, with a well developed root system formed by adventitious roots 20 mm in length. It is interesting to note that seedlings of *D. parishii* underwent the same annual cycle of development in vitro as adult plants under greenhouse conditions. During a 1- to 1.5-months period (November-December) they rested in the flasks: leaves fell and no symptoms of growth were visible. This can be explained by the fact that in nature *D. parishii* grows in deciduous forest in South-East Asia, where the climate is strongly seasonal with successive dry and wet periods (Lavarak et al. 2000).

In comparison with the other species studied, *D. parishii* had the shortest (about 300-day) period of cultivation *in vitro* – from seed sowing to transferring plants into glasshouse conditions; *Cattleyopsis lindenii* had the longest cultivation period (about 600 days). The formation of *Cattleya* seedlings, on average, took about 500 days. By this time

the most advanced seedlings had been transferred to glasshouse culture conditions. It was shown that, for successful acclimatization, the substrate for seedlings must provide sufficient aeration while holding enough moisture for root development.

Orchid capsules are dehiscent, and seeds for *in vitro* germination may be obtained after dehiscence or even before when the capsule is still unripe. Several studies have shown that the seeds from immature capsules can germinate *in vitro* much earlier (Arditti et al. 1981; Stancato et al. 1998). Our results showed that the capsules of all species studied ripened by 7 to 7.5 months (*C. aclandiae*, *C. bowringiana*, *Cattleyopsis lindenii*, *D. parishii*) or by 9 to 10 months (*C. granulosa*, *C. percivaliana*) after pollination, but seeds from unripe capsules germinated *in vitro* much earlier. Therefore the risk of seed loss and its contamination can be eliminated and the length of the reproductive cycle sufficiently shortened. Also, the utilization of seeds from unripe capsules allows to avoid the negative influences of sterilized substances.

The use of immature seed capsules as a seed source shortened the reproductive cycle by approximately 2 to 2.5 months. The results of these experiments showed that all of the species studied could be effectively propagated by *in vitro* seed culture.

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Tissue culture for elimination of lily viruses depending on explant type

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Abstract

The aim of the present work was to assess the potential of lily virus elimination by tissue culture *in vitro*. The medium used for explants was based on Murashige and Skoog medium. Plantlets regenerated from explants were examined by electron microscopy. Plantlets from basal parts of the petals and of basal pieces of leaves used as explants resulted in the best virus elimination. In conclusion, tissue culture is applicable for virus elimination in lily cultivars and hybrids but the success depends on the type of explants.

Key words: explant, lily *in vitro*, regeneration, virus elimination.

Introduction

Lithuanian breeders have created a great number of cultivars and hybrids of lilies, which constitute a part of the national treasure and ethnic culture (Dainauskaite, Indrišiūnaite 1997). Lilies introduced or created by local breeders are grown in special collection nurseries at the Department of Floriculture of Vilnius University Botanical Gardens (130 cultivars), Department of Plant Systematic and Geography (700 cultivars) and *in vitro* in the Laboratory of Plant Physiology and Biochemistry (48 cultivars). Seven viruses affecting lily have been isolated and identified in Lithuania: *Lily mottle potyvirus* (LMV), *Lily symptomless carlavirus* (LSV), *Tomato ringspot nepovirus* (ToRSV), *Cucumber mosaic cucumovirus* (CMV), *Tobacco rattle tobravirus* (TRV), *Tomato spotted wilt tospovirus* (TSWV) and *Lily X potexvirus* (LXV) (Dapkūnienė et al. 2000). According to some scientists, tissue isolation and cultivation leads to partial release of tissues from viruses (Allen 1974). It has been hypothesized that these cells may have a special system for eliminating viruses (Kubitz 1979). Different explants have been widely used for initial cultivation (Montezuma-de Carvalho, Quimares 1974; Niimi, Onozawa 1979; Globa-Mikhjlenko et al. 1986). The aim of the present work was to investigate the possibility of lily virus elimination by tissue culture *in vitro*, using micro bulbs from scale, bulbils from stem, and segments of the basal part of the petals and leaves as explants.

Materials and methods

The cultivars of the Asiatic hybrid lilies 'Aelita', 'Dzintars', 'Red Beauty' and 'Volchova' were used for cultivation in tissue culture. The bulbils from stem, bulblets from scale, basal pieces of leaves and basal pieces of the petals were used as the explants. The number of explants per variant was 20. Bulblets on the scales were obtained, separated, sterilized and were used as explants (Dapkūnienė et al. 2000). Other explants were sterilized with 0.1 % sublimate ($HgCl_2$) for 5 min and then three times rinsed in sterile distilled water for 15 min. The medium used for explants was based on Murashige and Skoog (1962; MS) medium: twice MS with addition of 1 mg l^{-1} 6-benzylaminopurine, 1 mg l^{-1} naphthaleneacetic acid for basal pieces of the petals by Takayama and Misawa (1982); for bulbils from stem – with addition of 5 mg l^{-1} naphthaleneacetic acid, 0.5 mg l^{-1} kinetin, 30 g l^{-1} sucrose and 10 g l^{-1} agar; MS with addition of 5 mg l^{-1} 6-benzylaminopurine, 0.1 mg l^{-1} naphthaleneacetic acid for bulblets from scale by Jakobsone and Andersone (1997) and MS with addition of 0.1 mg l^{-1} 6-benzylaminopurine, 1 mg l^{-1} naphthaleneacetic acid for the basal part of leaves as explants by Niimi and Onozava (1979). All mother material and plantlets were examined by electron microscopy (Robinson et al. 1987) and DAS-ELISA (Hagita 1989).

Results

The regeneration of explants differed depending on the type of explant and properties of the lily cultivar. Not all explants (bulblets from scale, bulbils from stem, basal pieces of the petals) regenerated at a full capacity (Fig. 1). The basal part of leaves and petals formed embryogenic calluses, from which regenerants were obtained.

The biggest number of regenerants were obtained from embryogenic calluses of basal pieces of leaves (from 166.7 to 309.1 %).

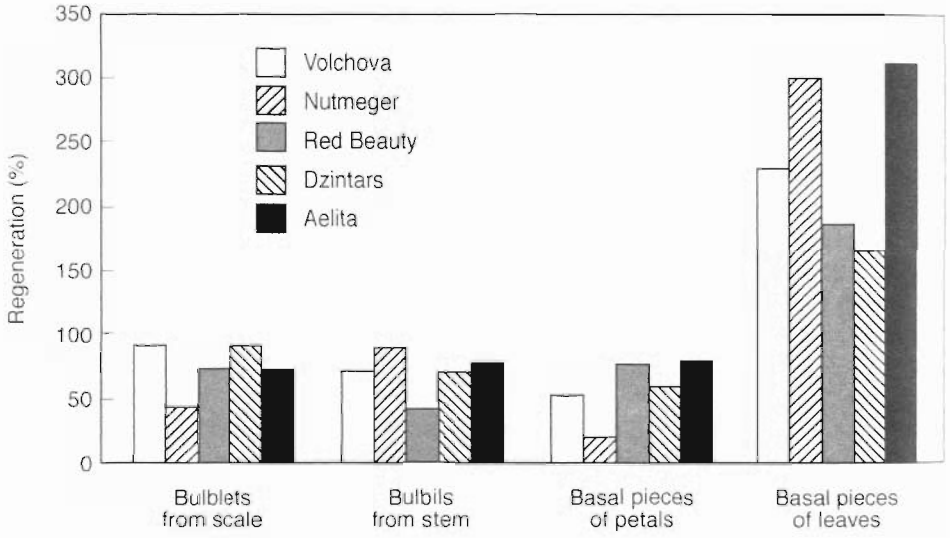

Fig. 1. Regeneration capacity of lily explants (%) of different cultivars.

Table 1. Electron microscopy observation of lily virus infection

Plant material	Morphology of virus particles in lily cultivars			
	'Aelita'	'Dzintars'	'Red Beauty'	'Volchova'
Scale of a mother plant	Isometric (30 and 80-120 nm in diameter), filamentous (lengths of 550, 640-650 and 750-770 nm)	Isometric (30 and 80-120 nm in diameter), filamentous (lengths of 550, 640-650 and 750-770 nm)	Isometric (30 and 80-120 nm in diameter), filamentous (lengths of 550, 640-650 and 750-770 nm)	Isometric (30 and 80-120 nm in diameter), filamentous (lengths of 550, 640-650 and 750-770 nm)
Plantlet of bulblets from scale	No particles	Isometric (30 nm in diameter), filamentous (lengths of 640-650 nm)	No particles	Isometric (30 nm in diameter), filamentous (lengths of 640-650 nm)
Plantlet of bulbils from stem	Isometric (30 and 80-120 nm in in diameter) filamentous (lengths of 750-770 nm)	Isometric (30 nm in diameter)	No particles	Isometric (30 and 80-120 nm in in diameter)
Plantlet of basal parts of the petals	No particles	Isometric (30 nm in diameter)	No particles	No particles
Plantlet of basal pieces of leaves	Isometric (30 and 80-120 nm in in diameter) filamentous (lengths of 750-770 nm)	No particles	No particles	No particles

All mother material and plantlets regenerated from different explants of the lily cvs. 'Aelita', 'Dzintars', 'Red Beauty' and 'Volchova' were examined by electron microscopy (Table 1). Some of the tested lily plantlets regenerated from basal pieces of leaves of cvs. 'Dzintars', 'Red Beauty' and 'Volchova', from the basal part of the petals of 'Aelita', 'Red Beauty' and 'Volchova', from bulb-scale explants of 'Aelita', 'Red Beauty' and from bulbils of 'Red Beauty', were found to be released from virus infection.

Discussion

In vitro propagation technology adopted for the floral industry was aimed initially to control virus and viroid disease problems in several important crops (Daub et al. 1997). For Asiatic hybrids, methods using adventitiously formed meristems on bulb-scale

explants resulted in 50 - 70 % LSV-free plants. No virus-free plants of *Lilium longiflorum* cultivars were obtained using this type of meristem. The culture of isolated vegetative stem apices of the bulbs can be used to release these lilies from LSV. Detection of LSV with ELISA during culture in vitro has a high reliability in contrast to the detection of LMV (Bloom-Batnhoorn, Van Aatrijk 1995). In our case, the bulblets from scale and bulbils from stem explants regenerated directly. The regeneration from the basal part of the petal and basal pieces of leaves proceeded through callus, but more plantlets from one explant were obtained from basal pieces of leaves (Fig. 1). According to virus particle morphology and symptom expression on test plants it was established that mother bulb scale material was infected by LSV, CMV, LMV, TSWV and LXV (Dapkūnienė et al. 2000). Using the tissue culture in vitro technique we obtained release from virus infection in some cultivars. Plantlets from basal parts of the petals and of basal pieces of leaves used as explants resulted in the best virus elimination. Tissue culture is suitable for virus elimination, but success depends on type of explants.

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Use of *in vitro* methods in intersection hybridisation of *Lilium* L.

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Abstract

For the study, 16 lily genotypes were chosen, including five Latvian varieties well adapted to the Latvian climate. The genotypes belong to three different sections and two intersection hybrids. Ovaries were cut in the 5th, 10th, 14th, 20th and 23rd days after pollination and cultured on Murashige and Skoog basal medium supplemented with 9 % sucrose, 0.7 % agar and 1.0 mg l^{-1} naphthaleneacetic acid. In 30 till 40 days, seeds were excised and cultured on embryo culture medium. The pollen fertility of tested genotypes was very different, ranging from 5 % till 83 %. The period of pollen germination depended on the genotype. Most of the genotypes had maximum pollen germination after 16 to 24 h, but for variety 'Pārgalvīgā' only after 40 h. For the major part of genotypes used in the investigation, 10-day-old ovaries were more successful. Hybrid embryos were established on 22 crosses, and bulblets were obtained from seven cross combinations, including four combinations from in-section hybrid crosses and three from intersection crosses.

Key words: intersection hybridisation, *in vitro*, *Lilium*, ovary culture.

Introduction

The conventional practice in lily breeding is in-section hybridisation. Most of widely grown cultivars of Asiatic hybrid lilies originated from crosses between species of the *Sinomartagon* section. The next popular hybrid group is the Oriental hybrid lilies, obtained from crosses within the section *Archelirion*. In the section *Leucolirion Lilium longiflorum* cultivars are an economically important group of lilies. *Aurelian* hybrids, originated from crosses between species *L. regale*, *L. sargentiae* and *L. henryi* of the section *Leucolirion* are suitable for outdoor conditions. Traditionally in lily breeding, cultivars from the *Sinomartagon*, *Archelirion* and *Leucolirion* sections are used for in-section crosses (De Jong 1974; Schenk 1990; Van Creij et al. 1990). However, this method cannot be used to combine traits from cultivars belonging to different sections.

To improve the assortment of lily by traits transmission from *Lilium* species of different sections, intersection breeding was begun in the 1970s. This was achieved in connection with the development of *in vitro* methods, because using crosses of genetically distant forms resulted in problems of fertilisation and growth ability of embryos. Several techniques, such as cutting of style, the grafted-style, *in vitro* isolated ovule pollination technique, embryo, ovary-slice and ovule *in vitro* culture methods, have been developed to overcome the pre-and post-fertilization barriers (Van Tuyl et al. 1991; Roh et al. 1996;

Van Tuyl et al. 1998).

Many lily breeding programmes has been carried out to obtain cultivars with improved flower longevity (Van der Meulen–Muisers, Van Oeveren 1990), highly ornamental flowers, and resistance to virus (Dapkuniene et al. 2000), bulb rot (caused by a fungus *Fusarium oxysporum* f. sp. *lilii*) (Löffler et al. 1990) and *Botrytis* (Doss et al. 1986; Koster, Meer 1993) etc. In the Latvian climatic conditions breeding for resistance against *Botrytis* is especially important. Ecological plasticity and flower longevity together with special flower colour and shapes also are important traits for Latvian lily breeders. The aim of the investigation was to elaborate a tissue culture method for obtaining in a short time new initial material for ornamental lily cultivars with good resistance to *Botrytis* and ecological plasticity.

Materials and methods

For the study 16 lily genotypes were chosen, including five Latvian varieties well adapted to the Latvian climate. The genotypes belong to three different sections and include two inter-section hybrids (Table 1). All of the used varieties were complex in-section hybrids. The bulbs of species *L. henryi*, *L. henryi* var. *citrinum* and Latvian lily varieties were obtained from the collection of the Botanical Garden of the University of Latvia. The hybrids *L. longiflorum* × *L. auratum* was obtained from Lindel Lilies Ltd, Canada, and the cultivars 'Casa Blanca', 'Gran Paradiso', 'Leslie Woodriff', 'Tetra Oriental', 'Aristo', 'Longistar', 'LA hybrids' from Bishoff-Tulleken Lilies, Holland. Some of the used genotypes ('Aristo', 'Eksotika', *L. henryi*, *L. henryi* var. *citrinum*) have a high level of resistance against *Botrytis* infection. Chromosome analysis was carried out for determination of chromosome number in root-tip cells (De Jong 1974).

Before pollination the pollen viability was checked. The pollen was collected from all genotypes used in the hybridisation. The pollen was put on Petri dishes in a culture medium (20 g l^{-1} sucrose, 75 mg l^{-1} gibberellic acid, 7 g l^{-1} agar) and cultivated 16 to 40 h at 25 °C in the dark. Germinated pollen was counted after 16, 24, 32 and 40 h. The viability of pollen was expressed as a percentage. In hybridisation only genotypes with a pollen viability 5 % and higher were used. In total 22 different crosses were made (Table 2). Hybridisation was conducted from mid July till early August both in a greenhouse and outdoor. For better pollination efficiency, stigmata were sprayed with proline solution in water (200 mg l^{-1}) two days after castration. Pollination was done on the third day after

Table 1. Lily genotypes used for hybridisation. *, lily varieties of Latvian origin; **, intersection hybrids

Section	Genotype
Sinomartagon	'Aristo', 'Banga', 'Gran Paradiso', 'Orfejs'*
Archelirion	'Casa Blanca', 'Liesma'*, 'Tetra Oriental', 'Leslie Woodriff'
Leucolirion	'Eksotika'*, 'Līksna'*, 'Pārgalvīgā', *L. henryi*, *L. henryi* var. *citrinum*
Sinomartagon × *Leucolirion*	'Longistar'**, 'LA hybrid'**
Leucolirion × *Archelirion*	*L. longiflorum* × *L. auratum*

castration. Repeatedly three days after pollination, stigmata were sprayed with a 75 mg l^{-1} gibberellic acid solution and 15 mg l^{-1} boric acid in water. The temperature ranged from 20 °C to 25 °C during pollination. For each cross, one to three ovaries were cut on stage when ovaries looked soft and were intensively green, which was found to be optimal in preliminary experiments (Table 2).

Sterilisation methods described earlier were used (Ornicāne, Rashal 1997). For *in vitro* cultivation the method described by Van Creij et al. (1990) with some modification was used. Ovaries were transversely cut in six to eight 2-to 3-mm-thick slices. Six to eight slices were placed on a Petri dish with Murashige and Skoog (1962; MS) basal medium, supplemented with 9 % sucrose, 0.7 % agar and 1.0 mg l^{-1} naphthaleneacetic acid. The medium was adjusted to pH 5.8 by NaOH and sterilised two times by autoclaving for 20 min. Ovary slices were incubated at 25 °C in the dark. After 30 to 40 days culture, establishing seeds were excised from the ovary slices and cultured on germinating medium: MS medium with 5 % sucrose, 0.7 % agar, 1.0 mg l^{-1} naphthaleneacetic acid (pH 5.5 before autoclaving). Seeds were incubated at 17 °C in the dark about a month, till embryos formed. Embryos were transferred on Petri dishes with growth stimulation

Table 2. Hybrid combinations used and the time after pollination, when ovaries were cut. First member of a combination represents a mother plant

Cross combination	Time after pollination (days)				
	5	10	14	20	23
'Aristo' × 'LA hybrid'					×
'LA hybrid' × 'Aristo'					×
'Banga' × 'Aristo'	×				×
'Orfejs' × 'Aristo'				×	
'Longistar' × 'Aristo'				×	
'Pārgalvīgā' × 'Casa Blanca'	×		×		
'Pārgalvīgā' × *L. henryi* var. *citrinum*	×		×		
'Tetra Oriental' × 'Līksna'	×				
'Liesma' × 'Tetra Oriental'	×				
'Tetra Oriental' × 'Gran Paradiso'		×			
L. henryi var. *citrinum* × 'Pārgalvīgā'		×			
'Pārgalvīgā' × (*L. longiflorum* × *L. auratum*)		×			
(*L. longiflorum* × *L. auratum*) × 'Pārgalvīgā'		×			
'Eksotika' × 'Pārgalvīgā'		×			
'Eksotika' × *L. henryi* var. *citrinum*		×			
'Casa Blanca' × 'LA hybrid'		×			
'Casa Blanca' × *L. henryi* var. *citrinum*		×			
'Casa Blanca' × 'Tetra Oriental'		×			
'Tetra Oriental' × 'Pārgalvīgā'		×			
'Tetra Oriental' × 'Eksotika'		×			
'Leslie Woodriff' × 'Eksotika'		×			
'LA hybrid' × 'Pārgalvīgā'		×			

medium: MS medium with 2 % sucrose, 0.7 % agar, 1.0 mg l^{-1} naphthaleneacetic acid (pH 5.0 before autoclaving). Embryos were incubated at 25 °C in the light (16 h day, light 3000 lx). After developing a leaf and root system, bulblets were transferred to pots with soil mix (one part sand / one part peat; v/v) and placed in a growth chamber (16 h day, light 3000 lx, 18 °C to 22 °C). In three to four weeks bulblets were planted in a greenhouse.

Results

The number of chromosomes was determined for genotypes with absent preliminary information (Table 3). It was found that most of those genotypes had 24 chromosomes (2n), three of them were mixoploids with fractions of cells with 24 and 36 chromosomes (3n).

The pollen fertility of genotypes included in the experiment was very different and ranged from 5 % ('Pārgalvīgā', 'Leslie Woodriff') to 83 % (*L. henryi* var. *citrinum*). The period of pollen germination depended on the genotype. Most of the genotypes had the maximum pollen germination after 16 to 24 h, but the variety 'Pārgalvīgā' only after 40 h.

Twelve percent of the ovaries were infected on the growth medium regardless whether the source plants were grown in a greenhouse or in outdoor conditions. Seventeen percent of the planted ovaries either did not start to grow and were damaged after about one week, or formed only calli and did not form seeds. Twenty one percent of the ovaries started to grow and formed seeds, but seeds were without embryos. Fifty percent of the ovary cultures formed seeds with hybrid embryos. Hybrid embryos were established on 14 hybrid combinations (Table 4). Plantlets were obtained only from seven hybrid combinations, including four combinations from in-sections crosses and three from intersection crosses. Hybrid embryos from the other seven crossing combinations were underdeveloped and dead after placing on embryo growth stimulation medium. It was extremely important to determine the stage of ovaries. When ovaries were too young, after planting on medium they failed start to grow or calli were formed. When ovaries were too old, they formed seeds without embryos. For the major part of genotypes used in the investigation, the most successful were 10-day-old ovaries. Our experience showed that cutting time depends both on genotype and growth conditions.

Table 3. Estimated chromosome numbers of different lily genotypes

Genotype	Number of chromosomes (2n)
'Banga'	24
'Līksna'	24
'Liesma'	24
'Orfejs'	24
'Eksotika'	24 + 36
'Pārgalvīgā'	24 + 36
L. longiflorum × *L. auratum*	24 + 36
L. henryi var. *citrinum*	24

Table 4. Embryo formation and number of obtained bulblets of different lily cross combinations. ×, embryos formed; -, no emryo formation

Cross combination	Embryo formation	Number of obtained bulblets
'Aristo' × 'LA hybrid'	×	0
'LA hybrid' × 'Aristo'	-	0
'Banga' × 'Aristo'	-	0
'Orfejs' × 'Aristo'	×	3
'Longistar' × 'Aristo'	-	0
'Pārgalvīgā' × 'Casa Blanca'	×	0
'Pārgalvīgā' × *L. henryi* var. *citrinum*	×	2
'Tetra Oriental' × 'Līksna'	×	1
'Liesma' × 'Tetra Oriental'	-	0
'Tetra Oriental' × 'Gran Paradiso'	-	0
L. henryi var. *citrinum* × 'Pārgalvīgā'	×	0
'Pārgalvīgā' × (*L. longiflorum* × *L. auratum*)	-	0
(*L. longiflorum* × *L. auratum*) × 'Pārgalvīgā'	×	0
'Eksotika' × 'Pārgalvīgā'	×	2
'Eksotika' × *L. henryi* var. *citrinum*	×	6
'Casa Blanca' × 'LA hybrid'	×	12
'Casa Blanca' × *L. henryi* var. *citrinum*	-	0
'Casa Blanca' × 'Tetra Oriental'	-	0
'Tetra Oriental' × 'Pārgalvīgā'	×	0
'Tetra Oriental' × 'Eksotika'	×	0
'Leslie Woodriff' × 'Eksotika'	×	8
'LA hybrid' × 'Pārgalvīgā'	×	0

Discussion

The success of intersection crosses depends on many different factors, such as genotype, hybrid combination, poilen viability, time of ovary cutting, ovary sterilisation method, *in vitro* cultivating conditions, plantlet growth conditions etc.

Knowledge of the chromosome number is critical for choosing genotypes for hybridisation (De Jong 1974). Accessions with 36 chromosomes (3n) usually have unbalanced meiosis and do not produce viable gametes therefore: can not be used for hybridisation (Van Tuyl et al. 1991). Using genotypes that contain both 2n and 3n cells was successful and embryos were obtained in such crosses. The variety 'Pārgalvīgā', used in various combinations, was never successful in outdoor crossings. Probably, this is related to a very long period of pollen germination. In our experiment we obtained embryos from crosses with this genotype. After pollination, stigmata were sprayed with gibberellic and boric acid, which prolonged the time of effective pollination and also stimulated the pollen germination.

In our investigation we mainly used genotypes with a chromosome number 2n or 4n.

One 3n variety ('Longistar') was used as a mother plant only in one cross with 'Aristo', but this cross was not successful: hybrid seeds were without embryos. Probably, sterility in this cross was associated with ploidity of the mother plant, because the cross between 'Aristo' and 'LA hybrid', which is from the same group that 'Longistar', resulted in formation of germinating seeds.

Even in the same hybrid combination the direction of crosses is important, shown by comparison of reciprocal crosses. For example, in the hybrid combination 'Aristo' × 'LA hybrid' and (*L. longiflorum* × *L. auratum*) × 'Pārgalvīgā', embryos were formed, but did not in the reciprocal combinations.

In our investigation, we did not observe a big difference in embryos and plantlets obtaining between in-section and intersection crosses, probable because the genotypes used in in-group crosses were from different species and in nature did not interbreed. The study results showed that it was possible to obtain intersection hybrids from Latvian breeding material, which could be used for creating varieties of lilies with wide ecological plasticity and good disease resistance.

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Micropropagation of onion (*Allium cepa* L.)

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Abstract

Bulblet formation, dormancy of plantlets, vitrification of tissue and decreasing regeneration ability are the main factors limiting the efficiency of onion micropropagation. The aim of the study was experimental micropropagation of onion cvs. 'Lietuvos didieji', 'Stutgarten Riesen' and 'Centurion' F1. Onions after desinfection were split radially into four equal sectors and cross-section. Murashige and Skoog medium, supplemented with 1 mg l^{-1} naphthaleneacetic acid, 0.9, 4.4, 8.9, 13.1 μM concentrations of 6-benzylaminopurine (BAP), kinetin (1.1, 5.3, 10.6, 5.8 μM) and 30 g l^{-1} sucrose were used for plant micropropagation. The efficiency for onion micropropagation of the investigated cultivars and type of an explant was assessed according to the ability of explants to regenerate microshoots. The highest number of microshoots (1.8 to 2.4 microshoots per explant) were formed by 'Centurion' F1 and 'Lietuvos didieji' explants, containing stem dome plus basal plate. Experiments with growth regulators showed that the number of microshoots increased when the BAP concentration was raised from 0.9 to 4.4 μM, respectively from 1.0 to 2.1 microshoots per explant. The lower concentration of BAP had a higher efficiency while raised BAP concentration significantly decreased regeneration. The highest micropropagation frequency using kinetin (1.9 to 2.1 microshoots per explant) was obtained at a moderate (10.6 μM) concentration. The regeneration intensity (output of microshoots) was 68 % higher using kinetin in comparison with BAP.

Key words: *Allium cepa* L., explant, growth regulators, micropropagation, onion set.

Introduction

A real applied need for multiple *Allium* crops is in clones of economically important species like garlic (*Allium sativum* L.) and shallots (*Allium ascalonicum* L.) as well as leek (*Allium porrum* L.) and onion (*Allium cepa* L.). These species generally do not propagate by seeds. However, several tissues of onion are capable of a high potential for shoot regeneration (Mohamed-Yasseen et al. 1993), allowing to multiply a large number of useful accessions within a short time and using a small amount of a propagation material.

The first successful reports on micropropagation of onion have been described by several authors (Hussey 1978; Dunstan, Short 1979). Different parts of bulb, immature inflorescences and flowers have been used for vegetative micropropagation. Bulblet formation, dormancy of plantlets, vitrification of tissues and decreasing regeneration ability are the main factors limiting the efficiency of onion micropropagation.

The aim of the experiment was to determine the frequency of onion microshoot regeneration depending on type of the explant and concentration of different growth regulators.

Materials and methods

The experiment was carried out with two onion (*Allium cepa* L.) cultivars 'Lietuvos didieji', 'Stutgarten Riesen') and one hybrid ('Centurion' F1). Onions were grown under open field conditions with diameter from 5.0 to 10.0 mm. In total, 3840 explants of onions of different origin were plated on the regeneration medium with different concentrations of benzylaminopurine (BAP) and kinetin. Fourty explants of different types were used per treatment.

For desinfection, outer peels, roots and part of the stem dome were removed from bulb and ethanol (70 %) and mercuric chloride (0.1 %) solution (12 min) were used. After being rinsed three times in sterile water, the bulbs were split radially into four equal sectors and four cross-sections were made, dividing the bulbs into stem dome, basal plate without stem dome, stem dome with basal plate and therminal part of sheaths. Explants were planted in Petri dishes and placed in a growth chamber at 25 °C temperature, 16 h photoperiod and illumination of 30 μmol m^{-2} s^{-1}. At the first stage explants were planted onto Murashige and Skoog (1962) medium, supplemented with 1 mg l^{-1} naphthaleneacetic acid (NAA) (in all experimental variants), 0.9 to 13.1 μM BAP, 1.1 to 15.8 μM kinetin and 30 g l^{-1} sucrose. After regeneration, explants and shoots were scored and multiplied on a hormone free medium.

Results

Influence of growth regulators

The first micro shoot buds, visual with the naked eye appeared 18 to 32 days after explants were plated on the regeneration medium, respectively after 25 to 45 days, representing the first easily countable micro shoots.

Experiments with growth regulators showed that the number of microshoots increased (from 1.0 to 2.1 microshoots per explant) when the BAP concentration was raised from 0.9 to 4.4 μM. The lower BAP concentration had a higher efficiency while increasing the BAP amount insignificantly decreased regeneration. The highest micropropagation frequency, 1.9 to 2.1 microshoots per explant, was observed using the medium (10.6 μM) kinetin concentration. The output of micro shoots was 68 % higher using kinetin in comparison with BAP.

A similar increase of micro shoot regeneration has been shown by other researchers (Kahane et al. 1992). The investigated varieties differed in onion microshoot regeneration in response to growth regulators. A higher yield of shoots was obtained for explants of the cv. 'Lietuvos didieji' (mean 1 microshoot per explant) using BA, and a mean 1.5 microshoots per explant using kinetin.

Effect of explant type

Explants of the cvs. 'Centurion' F1 and 'Lietuvos didieji' had the highest frequency of regeneration, respectively 1.8 and 2.4 micro shoots per one explant. The highest number of microshoots (1.8 to 24 microshoots per explant, Table 1) were formed by 'Centurion' F1 and 'Lietuvos didieji' explants containing stem dome plus basal plate.

Morphogenesis almost did not occur from explants that consisted of the terminal part of sheaths (0.01 shoots per explant), and these explants did not produce any axillary or

Table 1. Onion (*Allium cepa*) microshoot regeneration (number of microshoots per explant ± SD) in relation to the concentration of BAP and kinetin and the type of the explant. In total 3840 explants (40 explant per treatment) were used in investigation. Data on the effect of the explant type is presented as a pooled results from all of the used concentrations of cytokinins

Variant	'Lietuvos didieji'	'Stutgarten Riesen'	'Centurion' F1
BAP 0.9 μM	0.9 ± 0.2	0.5 ± 0.1	0.7 ± 0.2
BAP 4.4 μM	1.6 ± 0.2	0.9 ±0.1	1.0 ± 0.2
BAP 8.9 μM	0.7 ± 0.1	0.5 ± 0.1	1.0 ± 0.1
BAP 13.3 μM	0.3 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
Kinetin 1.1 μM	1.1 ± 0.1	0.6 ± 0.1	1.0 ± 0.1
Kinetin 5.3 μM	1.3 ± 0.1	1.0 ± 0.1	1.6 ± 0.2
Kinetin 10.6 μM	2.1 ± 0.2	1.5 ± 0.2	1.9 ± 0.1
Kinetin 15.8 μM	1.1 ± 0.1	1.2 ± 0.4	0.9 ± 0.1
Stem dome	0.1 ± 0.0	0.5 ± 0.0	1.0 ± 0.1
Stem dome plus basal plate	2.4 ± 0.2	1.4 ± 0.2	1.8 ± 0.2
Basal plate without stem dome	2.3 ± 0.2	1.4 ± 0.1	1.7 ± 0.2
Terminal part of sheaths	0.01	0.01	0.01

adventitious buds. However, they elongated and grew as single plants and some of them had intensive rhizogenesis.

Discussion

Our experiments assessed the character of onion reproduction, depending on the method of vegetative micro propagation *in vitro*. The regeneration ability of onion micro shoots depended on the concentration of cytokinin in culture medium and the type of explant. Almost no buds were observed on explants containing the terminal part of sheaths. Similarly Dunstan and Short (1979) reported that shoots were not produced from onion explants that did not include a part of apical dome. This might be related to the degree of cell differentiation in tissues of the abaxial sheath base.

Hussey (1978) suggested that axillary buds of onion could be stimulated by rupture of apical dome plus induction by cytokinin. We determined the optimal concentration of growth regulators for microshoot regeneration of onion. BAP had a higher efficiency to stimulate proliferation at the lowest concentration (< 4.4 μM). Kinetin also induced microshoot regeneration and was more effective in comparison with BAP.

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Clonal propagation of *Yucca aloifolia* L.

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Abstract

The optimal nutrient media and concentrations of growth regulators for obtaining *Yucca aloifolia* L. plants from seed culture, axillary branching and rooting of microshoots were determined. The common duration of germination of seeds of yucca *in vitro* on the hormone-free Monnier medium was 152 days to achieve 100 % germination. For the first time, modified Quorin and Lepover medium supplemented with 6-benzylaminopurine (1.5 mg l^{-1}) and naphthalene acetic acid (0.06 - 0.1 mg l^{-1}) was used for adventive shoot formation (7 to 8 shoots per explant) in *Y. aloifolia* epicotyl culture. The effects of indole-3-acetic acid, naphthalene acetic acid indole-3-butyric acid, 2,4-dichlorophenoxyacetic acid and its combinations on root proliferation were determined. The best rooting of microshoots of *Y. aloifolia* was found on Murashige and Skoog medium supplemented with 1 mg l^{-1} indole-3-butyric acid and light intensity 800 to 1200 lux. The rate of regenerant survival reached 87.1 % in a soil mixture of garden soil / peat / sand (2:1:1). The obtained results were useded to develop a the scheme of clonal propagation of *Y. aloifolia*.

Key words: growth regulators, *in vitro* propagation, yucca.

Introduction

Aloe Yucca (*Yucca aloifolia* L. – fam. *Agavaceae*) is a small tree with a natural geographic distribution from the North Carolina coast to central Florida and along the Gulf Coastal Plain to Louisiana. It is also occurs in the West Indies and southeastern Mexico (Elias 1989).

In North America, Aloe Yucca has been used as a source of fibres for making ropes, cord etc. (Anisimova et al. 1939). Extracts from *Y. aloifolia* possess oxytoxic and anti-inflammatory properties (Bahuguna et al. 1991). Steroid saponins like smilogenin, tigogenin, neotigogenin, sarsapogenin and gekogenin have been found in leaves of *Y. aloifolia* (Elmunajje et al. 1965; Waclov-Rozkrutowa 1972). Callus obtained from leaves of Aloe Yucca contains 0.82 % sapogenin, including 75 % tigogenin, and significant amounts of gekogenin, gitogenin and cholesterol (Kaneda et al. 1987; Miura et al. 1987). The Aloe Yucca fruit pulp is used in traditional medicine as a laxative and detergent (Kishor, Sati 1990)

Commonly, successful propagation of *Yucca* L. *in vitro* is achieved by induction of proliferation of axillary buds. (Litz, Conover 1978; Kukufchanka, Kromer 1984) At the University of Florida mass propagation of *Yucca* sp. has been achieved through proliferation of adventive buds on Murashige and Skoog (1962; MS) medium containing

naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) (Litz, Conover 1978). The formation of adventive shoots has been observed upon germination of seeds of *Y. shidigera* on MS medium supplemented with 0.3 mg l^{-1} BAP (MacCarthy, Staba 1985). Scientists from the Netherlands developed a method of mass clonal micropropagation of chimerical *Y. elephantipes* Regel., which was also based on the induction of axillary branching by applying MS medium in the presence of 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA (Pierik, Steegmans 1983). Earlier, in our laboratory we developed procedures of mass propagation of *Y. torreyi* Shafer based on induction of axillary branching on Quorin and Lepover (1977; with 2 mg l^{-1} BAP and 0.4 mg l^{-1} NAA) and MS (with 1 mg l^{-1} BAP and 0.1 mg l^{-1} NAA) nutrient media, which allowed to receive 6 to 14 adventive shoots from one explant (Karpov 2000).

Being the only representative of a genus capable of forming seeds without artificial pollination, *Y. aloifolia* is a potential source for industrial production of steroid saponins. Also, the application of *in vitro* clonal micro-propagation opens new opportunities for duplicating selection forms and hybrids of yucca (Mitrofanova 1997; Butenko 1999; Pierik 1999).

Materials and methods

Juvenile plants obtained *in vitro* in seed culture were used in the present investigation. Isolated seeds were placed on hormone-free Monnier (1968) medium (M). The seed germination was estimated as the ratio of germinated to planted seeds. The frequency of regeneration (R) was measured as a percentage of epicotyls forming adventive shoots.

The efficacy of clonal micropropagation was estimated according to the following formula:

$$K = \frac{N - n}{n};$$

where: N – final number of microshoots;
n – initial number of explants;
K – efficiacy of micropropagation.

Seeds were sterilized in two stages: (i) superficial sterilization of a fruit with 96 % ethanol and (ii) sterilization of the isolated seeds with 70 % ethanol (1 min), followed by rinsing in sterile distilled water. Seeds (10 to 17 seeds per 200 ml flask) were placed on hormone-free M medium.

In the experiments on adventive shoot formation epicotyl culture was used. Microshoots were cultivated on QL medium in two variants with full or a half concentration, supplemented by BAP (0.0; 0.5; 1.0; 1.5; 2.0 and 2.5 mg l^{-1}), 0.04 mg l^{-1} indole-3-acetic acid (IAA), 0.02 - 0.4 mg l^{-1} indole-3-butyric acid (IBA), 0.02 - 0.4 mg l^{-1} naphthalene acetic acid (NAA) and 0.04 mg l^{-1} 2.4-dichlorophenoxyacetic acid (2.4-D) containing 6 % of agar, pH 5.8. Cultures were incubated in a growth cabinet at 22 - 24 °C under a 16-h photoperiod (1000 - 1200 lux).

For microshoot rooting half-strength MS medium was used, which contained 25 mg l^{-1} of iso-inositol, 15 g l^{-1} of sucrose, 55.6 mg l^{-1} $FeSO_4$ $7H_2O$ + 74.6 mg l^{-1} Na_2EDTA, pH 6.5. Auxins were added in the following combinations:

(i) 1 mg l^{-1} IAA; (ii) 1 mg l^{-1} NAA;

(iii) 1 mg l^{-1} IBA; (iv) 2 mg l^{-1} IBA;
(v) 1 mg l^{-1} 2.4-D; (vi) 0.5 mg l^{-1} IAA + 0.5 mg l^{-1} NAA;
(vii) 0.5 mg l^{-1} IAA + 0.5 mg l^{-1} IBA; (viii) 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} IBA;
(ix) 0.9 mg l^{-1} IAA + 0.1 mg l^{-1} 2.4-D; (x) 0.9 mg l^{-1} NAA + 0.1 mg l^{-1} 2.4-D;
(xi) 0.9 mg l^{-1} IBA + 0.1 mg l^{-1} 2.4-D.
MS medium without growth regulators was used as a control.

Results

Germination of the first seeds started on the 9th day of cultivation, and finished on the 152nd day with the 100 %-level of germination. The epicotyls from these seedlings were placed on QL medium containing 0.4 mg l^{-1} of NAA and BAP in various concentrations. Growth of the isolated epicotyls was observed in a range of BAP concentration varying from 0.5 up to 2.5 mg l^{-1}, and axillary branching was observed only in BAP concentration range of from 1.0 to 2.0 mg l^{-1}. The most active axillary branching was observed on QL medium containing 1.5 mg l^{-1} BAP and 0.4 mg l^{-1} NAA. At a decrease of NAA concentration to 0.04 mg l^{-1} and addition of BAP in concentrations of 0.5, 1.0 and 1.5 mg l^{-1}, axillary branching was observed only on medium, containing 1.5 mg l^{-1} of BAP.

Decreasing the concentration of auxin from 0.04 to 0.02 mg l^{-1} and replacing NAA by IBA, IAA or 2.4-D, the most active axillary branching occurred on QL medium containing 1.5 mg l^{-1} BAP and 0.02 mg l^{-1} IBA. The QL medium containing 1.5 mg l^{-1} BAP and 0.02 mg l^{-1} NAA appeared to be ineffective for axillary branching.

When auxins was used in the ratio 1.5 mg l^{-1} BAP to 0.04 mg l^{-1} auxin (NAA, IBA, IAA and 2.4-D), axillary branching was noted only on media, containing NAA or IBA. Thus, the level of efficacy of micropropagation was higher on the medium with NAA (K = 0.8) than on medium with IBA (K = 0.73). However, the frequency of regeneration (R) in the last case was higher (42.3 % against 29.3 % in the variant with NAA).
We also investigated the effect of half diluted QL medium on axillary branching. The pH, concentration of sucrose, iso-inositol and growth regulators remained stable. Axillary branching was seen only on QL medium supplemented with 1.5 mg l^{-1} BAP and 0.04 mg l^{-1} IBA (K = 0.4, R = 40 %). In all other variants (BAP/NAA, BAP/IAA, BAP/2.4-D), axillary branching was not observed.

Using QL medium containing 0.06 mg l^{-1} NAA and 1.5 mg l^{-1} BAP, the number of axillary shoots reached seven (K = 0.83, R = 56.5 %). Replacing NAA by IBA appeared not to be effective (K = 0.38, R = 40 %).

The most active formation of axillary shoots was on QL media supplemented with 1.5 mg l^{-1} BAP + 0.08 mg l^{-1} NAA (max – 8 shoots; K = 0.95, R = 64.2 %) and 1.5 BAP + 0.1 mg l^{-1} NAA (max – 7 shoots; K = 1.04, R = 76 %) (Table 1, Fig. 1).

To determine the optimal medium for rooting of microshoots, the effects of different growth regulators were compared alone and in combinations (Table 2).

Optimal root and shoot growth was observed on MS medium containing 1 mg l^{-1} IBA. Increasing the IBA concentration to 2 mg l^{-1} resulted in callus formation instead of rooting. Light intensity affected rooting of shoots with normal rooting observed in a range from 800 to 1200 lux. An increase of light intensity to 2000 lux resulted in callus formation. The rate of regenerant survival reached 87.1 % in mixture of garden soil / peat / sand at 2:1:1 ratio.

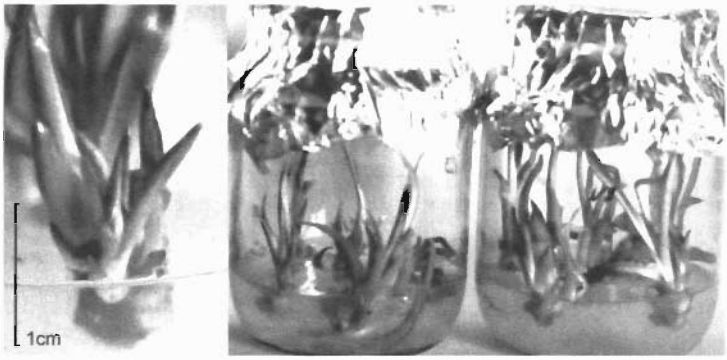

Fig. 1. Axillary branching of *Yucca aloifolia* on QL medium containing 1.5 mg l^{-1} BAP and 0.08 mg l^{-1} NAA.

Table 1. Basic characteristics of axillary shoot-producing cultures of *Yucca aloifolia*. Means ± SE are indicated

Medium and concentrations of growth regulators (mg l^{-1})	Length of maternal shoot (cm)	Characteristics of adventive shoots			K	R (%)
		n_{max}	N ± n	length (cm)		
QL, 1.5 BAP + 0.06 NAA	9.0 ± 3.0	7	3.5 ± 2.3	2.9 ± 2.3	0.83	56.5
QL, 1.5 BAP + 0.08 NAA	7.6 ± 3.2	8	4.0 ± 4.0	3.8 ±3.1	0.95	64.2
QL, 1.5 BAP + 0.1 NAA	6.5 ± 3.5	7	3.5 ± 3.5	4.6 ± 4.0	1.0	76.0

Table 2. Rooting of microshoots of *Yucca aloifolia* and their morphology under the influence of various auxins on the 30th day from the beginning of cultivation. *, abortive roots

Medium and concentrations of growth regulators (mg l^{-1})	Rooting			Callus-forming shoots (%)	Number of chlorotic leaves
	Rooted shoots (%)	Total number of shoots	Number of rooted shoots		
MS	83	12	10*	0	4
MS, 1 IAA	13	32	4	0	2
MS, 1 NAA	0	40	0	70	4
MS, 1 IBA	71	34	24	0	1
MS, 1 2,4-D	0	34	0	100	2
MS, 0.5 IAA + 0.5 NAA	0	22	0	100	2
MS, 0.5 IAA + 0.5 IBA	36	44	16	0	2
MS, 0.5 NAA + 0.5 IBA	0	42	0	43	2
MS, 0.9 IAA + 0.1 2,4-D	0	36	0	39	2
MS, 0.9 NAA + 0.1 2,4-D	13	32	4	33	3
MS, 0.9 IBA + 0.1 2,4-D	0	18	0	100	2

Discussion

The present investigation was conducted in the Nikita Botanical Garden (Yalta), in the Department of Plant Biotechnology and Biochemistry, as a part of the programme on introduction and propagation of yuccas and other arboreal monocotyledonous plants. The main reason complicating the introduction of yuccas is the absence of the natural pollinators – moths of the genera *Tegeticula* and *Parategeticula* (Pellmyr 1999). Therefore, mass seed propagation of these plants are impossible. Vegetative propagation of the most decorative yuccas is also complicated by very low rate of propagation (Novikova 1998).

Y. aloifolia is the sole representative of the genus able to form the seeds in the absence of natural pollinator. Therefore, Aloe Yucca was considered to be the best model object for the numerous experiments on *in vitro* clonal micropropagation of yuccas. Our previous investigations indicated that the easiest way to establish aseptic culture of yucca is to use seed and isolated embryo culture (Novikova et al. 1998, Karpov 2000). In the present experiment, the common duration of *in vitro* seed germination of *Y. aloifolia* on hormone-free Monnier medium (1968) takes 152 days to achieve 100 % germination.

The best formation of axillary shoots of *Y. aloifolia* (max 7 - 8 shoots, K = 0.38 - 1.04, R = 40 - 76 %) in epicotyl culture was observed on QL medium supplemented by 1.5 mg l^{-1} BAP and 0.06 - 0.1 mg l^{-1} NAA. The results are consistent to those obtained earlier in Netherlands for chimerical *Y. elephantipes* Regel., for which the quantity of formed axillary shoots was 7 - 8 per explant (Pierik, Steegmans 1983). As shown by the present investigation, our earlier results (Karpov 2000) and other studies, the best formation of axillary shoots of yucca is usually noted on nutrient media modified with BAP and NAA addition (Litz, Conover 1978; Pierik, Steegmans 1983, Kukufchanka, Kromer 1984). The Quorin and Lepover medium (1977) was used for the first time for axillary shoot-formation in yuccas.

The best rooting of microshoots of *Y. aloifolia* was observed on Murashige and Skoog (1962) medium with 1 mg l^{-1} IBA and light intensity from 800 to 1200 lux.

In accordance the obtained results, optimal clonal propagation of *Y. aloifolia* can be presented as a scheme (Fig. 2).

In conclusion, the results of present study can be used as the basis for clonal propagation of hard-breeding representatives of genus *Yucca* and hybrids that are not able to form the seeds and have a low rate of traditional vegetative propagation.

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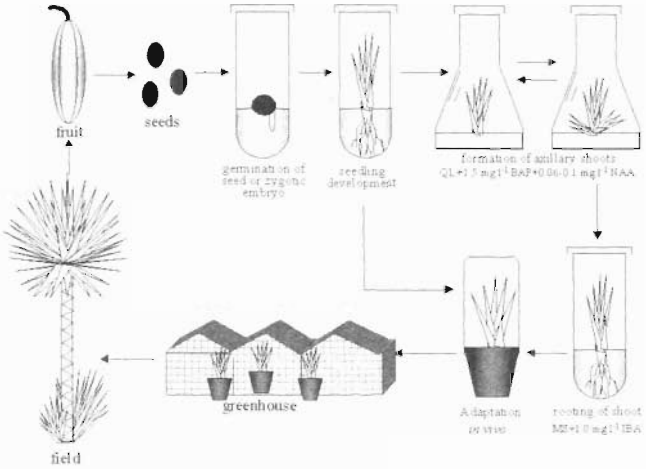

Fig. 2. Scheme of clonal propagation of *Yucca aloifolia* L.

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Tissue culture technology in conservation of threatened plant species of Latvia

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Abstract

To meet the requirements of the Convention on Conservation of Biological Diversity and internationally accepted botanical garden targets, the National Botanic Garden of Latvia started the elaboration of *in vitro* conservation methods for threatened plant species of Latvia. The objective of the present work was to develop a reliable protocol for *in vitro* conservation of threatened plants. Seeds and other materials of 40 threatened plant species from natural habitats were collected. In total 37 species were introduced in sterile conditions. For 20 species culture was initiated by seed germination and for six species – from shoot apex explants. Seed germination in sterile culture was not observed for 11 of experimental species. In five cases seed germination failure might be due to incomplete development of seeds. Normal organogenesis was observed in medium without hormones. In culture, the introduced species had different growth potentials *in vitro*. Development of germplasm conservation technique by minimal growth storage was started for species with a high multiplication rate.

Key words: conservation of biological diversity, endangered wild plants, establishment *in vitro*, multiplication, rooting.

Introduction

As a result of the degradation and destruction of natural habitats, many species have become threatened *in situ* and need conservation. One of the main tasks of Botanical Gardens is local flora conservation, including the elaboration of *ex situ* conservation measures (Wyse Jackson 2000). In accordance to the *ex situ* conservation strategy, it is not desirable to propagate plants from small and genetically restricted samples, because depletion of genetic variability may reduce the ability to adapt in habitats. Therefore, the material obtained from natural populations must represent a wide genetic base (Malda et al. 1999). Tissue culture is used for conservation of biological diversity for multiplying of endangered species that have extremely small populations, for species with reproductive problems and for recovery and reintroduction (Bromwell 1990).

The development of a reliable *in vitro* protocol are of great importance for producing plant material and for conservation of rare plant species, and to offset the pressure on the natural populations as well as plants for medical and ornamental purposes. The selection

of plant material and media for *in vitro* propagation and conservation requires considerable empirical research. The present investigations are focused on the development of *in vitro* techniques for collecting and preserving germplasm of threatened species.

Materials and methods

Material from 40 species from susceptible natural habitats of Latvia including a narrow endemics or species on their border of distribution was collected (Table 1). Explants from aseptically germinated seedlings were used to establish *in vitro* cultures. In several cases when seeds were not available actively growing shoots were excised from plants grown in the field. Explants were surface sterilised with commercial bleach ACE, in case of seeds – without dilution, in cases of shoot apexes – half diluted, for 7 to 20 min, then rinsed in sterile distilled water for three times. The sterile explants were placed in culture vessels on hormone free Murashige and Skoog (1962; MS) basal medium or on MS medium supplemented with growth regulators [0.1 to 0.5 mg l^{-1} 6-benzylaminopurine (BAP), 0.1 to 0.5 mg l^{-1} kinetine, 0.1 to 0.5 mg l^{-1} indole-3-acetic acid (IAA)]. They were stored in a growth chamber at 24 °C with a 16-h photoperiod.

Results and discussion

In total, 37 species were introduced in aseptic conditions. From these, 22 species were germinated in sterile conditions but in six cases culture was initiated from aseptic apexes. Seeds of 11 species failed to germinate due to incomplete development of seeds in five cases and perhaps due to the rest period and dense seed coats. For such species the scarification of the seed coats is essential to obtain good germination or by pre-treatment by immersion in water at 90 °C and soaked for 24 h (Iriondo et al. 1995).

Table 2 provides information on seed germination of various species in aseptic conditions. The percent germination and time for germination differed considerably for each species. In most cases, mature seeds were used and it was found that for germination of some species it is very important to use freshly harvested seeds (*Scrophularia umbrosa*, *Onobrychis arenaria*). Water supply and moisture conditions were very important in the germination process (Oboucheva 1999). Low germination was noted for some species (D'Antuono et al. 2003).

Growth characteristics of species *in vitro* are presented in Table 3. The growth character of species in aseptic conditions was similar to growth in wild conditions: growth in height or formation of rosettes and clumps of multiple shoots. According to multiplication potential, the cultivated species could be divided in three groups: species with a high multiplication rate, species with a medium or low multiplication and species which failed to multiplicate so far. The growth features on medium, mostly without hormones, are summarized in Table 3. The highest proliferation rate (10 to 30) was obtained for *Dianthus arenarius* (both subspecies), *Galium tinctorium* and *Dracocephalum ruyschiana* on MS medium without growth regulators. For propagation of these species with high multiplication rate, growth regulators were not necessary. In contrast, for the Czech Republic endemic plant *Dianthus arenarius* ssp. *bohemicus*, a relatively high BAP concentration (1 mg l^{-1}) was used to obtain a comparably high multiplication (Kovac 1995). For species with a medium or low multiplication potential, considerably increased proliferation was observed only

Table 1. Threatened species of Latvia for conservation *in vitro*. RDB, species listed in the *Red Data Book of Latvia* (category). ED, species protected by *The Europe Union Directive on Conservation of Species and Habitats*. NM, species included in the *National Biological Diversity Monitoring Programme*. (+) only for limited time

Species	Status of protection			Initiation *in vitro*	
	RDB	ED	NM	Seed germination	By apex culture
Agrimonia pilosa	+	+		–	
Ajuga pyramidalis	1			+	
Alisma gramineum	1		+	(+)	
Allium ursinum	3		+	–	
Angelica palustris	1	+	+	+	
Arenaria procera	2			+	
Armeria maritima	1			+	
Carex davalliana	3			+	
Carex ornithopoda	3			+	
Carex pilosa	1		+	–	
Carex supina	1		+	–	
Cinna latifolia	3	+		+	
Corydalis cava	1				
Cypripedium calceolus	2	+	+	–	
Delphinium elatum	2		+	–	
Dianthus arenarius ssp. *arenarius*	–	+		+	
Dianthus arenarius ssp. *borussicus*	+			+	
Dracocephalum ruyschiana	2		+	–	+
Equisetum telmateia	1				
Euphorbia palustris	2		+		(+)
Galium schultesii	2		+	+	
Galium tinctorium	1		+	–	+
Gladiolus imbricatus	3			+	
Helianthemum nummularium	3			+	
Ligularia sibirica	1	+	+	+	
Linaria loeselii	3	+		+	
Liparis loeselii	3	+	+	–	
Lunaria rediviva	4		+	–	
Onobrychis arenaria	3			+	+
Ophrys insectifera	1			–	
Pentaphylloides fruticosa	1		+		+
Peucedanum oreoselinum	3			+	
Polygonatum verticillatum	3			+/–	
Prunella grandiflora	1			+	
Pulmonaria angustifolia	2		+		
Pulsatilla patens	4	+	+	+	
Saussurea esthonica	1	+	+	–	
Scrophularia umbrosa	1		+	+	(+)
Trifolium fragiferum	2			+	
Veronica montana	1		+		+

Table 2. Seed germination *in vitro* of some threathened species. *, results from a separate experiment; **, results from the same experiment

Species	Germination (%)	Germination time (days)
Ajuga pyramidalis	25	78
Angelica palustris	40	28
	55*	112*
Arenaria procera	50	7
	92**	66**
Carex davalliana	28	132
	44*	266*
Carex ornithopoda	60	73
	73*	115*
Cinna latifolia	43	48
Dianthus arenarius ssp. *arenarius*	100	44
Dianthus arenarius ssp. *borussicus*	83	24
Galium schultesii	50	51
Gladiolus imbricatus	17	51
Helianthemum nummularium	100	48
Ligularia sibirica	67	30
Linaria loeselii	13	98
Onobrychis arenaria	88	38
Peucedanum oreoselinum	20	75
Pulsatilla patens	62	96
Scrophularia umbrosa	85	28
Trifolium fragiferum	11	26

after addition of cytokinins to the growth medium (*Arenaria procera*, *Carex ornithopoda*, *Pulsatilla patens*). For species without regeneration (*Ajuga pyramidalis*, *Angelica palustris*, *Gladiolus imbricatus*, *Peucedanum oreoselinum*, *Trifolium fragiferum*) various combinations of growth regulators will be tested for induction of propagation. In contrast to commercial propagation, for endangered species we must avoid elevated cytokinin levels in proliferation optimisation, as it is necessary to produce relatively few plantlets of each genotype for reintroduction or tissue culture collection. Minimal cytokinins and auxins in culture media would avoid somaclonal variation and efficiently produce true-to-type plantlets (Edson et al. 1996). Some authors have suggested the possibility of widening the genetic base of a species by somaclonal variation *in vitro* as a method of generating new vigour into natural populations of endangered species (Bromwell, 1990). However, this conservation strategy is disputable.

In all cases rooting occurred on MS medium without growth regulators and no special rooting medium was used. Nearly all species had good rooting potential, except *Onobrychis arenaria* introduced by shoot apex and *Pulsatilla patens* after the seedling stage. Similarly, some authors have reported a high rooting percetage of *Dianthus* sp.

Table 3. Growth *in vitro* of some threatened species. *, with citokinin BAP. Proliferation rate: ax., the number of axillary shoots obtained from one explant; nod., the number of nodes obtained from one explant. Rooting: + poor; +++ exelent

Species	Growth type	Proliferation rate	Rooting
Ajuga pyramidalis	rosette	1	++
Angelica palustris	rosette	1	+
Arenaria procera	in height	1.3 / 6.0*	+++
	clusters		(81 %)
Carex davalliana	clusters	7.0*	+++
Carex ornithopoda	clusters	1.5 / 4.2*	+++
			(100 %)
Dianthus arenarius ssp. *arenarius*	in height	4.8 ax.	+++
	axillary shoots	10.4 nod.	(100 %)
Dianthus arenarius ssp. *borussicus*	in height	5.0 ax.	+++
	axillary shoots	14.5 nod.	(100 %)
Dracocephalum ruyschiana	in height	4.2 ax.	++
	axillary shoots	14.5 nod.	(61 %)
Galium schultesii	in height	4	++
	axillary shoots		
Galium tinstorium	in height	7 ax.	++
	axillary shoots	30 nod.	
Gladiolus imbricatus	rosette	1	+
Helianthemum nummularium	in height	2 - 4 nod.	++
	axillary shoots		
Ligularia sibirica	clusters	1.3	++
Linaria loeselii	in height	2.7 ax. + nod.	++
	axillary shoots		
Onobrychis arenaria	clusters	1 - 3	
Peucedanum oreoselinum	rosette	1	+ (from seeds)
			+/– (from apex)
Pentaphyloides fruticosa	in height	4 ax. + nod.	+
	axillary shoots		
Pulsatilla patens	rosette	2 / 7*	+/–
Scrophularia umbrosa	in height	2 ax.	+++
	axillary shoots		
Trifolium fragiferum	rosette	1	+
Veronica montana	in height	1.4 ax.	++
	axillary shoots	3.4 nod.	

with different auxines or without them (Kovac 1995; Prolic et al. 2002).

Several species have been introduced *ex vitro*. After transferring the plantlets to the greenhouse, a 28 to 100 % survival rate was achieved (*Dianthus arenarius* 69 %,

Galium tinctorium 84 %, *Dracocephalum ruyschiana* 28 %, *Carex ornithopoda*, *Prunella grandiflora* 95 %, *Ligularia sibirica*, *Helianthemum nummularium* 90 %).

A total of 37 threatened plant species of Latvia have been introduced in sterile conditions. Seed germination time and percent *in vitro* differed considerably among the species. Seedlings for shoot culture initiation were obtained for 20 species. Shoot culture was initiated from shoot type for five species. Cultivated species can be conditionally divided in three groups: species with a high proliferation rate, with a medium or low proliferation rate and species which failed to multiplicate so far. Multiplication potential differed considerably on medium without growth regulators. Introduction of seven species *ex vitro* in greenhouse conditions was successful. Elaboration of slow growth conditions for species with a high multiplication potential is in progress.

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Development of recipient system of woody subtropical plants *in vitro*

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Abstract

The objecive of this study was to develop recipient systems of woody subtropical species and to obtain asimina (*Asimina triloba* Dun.), feijoa (*Feijoa sellowiana* Berg.), kiwi (*Actinidia deliciosa* (Chev.) Liang, Ferguson), persimmon (*Diospyros kaki* L.), and zizyphus (*Zizyphus jujuba* Mill.) plants through direct and indirect regeneration *in vitro*. The morphogenetic capacity of organs and tissues in selected woody subtropical plants was realized via five ways: activation of apical and lateral meristems; direct regeneration of plants from leaves, shoot segments and cotyledons; direct regeneration of seedlings from zygotic embryos; and direct and indirect somatic embryogenesis. High effective microshoot regeneration was induced by using direct regeneration of plants from leaves, shoot segments and cotyledons on modified Murashige and Skoog medium with 1.0 to 6.0 μM thidiazuron. The developed *in vitro* systems of woody subtropical plants allow us to use them for different purposes of plant biotechnology: multiplication, genetic transformation and conservation *in vitro* of valuable genotypes of asimina, feijoa, kiwi, persimmon and zizyphus.

Key words: *Actinidia deliciosa*, *Asimina triloba*, *Diospyros kaki*, *Feijoa sellowiana*, *Zizyphus jujuba*, *in vitro* regeneration.

Introduction

The Nikitsky Botanical Gardens – National Scientific Center (Crimea, Ukraine) has a large plant collection of asimina (*Asimina triloba* Dun.), feijoa (*Feijoa sellowiana* Berg.), kiwi (*Actinidia deliciosa* (Chev.) Liang, Ferguson), persimmon (*Diospyros kaki* L.) and zizyphus (*Zizyphus jujuba* Mill.). The climatic conditions of the Crimean region (Southern part of Ukraine, coast of the Black See) allow us to cultivate most of the woody subtropical plants. Micropropagation via axillary shoot proliferation has been applied to the commercial propagation of fruit trees since the beginning of 1980s (Zimmerman 1991). Recent advances in plant biotechnology have revealed the vast potential of tissue culture for propagation and breeding *in vitro* of fruit plants. Efficient methods for the micropropagation of important tropical fruits (banana, papaya, passion fruit, mango and pineapple) already exist (Litz, Jaiswal 1991; Grosser 1994). The skillful combination of biotechnology methods with classical methods of breeding considerably accelerates the breeding process of subtropical fruit trees (Litz, Jaiswal 1991; Oliveira, Pais 1992; Chalak, Legave 1996; Mitrofanova et al. 1997; Mitrofanova et al. 1998; Zdruikovskaya-Richter 2003).

This paper reports on several ways of plant regeneration *in vitro* in selected cultivars and hybrids of asimina, feijoa, kiwi, persimmon and zizyphus.

Materials and methods

Experiments were carried out with two-year-old field-grown plants of asimina, five to six-year-old field-grown plants of zizyphus (cvs. 'Ya-dzao', 'Kitaiskii 2A', 'Ta-yang-dzao'), feijoa (forms 1 and 2), kiwi (cvs. 'Bruno', 'Monti', 'Tomuri', 'Saanishton' and hybrids 'Monti' × 'Tomuri', 'Bruno' × 'Tomuri'), persimmon (cvs. 'Rossianka', 'Odnodomnaja', 'Korejskaja neterpkaja', 'Bordovaja', 'Meader') and *in vitro* cultivated plants of selected subtropical fruit trees. Mother plant material was collected on the strain testing plot of Nikitsky Botanical Gardens.

Explants (single nodes with bud) were surface disinfected for 1 min in ethanol (70 %), followed by 15 min in a solution containing 1 % Thimerosal (Sigma, USA), and subsequently for 3 to 5 min in a solution containing 0.08 % $AgNO_3$ plus three drops of Tween 80, then rinsed three times in sterile distilled water. In order to induce bud development and future shoot proliferation, isolated buds from field-growth plants of asimina, zizyphus, persimmon, kiwi and feijoa were placed on modified Murashige and Skoog (1962; MS) culture medium and Pierik (1976) medium, supplemented with 0.89 to 8.90 μM benzyladenin (BA), 0.93 to 9.30 μM kinetin and 0.91 to 9.12 μM zeatin.

Seeds were taken from mature fruits of kiwi and persimmon and surface sterilized for 20 min in 1.5 % (w/v) sodium hypochlorite followed by three rinses in sterile distilled water. Fruits of zizyphus and feijoa were submerged in 96 % ethanol and treated in a spirit-lamp flame. The embryo was separated from the surrounding tissues and placed on Monnier (1973) culture medium in a refrigerator at 5 ± 1 °C (10 to 60 days) and then transferred to cultivation room (25 ± 1 °C, 16 h photoperiod, illuminance – 25 μmol m^{-2} s^{-1}).

Leaf discs from *in vitro* cultured microshoots of kiwi were placed with abaxial and adaxial surface on modified MS medium, supplemented with different concentrations of BA (4.40 to 44.00 μM) and indol-3-acetic acid (5.71 to 57.08 μM; IAA). For direct regeneration, explants such as cotyledons (zizyphus), shoot segments (kiwi) and leaf discs (asimina, feijoa, kiwi, persimmon, zizyphus) were cultured on MS medium with different concentrations of thidiazuron (1.0 - 9.0 μM; TDZ).

To produce and cultivate of zizyphus somatic embryos, half-strength MS medium containing 1.1 to 15.8 μM 2,4-dichlorphenoxyacetic acid (2.4-D) and 2.6 to 16.1 μM α-naphthaleneacetic acid (NAA) was used (Mitrofanova et al. 1997).

Microshoots of all investigated plants were rooted on a half-strength MS culture medium, supplemented with different contents of β-indole-3-butric acid (0.98 to 4.90 μM; IBA) and NAA (1.07 to 5.37 μM). The pH of the medium was adjusted to 5.7 before sterilization (120 °C, 15 to 20 min). Plant material was subcultured onto fresh medium at 30-day intervals. Cultures of buds, shoots, germs, leaf discs and cotyledons were grown under a 16-h photoperiod (25 ± 1 °C) and photosynthetic photon flux of 25 μmol m^{-2} s^{-1} provided by Philips cool-white 36 W fluorescent tubes.

Data processing was carried out using STATISTICA for Windows, Release 5.1. Means ± SE for three replications of 50 explants used for each combination were determined

Results and discussion

The ability of isolated asimina, feijoa, kiwi, persimmon and zizyphus buds, leaf discs and embryos to produce microshoots and plants depended on the plant genotype and the applied cytokinin and auxin combination in the culture medium. The morphogenesis of tissues and organs of selected woody subtropical plants was realized via five ways: activation of apical and lateral meristems; direct and indirect somatic embryogenesis; direct regeneration of seedlings from zygotic embryos; direct regeneration of plants from leaves, shoot segments and cotyledons.

BA, kinetin and zeatin are well known as growth regulators that can induce microshoot regeneration. We observed that kinetin in all investigated concentrations (0.93 to 9.30 μM) retarded microshoot formation and induced active callus growth. The 0.88 μM and 4.40 μM concentrations of BA were effective for microshoot development from isolated buds of zizyphus and kiwi, respectively (Table 1). The cultivation of isolated persimmon buds on MS medium containing 2.22 μM BA stimulated the active regeneration of microshoots after three weeks of cultivation (Table 1). Addition of zeatin (4.56 μM and 9.12 μM) to MS culture medium induced the primary regeneration of microshoots from isolated buds of asimina and feijoa, respectively (Table 1). High concentrations of cytokinin (9.30 μM kinetin, 9.12 μM zeatin or 8.90 μM BA) in media caused shoot vitrification in all selected species.

The results of previous experiments with kiwi explants showed that the cultivation of germs and embryos on modified MS medium supplemented with 2.22 μM BA induced the development of axillary meristems and growth of microshoots. In callus culture of kiwi, alongside with vegetative gemmagenesis, the embryogenic zones were also observed (Vijeshwar et al. 1997). During eight weeks of cultivation on MS culture medium supplemented with BA and IAA, 80 to 90 % of leaf discs of kiwi isolated from one-year

Table 1. Effect of BA and zeatin on microshoot regeneration from isolated buds of kiwi (*Actinidia deliciosa*), zizyphus (*Zizyphus jujuba*), feijoa (*Feijoa sellowiana*), persimmon (*Diospyros kaki*) and asimina (*Asimina triloba*) after three weeks of cultivation *in vitro*

Cytokinin (μM)	Average number of shoots per isolated bud				
	Actinidia deliciosa	*Zizyphus jujuba*	*Feijoa sellowiana*	*Diospyros kaki*	*Asimina triloba*
Control	0	0.8 ± 0.1	0.2 ± 0.1	0	0
Zeatin 0.91	0	0.9 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.2 ± 0.1
Zeatin 1.36	0	1.1 ± 0.1	2.5 ± 0.1	3.5 ± 0.6	1.7 ± 0.1
Zeatin 2.73	0.6 ± 0.1	1.2 ± 0.2	2.9 ± 1.3	2.3 ± 0.9	1.9 ± 0.3
Zeatin 4.56	1.1 ± 0.1	0.7 ± 0.0	3.1 ± 0.8	2.7 ± 0.0	2.3 ± 0.4
Zeatin 9.12	1.6 ± 0.1	0	3.7 ± 1.2	2.9 ± 0.3	3.1 ± 0.6
BA 0.88	0	2.9 ± 1.3	0	1.4 ± 0.1	0
BA 1.33	1.5 ± 0.1	2.7 ± 1.3	0	2.3 ± 0.4	1.2 ± 0.1
BA 2.22	2.1 ± 0.3	2.4 ± 0.8	1.3 ± 0.1	3.3 ± 0.3	1.4 ± 0.1
BA 4.40	2.4 ± 0.4	1.6 ± 0.7	1.8 ± 0.1	1.5 ± 0.1	1.7 ± 0.1
BA 8.90	1.3 ± 0.1	0.7 ± 0.1	1.7 ± 0.1	0.9 ± 0.1	1.2 ± 0.1

Table 2. Effect of different concentrations of BA and IAA on regeneration rate of leaf discs of kiwi (*Actinidia deliciosa*, cv. 'Saanishton') with abaxial position on MS medium during four and eight weeks of cultivation *in vitro*

BA (μM)	IAA (μM)	Leaf discs forming shoots (%)	
		4 weeks	8 weeks
4.40	5.71	10.0 ± 2.1	27.0 ± 1.8
8.90	5.51	28.0 ± 2.4	60.0 ± 5.7
8.90	8.56	50.0 ± 4.1	90.0 ± 8.5
8.90	11.42	35.0 ± 2.5	80.0 ± 6.5
13.35	17.13	25.0 ± 0.5	40.0 ± 5.2
22.20	28.64	10.0 ± 0.8	25.0 ± 1.9
44.00	57.08	0.0	20.0 ± 2.3

Table 3. Regeneration of adventitious microshoots from leaf discs of different cultivars of kiwi (*Actinidia deliciosa*) with adaxial position on MS medium supplemented with 8.90 μM BA and 8.56 μM IAA during four and eight weeks of cultivation *in vitro*

Cultivar	Leaf discs forming shoots (%)		No. of shoots per leaf disc	
	4 weeks	8 weeks	4 weeks	8 weeks
'Monti'	14.0 ± 6.1	40.0 ± 7.0	1.2 ± 0.6	6.0 ± 0.5
'Bruno'	11.8 ± 2.1	55.6 ± 8.2	1.6 ± 0.1	6.5 ± 0.5
'Tomuri'	18.6 ± 1.6	75.0 ± 9.5	2.5 ± 0.5	7.2 ± 0.1
'Saanishton'	50.0 ± 5.5	100.0 ± 0.0	4.3 ± 0.4	10.0 ± 0.4
'Monti' × 'Tomuri'	0.0	38.0 ± 11.0	0.0	5.7 ± 1.3
'Bruno' × 'Tomuri'	45.0 ± 4.3	100.0 ± 0.0	5.7 ± 0.2	10.0 ± 0.6

Fig. 1. The plantlets of kiwi (*Actinidia deliciosa*) cv. 'Saanishton' obtained from leaf discs on MS medium with 4.40 μM BA after three month cultivation *in vitro*.

cultivated *in vitro* plants formed adventitious buds. The optimum combination of BA (8.90 μM) and IAA (8.56 μM) concentrations was established (Table 2). With adaxial position of leaf discs of kiwi cv. 'Saanishton' and hybrid 'Bruno' × 'Tomuri', the frequency of microshoot regeneration reached 100 % (Table 3). In the case of the microshoot regeneration from leaf discs of kiwi, callus was not formed. Normal plants were obtained during five month of cultivation on MS medium with 4.40 μM BA (Fig. 1). These plants can be used for future genetic transformation and other breeding investigations. The *in vitro* system via leaf discs in kiwi enabled us to create long-term shoot cultures with a high multiplication rate (8 to 10) and a 45-week subculture interval.

The use of zygotic embryos has opened the possibility of obtaining woody subtropical plants via direct and indirect somatic embryogenesis, direct regeneration of seedlings from zygotic embryos, and direct microshoot regeneration from cotyledons. We were the first to induce direct somatic embryogenesis from cotyledons of zygotic embryos and to produce zizyphus plants, when one culture medium (half-strength MS medium supplemented with 2.4-D) was substituted for another (modified Pierik medium without growth regulators). The largest number of somatic embryos per explant (7.2 ± 1.3 in cv. 'Ya-dzao', 15.5 ± 3.5 in cv. 'Kitaiskii 2A' and 3.0 ± 0.6 in cv. 'Ta-yang-dzao') was observed on ½ MS medium with 2.26 mM 2.4-D (data not shown; Mitrofanova et al. 1997). During 60 days of culture, most embryoids followed the stages of development of zygotic embryos: globular, heart-, and torpedo-shaped. The efficiency of somatic embryogenesis in zizyphus was increased by joint cultivation of primary and secondary embryoids. Within a year it was possible to obtain up to 630 somatic embryos from one cotyledon (Mitrofanova et al. 1997). Sometimes, indirect somatic embryogenesis was induced on the surface of cultured undeveloped zygotic embryos (torpedo-shape stage) of

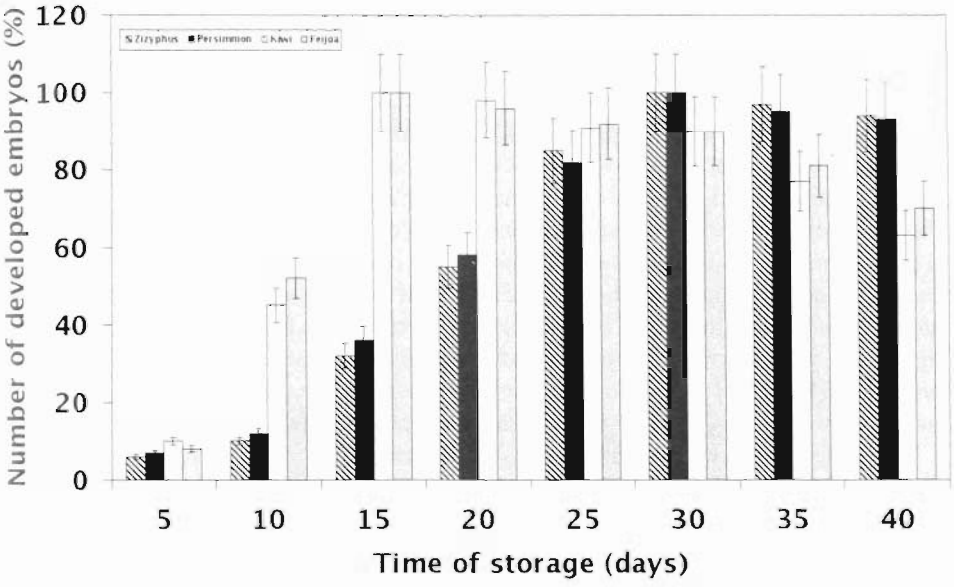

Fig. 2. Effect of the time of storage at 5 °C on development of zygotic embryos of zizyphus (*Zizyphus jujube*), persimmon (*Diospyros kaki*), kiwi (*Actinidia deliciosa*) and feijoa (*Feijoa sellowiana*) *in*

Table 4. Microshoot regeneration (no. of microshoots per explant) on culture medium with different concentrations of thidiazuron from different explants of *in vitro* cultivated zizyphus, asimina, feijoa, persimmon and kiwi

Species	Type of primary explant	Concentration of thidiazuron			
		1 μM	3 μM	6 μM	9 μM
Zizyphus	cotyledon	2.5 ± 0.1	callus	4.0 ± 0.1	7.0 ± 0.3
	leaf disc	1.2 ± 0.1	4.5 ± 0.3	2.0 ± 0.0	callus
Persimmon	cotyledon	1.4 ± 0.0	1.8 ± 0.1	2.6 ± 0.1	6.7 ± 0.4
	leaf disc	callus	0	10.0 ± 0.2	7.2 ± 0.3
Asimina	leaf disc	0	0	2.5 ± 0.1	callus
Kiwi	leaf disc	2.7 ± 0.3	callus	callus	callus
	shoot segment	0	callus	4.2 ± 0.1	callus
Feijoa	leaf disc	0	1.7 ± 0.1	3.7 ± 0.2	4.6 ± 0.1
	shoot segment	0	callus	3.7 ± 0.2	callus

feijoa on the modified MS medium with 4.56 μM and 9.12 μM zeatin. Unfortunately, even the use of different combinations of concentrations did not lead to induction of somatic embryogenesis in persimmon. Isolated zygotic embryos of three zizyphus cultivars arising from free pollination were also used for obtaining seedlings and microshoot regeneration. The size of isolated zygotic embryos differed from 4.0 mm to 10.0 mm. Zygotic embryos 6.1 to 10.0 mm in size produced well-developed plantlets. At a low positive temperature of 5 °C, the frequency of zygotic embryo development in feijoa, kiwi (15 days), persimmon and zizyphus (30 days) increased to 100 % (Fig. 2). The period of embryos storage of selected woody subtropical plants was considerably shorter than for peach, apricot, cherry plum and cherry (Zdruikovskaya-Richter 2003). Normal plants of feijoa, kiwi, zizyphus and persimmon were obtained after 1.5 to 2 months of zygotic embryos cultivation on Monnier medium.

Thidiazuron was selected among other cytokinins for its tremendous ability to stimulate *in vitro* shoot proliferation of woody species (Huetteman, Preece 1993). Today TDZ is a potent cytokinin for woody plant tissue culture. The results of our experiments (Table 4) showed that the number of regenerated microshoots per explant depended both on plant genotype and TDZ concentration in MS medium. Using 1 to 9 μM TDZ for direct and indirect regeneration of shoots allowed to induce microshoot development from cotyledons and leaf discs in zizyphus and persimmon, from leaf discs in asimina, feijoa, kiwi, persimmon and zizyphus, and from microshoot segments in feijoa, kiwi and persimmon (Fig. 3). However the number of regenerated microshoots from leaf discs of kiwi was lower in comparison with cultivation on medium containing BA and IAA.

To regenerate adventitious microshoots of selected subtropical plants, microcuttings of asimina, feijoa, kiwi, persimmon and zizyphus were placed on MS medium, supplemented with different concentrations of BA and zeatin. A high regeneration rate was obtained with cultivation of explants of kiwi and zizyphus on medium with 4.40 μM BA. After the 4th subculture on the MS medium supplemented with 1.82 μM zeatin and 0.24 μM IBA, a regeneration rate of five microshoots per bud was achieved for asimina. Elongation of microshoots also occurred on this medium. Active adventive microshoot regeneration in

Fig. 3. Microshoot regeneration from cotyledon explants of persimmon (*Diospyros kaki*) on MS culture medium with 6 μM TDZ after fore weeks of cultivation.

Fig. 4. Active regeneration of microshoots from microcuttings of feijoa (*Feijoa sellowiana*) on MS medium with 4.56 μM zeatin during two weeks of cultivation.

feijoa and persimmon was observed on media containing 4.56 μM and 3.19 μM zeatin, respectively (Fig. 4).

The obtained microshoots of selected woody subtropical plants were placed on ½ MS medium with 0.98 - 4.90 μM IBA and 1.07 - 5.37 μM NAA to promote root formation. When microshoots were cultured on ½ MS medium, supplemented with 4.90 μM IBA and 2.15 μM NAA, the rooting frequency increased and the average root number in zizyphus and asimina was about 2.3 ± 0.1 per microshoot (Fig. 5). A 0.98 μM IBA stimulated

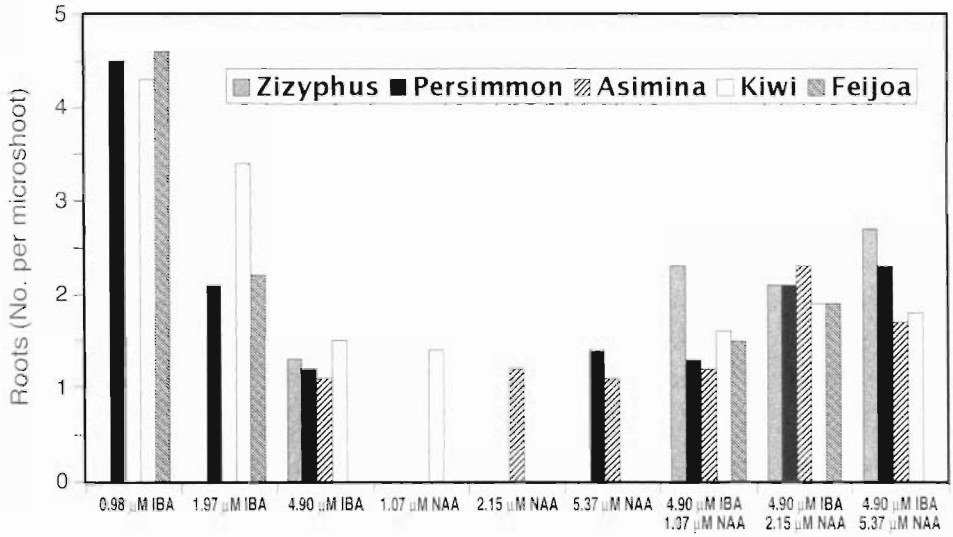

Fig. 5. Effect of IBA and NAA concentrations on root formation of microshoots in zizyphus (*Zizyphus jujube*), persimmon (*Diospyros kaki*), kiwi (*Actinidia deliciosa*), asimina (*Asimina triloba*) and feijoa (*Feijoa sellowiana*) on ½ MS medium.

in vitro rooting (100 %) of feijoa, kiwi and persimmon microshoots, and the average root number of these three cultures was about 4.5 ± 0.2 per microshoot (Fig. 5). Higher concentrations of IBA and NAA induced callus formation on the base of microshoots and reduced the rooting process.

This study showed the possibility of direct and indirect regeneration of plants in asimina, feijoa, kiwi, persimmon and zizyphus from vegetative buds, leaf discs, shoots segments, embryos and cotyledons. The developed recipient systems of woody subtropical plants allows their use for different plant biotechnology purposes: multiplication, genetic transformation and conservation *in vitro* of valuable genotypes.

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Organogenesis of *Fraxinus excelsior* L. by isolated mature embryo culture

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Abstract

The *in vitro* organogenesis of common ash *Fraxinus excelsior* L. on isolated mature embryo culture was investigated. Mature seeds of three genotypes (maternal trees) were collected from different ash populations of Lithuania: 'Kedainiai' (K-59), 'Nemencine' (N-5) and 'Telsiai' (T-3). The non-stratified seeds were disinfected with 70 % sulphur acid 1 min and with 80 % ethyl alcohol 3 min followed by four rinses in sterile distilled water. Mature zygotic embryos were aseptically removed from endosperm and placed on solified Murashige and Skoog medium supplemented with 1 mg l^{-1} 6-benzylaminpurine, 1 mg l^{-1} Ca pantetonate and 30 g l^{-1} sucrose. Morphogenesis was observed every 10 days. The genotype of common ash had high effects on organogenesis. The T-3 maternal tree embryo showed the highest morphogenesis capacity under the same cultivation conditions, forming all organs within 20 days and at the highest frequency. Hypocotyls, embryonic leafs and embryonic stems could be used for direct organogenesis of *Fraxinus excelsior* L.

Key words: common ash, *Fraxinus excelsior* L., mature embryo, organogenesis, stratification.

Introduction

Common ash (*Fraxinus excelsior* L.) is one of the most important forest tree species in many European countries. In Lithuania this hard wood tree occupies 50.8 thousand ha, attributing 2.89 % of the land area covered by all forest tree species. Breeding activities for hard wood species in Lithuania were initiated in 1998 (Pliura 2000).

Adventitious shoot organogenesis is a type of *in vitro* propagation that can be used for clonal reproduction of mass propagation of plants. Organogenesis involves *de novo* production of adventitious shoots on explants from many different sources, and this tissue culture technique is widely used in horticulture and forestry for production of ornamental plants and timber species (Tzfira et al. 1998). Although somatic embryogenesis and organogenesis has the potential to be a very efficient method for forest plant micropropagation *in vitro*, embryonic cultures produce only from seeds of zygotic embryos (Peña, Séguin 2001). There are few published studies on the somatic embryogenesis of common ash (Chalupa 1990; Hammat, Ridout, 1992; Hammat 1994). The isolated mature embryo culture could be helpful for tree research, as it does not involve seed stratification.

The objective of this study was to induce organogenesis and to obtain plant regeneration in isolated mature embryos from non-stratified seeds of *Fraxinus excelsior*.

Materials and methods

Mature embryos, used as explants, were rescued from non-stratified seeds collected from common ash Lithuanian trees during autumn in 2000 and stored in the dark at 4 ± 2 °C. Mature seeds from three genotypes (maternal trees) were collected from different ash populations of Lithuania: 'Kedainiai' (K-59), 'Nemencine' (N-5) and 'Telsiai' (T-3).

Pericarps were removed and seeds were sterilized with 70 % sulphur acid 1 min and with 80 % ethyl alcohol 3 min, followed by four rinses in sterile distilled water. The embryos were rescued by cutting the seeds along the edges and put them on solidified Murashige and Skoog (1962; MS) medium supplemented with 1 mg l^{-1} 6-benzylaminpurine (BAP), 1 mg l^{-1} Ca pantetonate and 30 g l^{-1} sucrose. The pH was adjusted with KOH or HCl after dissolving gelling agents and before autoclaving (30 min at 120 °C). Fifteen explants per replicate (four replicates) were initially placed into 85 mm diameter plastic Petri dishes, each containing 5 ml solidified medium. Culture tubes (20 × 145 mm) containing 10 ml medium were then used for the further plant regeneration. All cultures in first experiment were incubated under two light treatments: at 23 ± 2 °C with a 16-h photoperiod under 80 W "cool-white" fluorescent lamps and in thermostat for darkness all day. The cultures for morphogenesis of different explants were cultivated at 23 ± 2 °C with a 16-h photoperiod.

The research data was analysed using ANOVA.

Results

The results demonstrate different morphogenesis frequencies for isolated mature embryo culture of common ash different maternal trees. Naturally, seeds germinate in darkness. Thus, we observed organogenesis in embryo culture of genotypes of common ash in the light and dark. Vitality of mature embryos was observed within ten days of culture. Fig. 1 presents morphogenesis success after 20 days cultivation under dark or light conditions. Leaves and callus were formed in the highest frequency under light, but dark conditions activated root and cotyledon formation in all genotypes. Embryos of the T-3 genotype formed callus and leaves in the highest frequency in comparison with the other two genotypes. In general, genotype T-3 seed embryos showed the highest organogenesis capacity in mature embryo culture.

The second experiment examined morphogenesis of isolated three types of explants (initial leaves, hypocotyls and embryonic stem) in tissue culture of the T-3genotype. Individual explants showed different response in tissue culture under the same conditions (Table 1). Hypocotyls formed roots in the highest frequency and they also formed callus. Initial leaves regenerated shoots, callus and roots. Embryonic stems formed shoots in the highest frequency.

Discussion

Dormant seeds of ash acquire the ability to germinate after 6 to 7 months of stratification (Vorobyova 1981). For mass propagation it is important to find a technique that involves non-stratified seeds. Tissue technique, as isolated mature embryo culture, is promising for this purpose. The clonal propagation of ash by cuttings is frequently unsuccessful (Dirr.

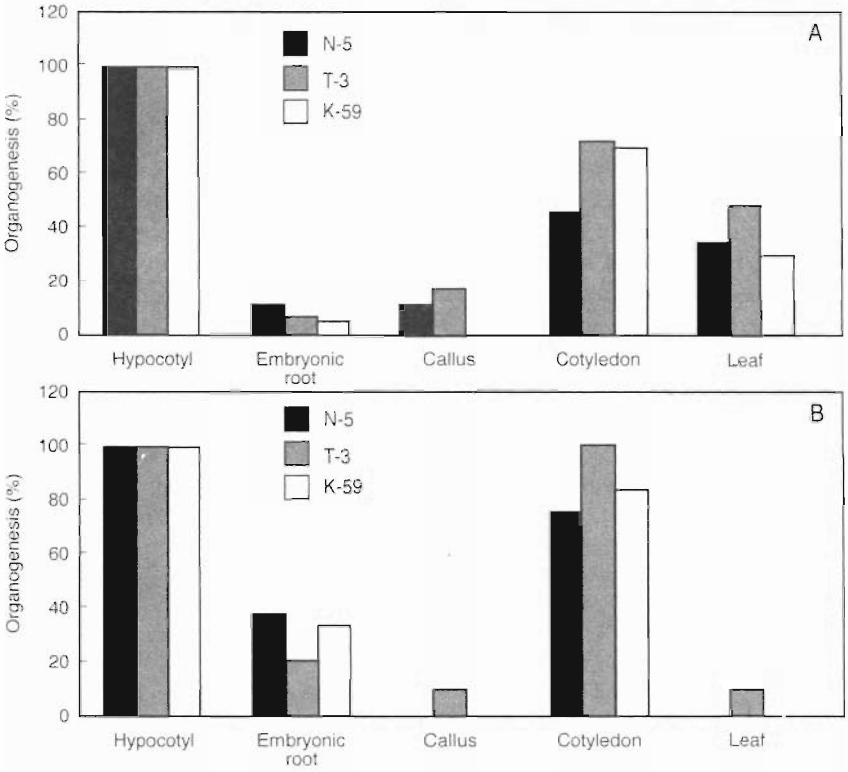

Fig. 1. Organogenesis in embryo culture of different genotypes of common ash in light (A) and darkness (B).

Heuser 1987), but there are some reports of *in vitro* regeneration of *Fraxinus* species. For example, both shoot organogenesis and somatic embryogenesis in white ash (*F. americana* L.) were achieved using cut mature seeds as primary explants (Bates et al. 1993; Bates, Preece 1995). In common ash (*F. excelsior*) micropropagation has been achieved from juvenile (Chalupa 1990; Hammat, Ridout 1992) and mature (Hammat 1994) plants. In our experiment, we attempted to induce *F. excelsior* organogenesis using by cutting sterile plantlets derived in mature embryo culture for receiving viable plants with roots.

In this work the medium was optimized for ash mature embryos culture initiation. After several days of cultivation, cotyledons, hypocotyls and embryonic stems developed.

Table 1. Effect of type of explant on morphogenesis of common ash genotype T-3

Type of explant	Number of explants	Callus formation		Root formation		Shoot formation	
		No.	%	No.	%	No.	%
Initial leaves	53	17	32.08	2	3.77	10	18.87
Hypocotyl	61	3	4.92	33	54.09	10	16.39
Embryonic stem	16	0	0	0	0	2	12.5
Sx %			1.15		1.49		3.47

This observation is similar to that observed by Preece et al. (1989) in white ash (*F. americana*), where they achieved somatic embryogenesis and bud organogenesis on white ash cotyledons.

Morphogenetic processes, like rooting, somatic embryogenesis and bud organogenesis are often described as complex phenomena characterized by different phases, each with specific nutritional requirements (Gaspar et al. 1992). In the present study, MS medium proved to be effective for the induction and initiation phases. For common ash organogenesis, the medium was based on MS solution supplemented by additional growth regulators: 1 mg l^{-1} BAP, 1 mg l^{-1} Ca pantetonate.

Under the used conditions it was possible to achieve plant regeneration in mature embryos culture from non-stratified seeds of *F. excelsior*. Also, hypocotyls, initial leaves and stems as explants could be used for direct organogenesis of common ash.

Acknowledgements

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Root initiation and development by auxin physiological analogue TA-12

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Abstract

The effect of the compound TA-12 (1-[2-chloroethoxycarbonylmethyl]-4-) naphthalene sulphonic acid calcium salt) on the rooting of difficult-to-root cherry (*Cerasus vulgaris* Mill.) cv. 'Zagarvysne' and various cultivars of hazel (*Coryllus avellana* L.) green cuttings was studied and compared to the activity of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). Under the effect of TA-12, IAA and IBA, cherry 'Zagarvysne' cuttings undergo complex anatomical and morphological changes in the basal and subbasal parts. Using wheat coleoptile cell plasmalemma as a model test object in IAA investigations, it was shown that TA-12 could be percieved in the plasmalemma under conditions similar to IAA and transduced to the nucleus. It is supposed that TA-12 can affect the plant cell through the IAA receptory system, but TA-12 is distinguished for having high activity in rhizogenesis of difficult-to-root cherry 'Zagarvysne', hazel 'Pervenets' and 'Akademik Jablokov' green cuttings, as compared to the activity of optimal concentrations of IAA and IBA.

Key words: cherry *Cerasus vulgaris* Mill., hazel *Coryllus avellana* L, indole-3-acetic acid, indole-3-butyric acid, rooting, TA-12.

Introduction

Organogenesis occurs in various plant tissue cultures and during vegetative propagation, in response to exogenously applied phytohormones, mainly auxin and cytokinin, and also on the ability of the tissue to respond to these phytohormone changes during culture (Sugiyama 1999). Lateral root formation plays a crucial role in plant development by permitting the construction of branched root systems. The process of lateral root formation consists of two major steps: cell cycle reactivation in the xylem pericycle and establishment of a new meristem (Laskowski et al. 1995; Himanen et al. 2002). The first formative divisions in the pericycle depend on the basipetal transport of auxin, whereas shoot-derived auxin regulates the outgrowth of lateral roots (Casimiro et al. 2001). Cuttings of the cherries 'Liubskaja' and 'Zagarvysne', as well as those of numerous varieties of culivated hazel, are difficult-to-root plants (Novickiene, Darginaviciene 2001). The objectives of the present work were to determine the physiological activity of an auxin analogue, TA-12 (1-[2-chloroethoxycarbonylmethyl]-4-naphthalenesulfonic acid calcium salt); (Merkys et al. 1993)], as well as to compare its effect on the rooting of cherry and hazel sprout cuttings with the effect of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), depending on the parent plant and sprout cutting age.

Materials and methods

Cuttings of cherry (*Cerasus vulgaris* Mill. cv. 'Zagarvysne') and hazel (*Corylus avellana* L.) served as the object of research. Cuttings for rooting were obtained in June from the upper, middle and basal part of annual first and second line sprouts of young five- to six-year-old parent plants. The rooting was carried out according to Turetskaja and Polikarpova (1968) with modifications. The basal ends of cuttings were immersed in compounds at optimal concentrations: TA-12 (1×10^{-3} M), IAA (1×10^{-3} M), IBA (2.5×10^{-4} M) for 15 h. Ninety green cuttings were used for each treatment. The cuttings were planted in a greenhouse and grown at a constant temperature (~26 °C) under conditions of artificial mist till October. Then their roots were analysed morphometrically. Samples for anatomical and cytological studies of callus and root formation were taken in the course of five weeks every seven days. Longitudinal and transverse microtomic slices were analyzed by light microscopy.

The plasmalemma vesicle fraction was obtained from wheat (*Triticum aestivum* L. cv. 'Nandu') coleoptiles according to Tichaja et al. (1986). A transmembrane potential was created as described by Maksimov et al. (2004). The physiological activity of IAA and TA-12 complexes with protein in the plasmalemma (pH 7.2) was evaluated from RNA-polymerase II activity in a system of nuclei isolated from wheat coleoptile cells. CTP, GTP, UTP and 8-^{14}C-ATP (ammonium salt; 2.11 GBq mmole^{-1} at a final concentration 0.1 mM) were added into the system (Novickienė, Darginavičienė 2001). The label incorporation was sensitive to RNA-polymerase II inhibitor α-amanitine. The label content in the vesicles was determined with the aid of an LS 1801 scintillation counter (Beckman, USA). Data were statistically evaluated. Differences were statistically significant at $p \leq 0.05$.

Results

The effect of TA-12 and IAA on rooting of cherry 'Zagarvysne' cuttings depends on the sprout age and its position in the tree crown

In our studies cuttings were prepared from the upper, middle and basal sprout parts of five-year-old plants and exposed to TA-12 (1×10^{-3} M) and IAA (1×10^{-3} M). Cuttings taken from the middle part were found to root best, while the rooting of cuttings from the upper and basal parts was the weakest. The experiments showed the advantage of the compound TA-12 over IAA. Under the effect of TA-12 and IAA the number of main and lateral roots increased in all parts of sprouts, especially in the midle part (Table 1). Under the effect of TA-12, the length of lateral roots in the upper, midle and basal part cuttings increased 5.2-, 11.9- and 10.0-fold as compared to the control, while under the effect of IAA the increase was 2.0-, 3.75- and 3.2-fold, respectively.

It seemed worthwhile to study the dependence of rooting on the sprout position in the tree crown. Cuttings were taken from the middle part of the sprout. In the control variant, no significant difference in the rooting of the first and second row sprouts was detected. However, under the effect of TA-12 and IAA the rooting of cuttings of the first row branching sprouts was higher by 14 % and 11 %, respectively, while the formation of main roots per cutting was higher by 66 % and 91 %, respectively, as compared to the cuttings of the second branching row sprouts (Table 1).

Table 1. Effect of TA-12 and indole acetic acid (IAA) on rooting of cherry 'Zagarvysne' green cuttings taken from different sprout parent plant parts (data are means ± SE from 90 cuttings)

Parent plant parts	Variant	Rooted cuttings (%)	Main roots		Lateral roots	
			Number	Length (mm)	Number	Length (mm)
Upper sprout part	Control	15.5	1.5 ± 0.2	19 ± 2	1.2 ± 0.1	2.1 ± 0.6
	TA-12 10^{-3} M	44.4	6.0 ± 0.5	68 ± 2	21.5 ± 1.2	11.0 ± 0.1
	IAA 10^{-3} M	34.4	4.6 ± 0.4	53 ± 2	17.0 ± 1.3	4.0 ± 0.2
Middle sprout part	Control	17.8	1.8 ± 0.1	25 ± 2	1.3 ± 0.1	2.6 ± 0.4
	TA-12 10^{-3} M	63.3	7.6 ± 0.4	79 ± 1	26.1 ± 2.1	19.0 ± 0.3
	IAA 10^{-3} M	53.3	6.3 ± 0.3	71 ± 2	32.0 ± 2.1	6.0 ± 0.1
Basal sprout part	Control	11.1	1.3 ± 0.1	16 ± 1	1.0 ± 0.1	1.9 ± 0.3
	TA-12 10^{-3} M	31.1	5.1 ± 0.5	61 ± 3	20.0 ± 2.2	19.0 ± 0.3
	IAA 10^{-3} M	26.7	3.9 ± 0.1	47 ± 3	21.0 ± 1.2	6.1 ± 0.3
Branching of the first row	Control	12.6	1.4 ± 0.2	39 ± 1	1.3 ± 0.1	2.7 ± 0.3
	TA-12 10^{-3} M	68.4	7.5 ± 0.3	131 ± 2	37.0 ± 2.1	17.4 ± 0.2
	IAA 10^{-3} M	61.6	6.9 ± 0.4	77 ± 2	32.0 ± 1.3	12.2 ± 0.2
Branching of the second row	Control	10.6	1.6 ± 0.3	41 ± 2	1.1 ± 0.1	2.7 ± 0.7
	TA-12 10^{-3} M	54.2	6.2 ± 0.3	116 ± 2	33.0 ± 3.4	14.9 ± 0.1
	IAA 10^{-3} M	50.8	5.3 ± 0.4	73 ± 2	29.0 ± 2.5	8.0 ± 0.2

The effect of TA-12 and IBA on rooting of cuttings of various hazel cultivars

The effect of TA-12 and IBA, the classic compound for stimulation of rooting, was studied on various cultivars of hazel green cuttings: 'Pervenec', 'Akademik Jablokov', 'Tambovskij rannij' and 'Moskovskij rubin'. The effect of the tested compounds on rooting was especially high in the cvs. 'Pervenec' and 'Akademik Jablokov'. No significant differences in rooting under the effect of TA-12 and IBA were detected, except for cuttings of hazel 'Akademik Jablokov' in which the effect of TA-12 on rooting was higher than that of IBA (Table 2).

Table 2. Effect of auxin physiological analogues on rooting of hazel green cuttings (average % from 100 cuttings)

Variant	Cultivar			
	'Pervenec'	'Akademik Jablokov'	'Tambovskij ranij'	'Moskovskij rubin'
Control	9	1	1	0
TA-12 1×10^{-3} M	71	9	6	2
TA-12 5×10^{-4} M	83	20	11	2
TA-12 5×10^{-5} M	58	7	1	0
IBA 1×10^{-3} M	88	10	8	4
IBA 2.5×10^{-4} M	77	8	9	2
IBA 1×10^{-4} M	43	8	1	2

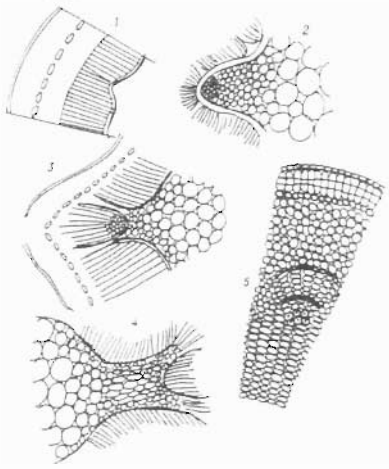

Fig. 1. Scheme of root formation of cherry 'Zagarvysne' green cuttings under the effect of IAA (1×10^{-3} M) and IBA (2.5×10^{-4} M). 1, secondary xylem layer (curvature); 2, 3, formation of initial cells in the area of the peripith; 4, streching of initial cells in transverse direction; 5, formation of secondary phloem.

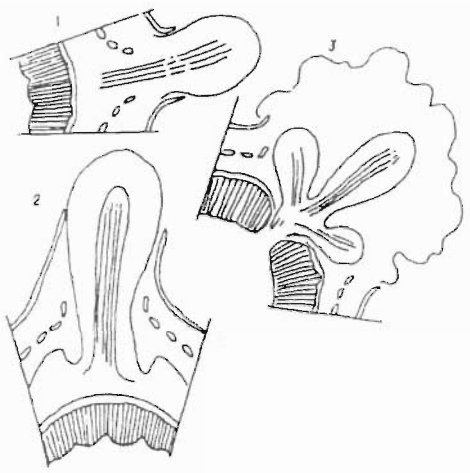

Fig. 2. Scheme of root formation in cherry 'Zagarvysne' green cuttings under the effect of IAA (1×10^{-3} M), IBA (2.5×10^{-4} M) and TA-12 (1×10^{-3} M). 1, 2, on day 21 and 28 roots are formed from secondary phloem under the effect of IAA and IBA; 3, on day 35 roots (by 3) are formed from the peripith area under the effect of TA-12.

Anatomical features of root primordium formation and root development

Root primordium formation as well as their further growth was strongly modified by the test compounds IAA, IBA and TA-12. On the 7th day from the beginning of rooting, parenchyma became narrow on the layer of cortex in transverse section of the cutting and a bend of certain tissues was notable. This was followed by formation of initial cells in the peripith area and stretching of initial cells in transverse direction. These initial cells in cutting segments were vascularized and intended specifically for root formation. In cuttings treated with IAA, also the semicircular secondary phloem with a thin secondary cambium layer was formed (Fig. 1). The structure of the control cutting sections remained the same and primordium initiation did not occur. Quantitative and qualitative differences between the test variants become significant only on day 21. On day 28, under the effect of TA-12, IAA and IBA, on average 3.7, 3.6 and 4.1 roots had formed on each sprout, respectively. After 35 days, under the effect of IAA and ABA the anatomical structure of roots did not change, while in the variant with TA-12, two to three roots from one group of initial cells were formed (Fig. 2). No such cases were found in other variants of the experiments.

Is the similarity in the action of TA-12 and IAA in the cell?

To answer this question, as a model object we used wheat coleoptile segments whose growth is controlled by IAA. TA-12 (1×10^{-4} M), like IAA, also stimulates their growth. We previously showed that the functional activity of IAA-protein complexes formed in the system *in vitro* is potential-dependent (Maksimov et al. 2004). What events are

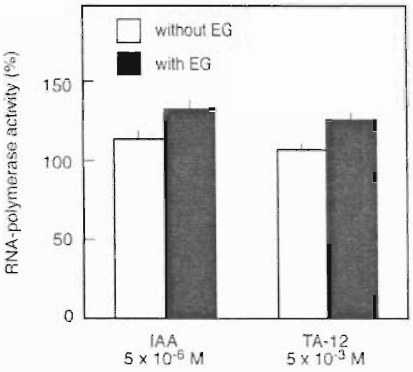

Fig. 3. Activity of RNA polymerase II (cpm 100 μg^{-1} plasmalemmal protein, %) in the system of isolated nuclei under the effect of IAA (5×10^{-6} M) or TA-12 (5×10^{-5} M), both 30 min *in vitro* treated plasmalemma. Control (non treated plasmalemma addition) = 100 %. White columns, elecrochemical gradient (EG) not created on membrane of plasmalemma vesicles. Black columns, the same in the conditions of a created electrochemical gradient.

related with TA-12 action on pasmalemma level? To generate an electrochemical gradient on the membrane, plasmalemma vesicles were filled (by means of osmotic shock) with 150 mM K_2SO_4, transferred into equimolar solution of Na_2SO_4, and to induce membrane potential generation valinomicin (8.3×10^{-9} M) was added. The resulting complexes of TA-12-plasmalemmal protein, upon addition into the system of isolated nuclei, activated RNA-polymerase II by 26 % above the control (without TA-12 treatment), whereas in the absence of the potential, the activity of TA-12 was weak (Fig. 3). The activity of the IAA-protein complexes exceeded the control by 14 % and 34 %, respectively.

Discussion

This study focuses on root primordium formation and further root development under the effect of a classical natural auxin IAA, its physiological analogue, IBA, and the newly synthesized compound TA-12 of the same class, in difficult-to-root cherry and hazel green cuttings. Under the effect of TA-12, root primordia are mostly formed from the peripith area, while under the effect of IBA and IAA they are formed also from secondary phloem. Under the effect of compound TA-12, root primordia are formed in a different way: two-three roots are formed from a group of initial cells. Despite the anatomical differences in the effects of the physiologically active compounds studied, under the effect of TA-12, IAA and IBA, the root regeneration stages occur three weeks earlier and are five to seven times more intensive than in the control (Novickiene, Darginaviciene 2001). The action of TA-12 on the cell level, as shown using wheat coleoptile cells as a model object, is like IAA. TA-12, as IAA, activates growth of coleoptile segments and simultaneously is able to compete for IAA binding sites in the cell plasmalemma during formation of complexes with proteins that realize physiological function in the nucleus. The events that occur in cell plasmalemma under the influence of TA-12 depended on the transmembrane potential, as in the case of IAA.

TA-12 and IBA molecules have two methylene groups, which most likely determine

the activity of these compounds. However, the molecules differ: the TA-12 molecule contains a naphthalene ring and IAA and IBA contain an aromatic indole ring.

In summary, TA-12 can affect the plant cell through the IAA receptory system, but TA-12 is distinguished for high activity in rhizogenesis of difficult-to-root cherry 'Zagarvysne', hazel 'Pervenets' and 'Akademik Jablokov' green cuttings, as compared to the activity of optimal concentrations of IAA and IBA.

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In vitro propagation of *Vaccinium* species

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Abstract

The aim of the work was direct regeneration of shoots from meristems, as well as adventitious shoot production from leaves for regeneration and effective production of highbush blueberry *Vaccinium corymbosum* L. and lingonberry *Vaccinium vitis-idaea* L. The results showed that the shoot proliferation intensity of individual cultivars differed. Therefore, optimisation of cytokinin type and concentration for each cultivar was necessary. For meristem regeneration, improved multiplication was achieved on medium with zeatin in comparison with N^6-Δ^2-isopentenyl adenine. Thidiazuron was effective in adventitious shoot regeneration from leaf tissue of highbush blueberry, and both thidiazuron and zeatin for lingonberry leaf tissue. Microshoot rooting was achieved on Anderson culture medium supplemented with indole-3-butyric acid or directly in peat after dipping of shoots into indole-3-butyric acid solution under *ex vitro* conditions.

Key words: adventitious organogenesis, meristem culture, regeneration *in vitro*, *Vaccinium corymbosum* L., *Vaccinium vitis-idea* L.

Introduction

Highbush blueberry (*Vaccinium corymbosum* L.) and lingonberry (*Vaccinium vitis-idaea* L.) are important and biologically valuable small fruit species. Both species have been succesfully grown in USA and in many European countries for a long period (Jaakola et al. 2001). Some introduced cultivars of these species are suitable also for cultivation in the conditions prevailing in Slovakia. Effective mass production of plants is needed for commercial plantation establishment (Ostrolucká et al. 2002). Vegetative propagation is not very successful and is considerably limited by seasonal growth. Generative propagation is not only time demanding, but also does not give the opportunity to obtain homogeneous progeny. Tissue culture propagation techniques can be used as a system for effective plant production, production of virus-free plants which are genetically identical with maternal plant, and also allow for induction of genetic variability (George 1993; Marcotrigiano et al. 1996). This paper presents our results obtained during experimental micropropagation of different cultivars of highbush blueberry *V. corymbosum* and lingonberry *V. vitis-idaea*.

Materials and methods

The regeneration ability of some cultivars in species *Vaccinium corymbosum* L. and *Vaccinium vitis-idaea* L. was tested by direct organogenesis from apical and axillary buds, and isolated meristems, and by adventitious organogenesis from whole leaves derived from microshoots. The primary explants were obtained from dormant shoots collected from the plantation at the Research Station, Krivá (Research Institute in Banská Bystrica, Slovakia). Shoot segments were sterilised in 70 % ethanol (2 min) and 0.1 % $HgCl_2$ (6 min). The explants were cultivated on Anderson (1980) culture medium (AN) supplemented with 3 % sucrose and the growth regulators zeatin, N^6-Δ^2-isopentyl adenine (2iP), thidiazuron (TDZ), indole-3-butyric acid (IBA) in different concentrations. The medium pH was adjusted to 5.0 in all variants of culture media with the exception of an experiment aimed at modification of the pH values for cultivar 'Duke' cultivation.

For *V. corymbosum* cv. 'Duke', the intensity of shoot proliferation on AN culture medium with 0.5, 1.0, 2.0 mg l^{-1} zeatin (in pH 5.0) was tested. Also effect of different medium pH on shoot regeneration was investigated. The medium pH was tested in the range from 3.0 to 5.5 using AN culture medium with 2.0 mg l^{-1} zeatin.

The effect of zeatin and 2iP was compared for *V. corymbosum* cvs. 'Blueray', 'Darrow', 'Berkeley', 'Bluecrop' and 'Duke' on AN culture medium with 2.0 mg l^{-1} zeatin or 15 mg l^{-1} 2iP in combination with 0.5 mg l^{-1} IBA.

Regeneration *in vitro* for *V. vitis-idaea* cvs. 'Red Pearl' and 'Koralle' was tested on AN culture medium supplemented with zeatin and 2iP in three different concentrations (0.25, 0.50, 1.0 mg l^{-1} zeatin and 2.50, 5.0, 10.0 mg l^{-1} 2iP). As a control, AN culture medium without cytokinins was used.

Explants were incubated at 25 °C, light intensity 50 μmol m^{-2} s^{-2} and 16/8 h photoperiod. Each experiment was repeated three times and the number of primary explants was 30 per experiment. The regeneration ability of cultivars was evaluated on the basis of shoot proliferation intensity parameters: mean number of shoots per explant, mean total number of regenerants per explant (mean number of shoots per explant and one-node segments per shoot) after five weeks of cultivation of *V. corymbosum* and after five subcultures of *V. vitis-idaea* with a subculture interval of 5 to 6 weeks. Isolated microshoots were rooted *in vitro* (AN culture medium with 0.8 mg l^{-1} IBA and 8 g l^{-1} charcoal) or *ex vitro* (directly in peat substrate after dipping into 0.5 to 0.8 mg l^{-1} IBA solution).

For induction of adventitious shoot regeneration from leaf tissue of cv. 'Duke' AN medium supplemented with TDZ (2.2 mg l^{-1}) was used. For the cvs. 'Red Pearl' and 'Koralle', AN medium with 2.2 mg l^{-1} TDZ or 2.19 mg l^{-1} zeatin was tested. Leaves were placed horizontally with the abaxial surface on the medium and cultivated under light.

The data were statistically evaluated by using Statgraphics one-way analysis of variance and multiple range analysis.

Results and discussion

Our observations showed that multiplication (shoot proliferation intensity) depends not only on the concentration of cytokinins in culture medium but also on the response of individual species and cultivars, previously shown by Popowich and Filipenya (1997).

Our experiments confirmed a positive and significant influence of the cytokinin

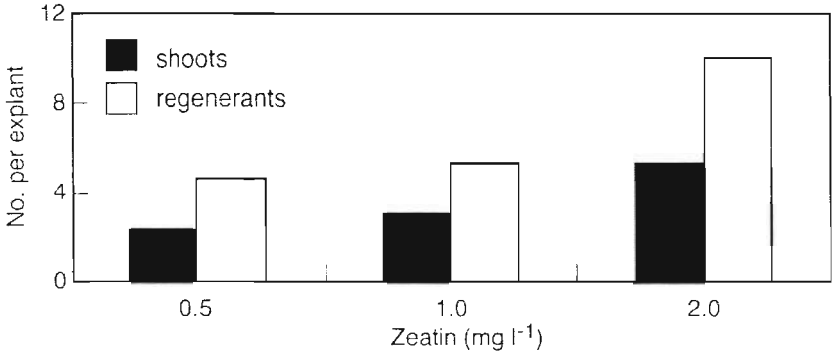

Fig. 1. Effect of different concentrations of zeatin on regeneration of *Vaccinium corymbosum* cv. Duke' on Anderson culture medium after 5 weeks of cultivation.

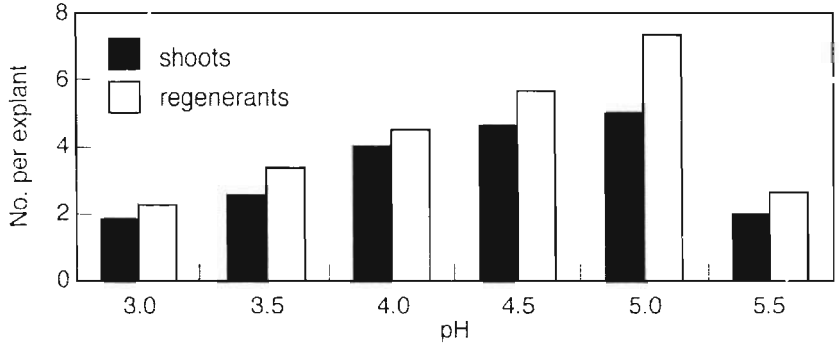

Fig. 2. Effect of medium pH on regeneration of *Vaccinium corymbosum* cv. 'Duke' on Anderson culture medium with 2 mg l^{-1} zeatin after 5 weeks of cultivation.

zeatin on shoot differentiation in cultivar 'Duke'. A higher concentration of zeatin was more effective. The highest mean number of shoots and total number of regenerants per explant was achieved on medium with 2 mg l^{-1} zeatin (Fig. 1). Shoot proliferation was absent on medium without zeatin. Our results are in agreement with work of other authors (Chandler, Draper 1986; Reed, Abdelnour-Esquivel 1991), who showed that zeatin is suitable for stimulation of shoot multiplication in *V. corymbosum*.

Several reports in the literature show that pH of the medium can influence *in vitro* shoot and root formation in some plants and that pH changes during culture (Finn et al. 1991; George 1993). It is known that some plants can tolerate a broader pH range, while in others pH tolerance is limited. Therefore, it is necessary to determine optimal pH levels. The *Vaccinium* sp. are acidophilic plants. *In vitro* screening system allows to investigate the response of plants to different pH levels. Our preliminary experiment with cultivar 'Duke' confirmed differences in shoot proliferation intensity depending on pH of the medium (pH 4.0 to 5.5). A higher multiplication effect was obtained at pH 5.0 and the lowest in pH 3.0 (Fig. 2).

Experiments confirmed that successful regeneration *in vitro* depends also on the reaction of specific cultivar to the cytokinin type. When the effect of zeatin and 2iP on

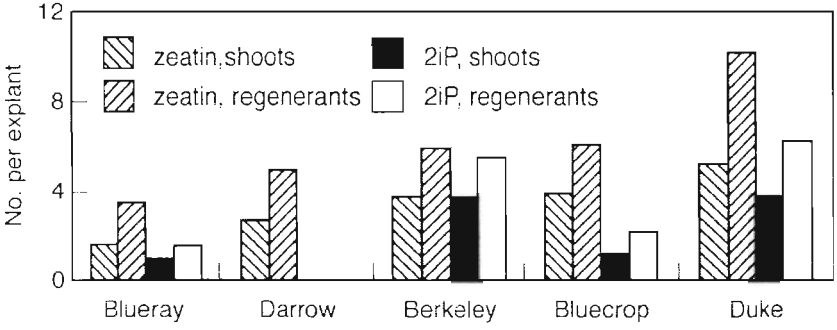

Fig. 3. Intensity of shoot proliferation in different cultivars of *Vaccinium corymbosum* on Anderson culture medium with 2 mg l^{-1} zeatin or 15 mg l^{-1} 2iP after five subcultures.

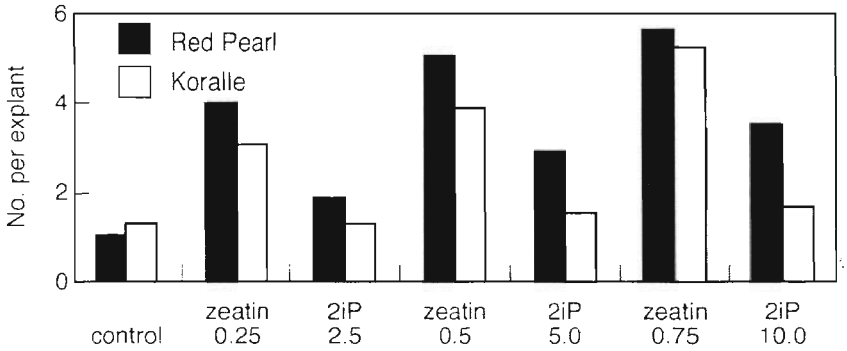

Fig. 4. Effect of different concentrations of zeatin and 2iP (mg l^{-1}) on regeneration of *Vaccinium vitis-idaea* cv. 'Red Pearl' and 'Koralle' on Anderson culture medium after five subcultures.

multiple shoot culture formation was studied in individual cultivars, differences (1.71 to 5.28 shoots per explant) among cultivars on the same medium were observed. On a medium with 2 mg l^{-1} zeatin, a higher intensity of shoot proliferation was achieved in 'Bluecrop' (3.94), 'Berkeley' (3.78) and the highest in cultivar 'Duke' (5.28). The lowest ability of regeneration was found for 'Blueray' (1.71). The differences in intensity of shoot proliferation between cultivar 'Duke' and the other cultivars were highly statistically significant. Similar results were obtained for total number of shoots per explant (3.50 to 10.06) – the lowest for 'Blueray' and the highest for 'Duke'. On medium with 2iP, lower multiplication (1.02 to 3.80 number of shoots per explant and 1.66 to 6.20 total number of regenerants per explant) were obtained in comparison with zeatin. The results confirm the importance of culture medium composition regarding shoot differentiation. The data for regeneration in cultivar 'Darrow' is absent because of contamination of cultures on the medium (Fig. 3).

There is little information about the micropropagation of *V. vitis-idaea* in literature (Hosier et al. 1985, Sidorowich et al. 1995, Jaakola et al. 2001). Our experiments on cultivation of cvs. 'Red Pearl' and 'Koralle' on medium with zeatin and 2iP showed the importance of cytokinins on regeneration (Fig. 4), demonstrated by significant differences

in the intensity of shoot proliferation between the control and AN medium with zeatin and 2iP. The exception was cultivar 'Koralle' where shoot proliferation in the control was similar to that on medium with 2iP, but zeatin had a stimulating effect on the intensity of shoot proliferation. Zeatin was more effective also in cultivar 'Red Pearl'.

Microshoot rooting was achieved on AN culture medium supplemented with IBA (0.8 mg l^{-1}) or directly in peat after dipping of shoots into IBA solution under *ex vitro* conditions (80 - 90 - 95 %), depending on the cultivar. Transfer from *in vitro* to *ex vitro* conditions was successful. Four thousand regenerants were provided to the Krivá Research Station for establishment of production plantations.

Adventitious organogenesis is an essential tool in the generation of somaclonal variants and in most methods of genetic transformation (Marcotrigiano et al. 1996). Our experiments on cultivation of leaf explants from micropropagated shoots of cvs. 'Duke', 'Red Pearl' and 'Koralle' showed an excellent alternative way of regeneration and reproduction of highbush blueberry and lingonberry. The concentrations of TDZ and zeatin used in our experiment were sufficiently effective to induce regeneration of adventitious shoots from leaf derived calli. In callus cultures, intensive production of anthocyanin pigment was observed.

Acknowledgements

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Interspecific hybridization and embryo rescue in breeding of lilies

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Abstract

This research investigates the hybridization of species *Lilium pumilum* Delile, *L. regale* Wils., *L. candidum* L., *L. monadelphum* Bieb., *L. martagon* L., *L. henryi* Baker. and cultivars of Asiatic, Trumpet and Oriental hybrids. To overcome fertilization barriers in incongruous crosses, cut-style pollination and pollination by mixed pollen were performed. The hybrid plants were obtained by *in vitro* cultivation of excised immature embryos. Cut-style pollination was efficient when Asiatic hybrids were pollinated by pollen of *L. pumilum*. Cut-style pollination and culture of excised embryo allowed receive few hybrids between Oriental hybrids and *L. henryi* or *L. regale*. Pollination of a female by a mixture of incompatible pollen was more efficient than cut-style pollination. Hybrid plants with random paternity were produced using the pollen mixture.

Key words: interspecific hybridization, pre-fertilization barriers, embryo rescue, *Lilium candidum*, *L. pumilum*, *L. monadelphum*, *L. regale*, *L. henryi*, *L. martagon*.

Introduction

The genus *Lilium* comprises more than 90 species. The involvement of wild species is promising for the production of novel cultivars (Van Tuyl et al. 1986; Van Tuyl et al. 2000).

The factors limiting interspecific crosses in *Lilium* can be separated into pre- and post-fertilization barriers (Van Tuyl et al. 1991). To overcome pre-fertilization barriers, various pollination methods have been tested: applying a mixture of pollens from several species, cut-style, grafted-style, placenta pollination and *in vitro* ovule pollination (Asano, Myodo 1977; Van Tuyl et al. 1982; Van Tuyl Jet al. 1991; Chi 2000). Embryo rescue, ovary slicing and ovule culture have been used to attempt to overcome post-fertilization barriers (Chi 2002).

Asiatic hybrid lilies (AH) are important today in the flower trade. However, they lack fragrance and are sensitive to *Botrytis* blight. The Oriental hybrids (OH) are ornamental but sensitive to virus. *Lilium pumilum* Delile is a hardy early flowering species. *L. regale* Wils. and *L. henryi* Baker. are outstanding and extensively used for breeding of Trumpet hybrids (TH). *L. candidum* L. and *L. monadelphum* Bieb. are rarely employed in breeding, but they possess value due to early flowering and fragrance. *L. martagon* L. is rare used in crosses within distantly related species. The goal of this research was to involve the

gene pool of distantly related lilies in Asiatic hybrids in breeding, and to obtain hybrids between *L. martagon* and cultivars from the divisions AH, TH or OH.

Materials and methods

Ten cultivars and hybrids from division 1 (AH), eight cultivars from division 7 (HT), nine cultivars from division 8 (OH) and the species *Lilium candidum* L., *L. monadelphum* Bieb., *L. regale* Wils., *L. pumilum* Delile, *L. henryi* Baker., and *L. martagon* L. were used for crossing. Plants were grown in field and in greenhouse. To prevent self-pollination, flowers were emasculated before opening. For pollination, the pollen was applied on the stigma or on the surface of a cut by 10 % sucrose solution smear. After pollination the stigma was isolated with an aluminium foil cap. The embryos were isolated 30 days after pollination from swelled ovary and cultured *in vitro* until germination on solid Murashige and Skoog (1962) medium supplemented with 3 % sucrose at 25 °C in dark.

Results

The combinations of performed distant crosses and the respective number of different pairs are presented in the Table 1. No less than five flowers were pollinated in each cross. All crosses were performed in both reciprocal directions, except when the apomictic species *L. pumilum*, *L. monadelphum* and *L. regale* participated. These species were used only as males.

The crosses of two cultivars ('Toscana' and 'Bell Ami') from division AH with *L. pumilum* were promising when the style was cut to assist pollination. Seedpods consistently developed when pollen of *L. pumilum* was applied on the native stigma. Hybrid plants were obtained only in the case when embryos were isolated before full ripening of the seedpod. In these cases, 20 hybrids were obtained: 15 from 'Toscana' × *L. pumilum* and five from 'Bell Ami' × *L. pumilum*. Six crossing combinations between AH and *L. monadelphum* were successful. The pollination of AH with *L. monadelphum* by the cut-style technique did not improve fertilization. Three hybrids were obtained in crosses AH × *L. regale* – one from 'Tiger Babys' × *L. regale* and two from 'Lollypop' × *L. regale*. Ten hybrids were produced after crossing of *L. martagon* with AH cultivars 'Lollypop', 'Stones' and 'Toscana'. All of these successful crosses involved native uncut-style pollination. No hybrids developed in reciprocal crosses of AH with *L. candidum*, *L. henryi* and cultivars from divisions TH and OH, nor in crosses between *L. martagon* and TH or OH. The hybridization of cultivars from division OH with *L. henryi*, *L. regale* and TH allowed to obtain a few hybrids in the cases 'Sorbone' × *L. regale* and *L. henryi* × 'Acapulco' after cut-style pollination.

To achieve greater success in distant crosses, pollination was performed using mixtures of incongruous pollen. The amounts of different pollen in the mixture was equal. The results in some crosses were promising and hybrids with random paternity were obtained (Table 2).

Discussion

The failure to produce interspecific hybrids may be explained in most cases by

Table 1. Fertility of crosses between distantly related species and cultivars of lilies. *, in parentheses – number of different pairs used in each crossing combination. **, confidence intervals (95 %) are given in parentheses

Cross combination*	Frequency of swelled ovaries after pollination (%)**		Viability of embryos (%)**	Obtained hybrids
	Usual pollination	Cut-style pollination		
AH × *L. pumilum* (2)	0	90.0 (35.6 - 100.0)	56.6 (25.0 - 8.2)	20
AH × *L. monadelphum* (6)	0	0	-	-
AH × L. *regale* (6)	9.7 (0 - 33.3)	0	10.3 (0 - 16.6)	3
AH × *L. candidum* (7)	0	0	-	-
L. candidum × AH (7)	0	0	-	-
AH × *L. henryi* (5)	0	0	-	-
L henryi × AH (5)	0	0	-	-
AH × *L. martagon* (5)	0	0	-	-
L. martagon × AH (3)	80.0 (9.3 - 100.0)	0	38.5 (9.2 - 49.5)	10
AH × TH (6)	0	0	-	-
TH × AH (7)	0	0	-	-
AH × OH (9)	0	0	-	-
OH × AH (7)	0	0	-	-
OH × TH (4)	0	20.0 (0 - 30.5)	0	-
TH × OH (5)	0	12.0 (0 - 25.0)	0	-
OH × *L. regale* (3)	0	50.0 (24.5 - 100.0)	1.0 (0 - 2.0)	1
OH × *L. henryi* (3)	0	0	-	-
L. henryi × OH (3)	0	25.5 (0 - 55.5)	0.1 (0 - 10.1)	1
OH × *L. martagon* (5)	0	0	-	-
L. martagon × OH (5)	0	0	-	-
TH × *L. martagon* (5)	0	0	-	-
L. martagon × TH (5)	0	0	-	-

incompatibility of style and growing pollen. Cut-style pollination has been used to overcome incongruity in interspecific crosses of lilies (Asano, Myodo 1977; Van Tuyl et al. 1986; Van Tuyl et al. 1991; Van Creij et al. 1993; Van Tuyl et al. 2000). We found that cut-style pollination was most efficient in crosses AH × *L. pumilum*, which may

Table 2. Fertility of incongruous crosses in lilies by pollination of style with mixed pollen of incompatible partners. *, in parentheses – number of different pairs used in each crossing combination. **, confidence intervals (95 %) are given in parentheses

Cross combination*		Frequency of swelled ovaries after pollination (%)**	Viability of embryos (%)**	Obtained hybrids
Maternal plant	Pollen plants			
AH (3)	*L. regale*, *L. monadelphum*	0	-	-
AH (4)	*L. regale*, *L. candidum*	25.0 (0 - 25.5)	8.8 (0 - 10.0)	3
AH (2)	*L. regale*, *L. martagon*	12.0	-	-
AH (3)	*L. candidum*, *L. monadelphum*	12.2	- (0 - 32.0)	-
AH (3)	*L. candidum*, *L. henryi*	11.1 (0 - 33.3)	0	-
AH (4)	*L. candidum*, *L. martagon*	0	-	-
AH (2)	*L. monadelphum*, *L. henryi*	8.0 (0 - 42.3)	0	-
AH (2)	*L. monadelphum*, *L. henryi*	60.0 (9.3 - 70.5)	0 (22.0 - 68.1)	-
AH (2)	*L. regale*, *L. monadelphum* *L. candidum*	70.3 (18.0 - 100.0)	70.5 (41.3 - 99.9)	63
AH (2)	TH, OH	50.0 (0 - 98.5)	73.6 (11.1 - 85.5)	29
L. martagon (2)	TH, OH	45.5 (0 - 48.0)	26.7 (0 - 45.6)	11

be explained by a short stigma: the pollen tube of *L. pumilum* can not penetrate a long distance and fails to reach the ovules if a seed parent has longer style than the pollen parent. However, when pollen from a male with a long tube was used to pollinate by cut-style the efficiency of fertilization was low. According to Chi (2000) in cut-style pollination, the generative pollen cell divides into two sperm cell later than pollen tube penetration in to the ovule micropyle.

It is supposed that the more compatible pollen induces receptivity in the stigma, allowing the penetration of less compatible pollen. This method is a variant of the so called "mentor pollination method" when pollen of incongruous species is mixed with killed compatible pollen (Van Tuyl et al. 1982). The use of mixed incompatible pollen allowed to overcome pre-fertilization barriers in incongruous crosses (Table 2). The ancestry of hybrids received after pollination by incongruous pollen mixtures will be analysed.

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The effect of photoperiod and growth regulators on organogenesis in thin-layer tissues

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Abstract

Thin layer tissue cultures are good model in studies of direct induction of various developmental programmes, particularly in floral determination. Despite an increasing number of related investigations, the factors that regulate flower formation *in vitro* are not yet clear. To evaluate the role of growth regulators in flower and stem meristem genesis, we determined the ratio of regenerating vegetative shoots and flowers as well as the dependence of the growth of regenerated organs on cultivation conditions. Genesis of vegetative organs ocurred after two to three days of cultivation in media with growth regulators. Flower buds appeared after three to four days of cultivation. The number of vegetative shoots continued to increase with the extension of growth regulator exposure from two to six days. However, the number of flowers did not change. The elimination of the stimulus after the initiation of the process showed an increase in the number of organs.

Key words: organogenesis, growth regulators, photoperiod, tobacco, thin-layer tissues.

Introduction

Plant growth and morphogenesis are controlled by meristems, organized tissues containing pluripotent stem cells whose identities and activities are regulated by intrinsic and environmental signals. Molecular studies have shown that the identity of the apical meristem and genesis of floral organs are governed by a network of gene expression, which depends on the interrelation between organs and tissues (Adams et al. 2001; Sharma, Fletcher 2002). However, these ordinary correlation links are disrupted in experiments performed with tissue explants cultivated *in vitro*. Thin-layer tissues are cell groups of the same differentiation level, e.g. subepidermal cells which *in vitro* can form roots, flower- or vegetative buds as well as irregular meristems without any intermediate callus, or can proliferate into callus without any subsequent organogenesis (Tran Thanh Van 1973). The aim of the present study was comparison of the effect of photoperiod and growth regulator exposure on flower and stem meristem genesis.

Materials and methods

The experiments were carried out with tobacco tissues having different photoperiodism

traits: long-day flowering garden tobacco – *Nicotiana alata* Link et Otto and photoperiodic neutral common tobacco – *Nicotiana tabacum* L. cv. 'Samsun'. Intact plants were cultivated under greenhouse conditions in a 16-h light photoperiod. Flower stalk thin-layer tissue explants of tobacco at stage IX of organogenesis (Kuperman 1984) were used. Thin-layer tissues composed of epidermis and three to six subepidermal cell layers were excised from flower stalks of *N. alata* and *N. tabacum*. The initial mass of the explant was 14 ± 3 mg. The tissues were cultivated for 30 days under two types of photoperiod: 16 h long-day and 8 h short-day light of 40 mM m^{-2} s^{-1} under cool white fluorescent tubes at 25 °C.

The basal cultivation medium contained Murashige and Skoog mineral salts (MS; 1962), 0.1 mg l^{-1} thiamine HCl, 100 mg l^{-1} myoinositol and 30 g l^{-1} glucose and 8 g l^{-1} agar (Altamura et al. 1998). A pH was adjusted to 5.7. The medium was supplemented with 1 μM indole-3-acetic acid (IAA) and 1 μM 6-benzylaminopurine (BA) for flower and vegetative bud induction. After 1, 2, 3, 4, 5, 6, 14 days of stimulation media with growth regulators, thin-layer explants were transferred to MS without growth regulators and cultivated up to 30 days. One of the samples of the performed experiments was cultivated in media supplemented with IAA and BA up to 30 days. The control consisted of tissues cultivated in media without growth regulators. Each experiment was carried out twice and at least 10 explants were used per test. The number of newly formed buds in an explant was estimated in relation to vegetative and to floral buds. The cultures were tested under the microscope to count the number of flower and vegetative buds formed and to determine the developmental stage of the regenerated organs. The average number of structures with differences statistically significant at $p \leq 0.05$ was estimated.

Results

The cultivation of thin-layer flower stem tissues with growth regulators in a long and short photoperiod indicated the dependence of flower regeneration on both photoperiodic reaction and photoperiod. In isolated tissue cultures the formation of flowers, similar

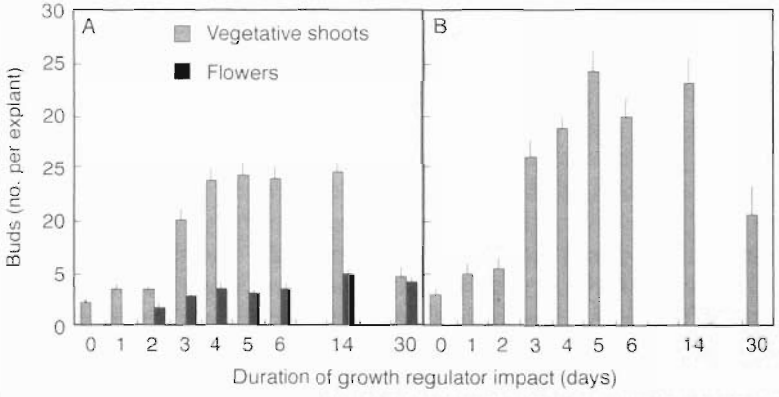

Fig. 1. Organogenesis in long day plant *Nicotiana alata* flower stalk thin-layer tissues in relation to the photoperiod and growth regulators. A, long day conditions; B, short day conditions. The number of days of thin-layer tissues cultivation on stimulation media with growth regulators is shown. After cultivation, the explants were transferred to MS medium without growth regulators and incubated up to 30 days. 0, control, explants 30 days cultivated in MS. Each experiment was carried out twice and at least 10 explants were used per test. The bars represent SE.

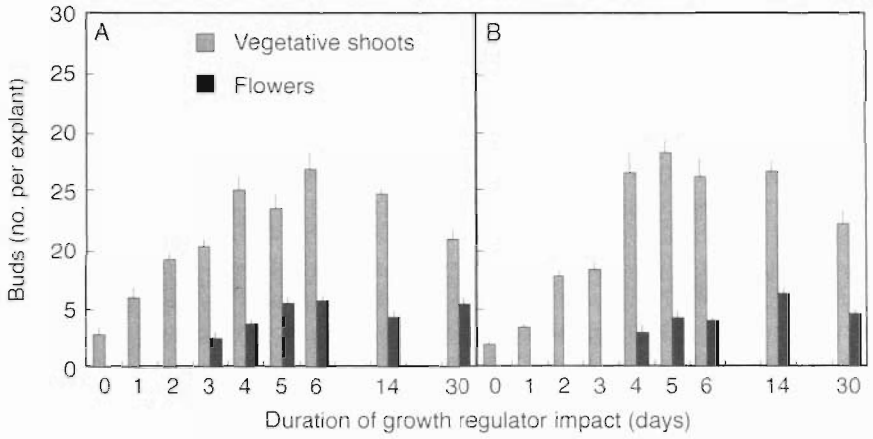

Fig. 2. Organogenesis in photoperiodic neutral *Nicotiana tabacum* 'Samsun' flower stalk thin-layer tissues in relation to the photoperiod and growth regulators. A, long day conditions; B, short day conditions. The number of days of thin-layer tissues cultivation on stimulation media with growth regulators is shown. After cultivation, the explants were transferred to MS without growth regulators and incubated up to 30 days. 0, control, explants 30 days cultivated in MS. Each experiment was carried out twice and at least 10 explants were used per test. The bars represent SE.

to vegetative shoots, needed an additional growth regulators stimulus. Initial (two to tree days) cultivation in media with IAA and BA was sufficient to evoke generation of vegetative organs, as compared with the control (Fig. 1, 2). Explants cultivated for three days in media with growth regulators produced five times more vegetative buds in long day and four times more in short day. Flower genesis was modified by a longer two-to-four day growth regulator exposure. In thin-layer flower-stem cultures the number of vegetative shoots continued to increase with the extension of growth regulator exposure from two to six days. However, the number of flowers did not change. A larger number of vegetative buds was observed after four days of cultivation in growth regulator media. Flower regeneration was less dependent on the media composition. Continuous cultivation of flower-stem tissues with growth regulators reduced regeneration of vegetative shoots.

Tissues of the long-day plant *N. alata* formed flowers (maximum was 4.58 ± 0.56 flowers per explant) only in a long day regime while only vegetative shoots were induced in a short day. Tissue of the *N. tabacum* 'Samsun', witch has a photoperiodic neutral reaction, formed flowers both under long- and short-day lighting, with maximum numbers 5.54 ± 0.33 and 4.36 ± 0.31 flowers per explant, respectively.

Discussion

Regions of dividing cells form meristematic centres and meristems, the morphogenesis of which depends on factors that modify the realisation of genetic information (Sharma, Fletcher 2003). Flower and vegetative shoot formation in thin-layer flower stem tissues requires a shorter exposure to growth regulators as compared with leaf tissues (Šauliene, Rakleviciene 2002). The observed increase in the number of vegetative organs as a response to removing growth regulators from the medium (Fig. 1, 2) suggests that growth regulator excess caused by *de novo* synthesis suppresses the morphogenesis. It is also possible that

growth regulator decomposition in the medium and the appearance of phenol compounds in the course of long-term cultivation could suppress the formation of new meristems (Scaramagli et al. 1999; Mizukami, Fischer 2000; Weyers, Paterson 2001; Torrigiani et al. 2003). Ways to enhance organogenesis may be to shorten the phytohormone effect of the medium as one of the possible reasons for the genetic instability of the regenerated organs and, after stimulation, to cultivate the tissues in hormone-free media.

Our experiments showed the importance of the photoperiodic reaction of a plant and photoperiod in flower genesis. The hypothesis that tobacco flower-stem tissues, in contrast to stems, possess a predetermined number of cells that can form flower meristems (Rajeevan, Lang 1993), is consistent with our results: as these cells are inactive under unfavourable photoperiod conditions and when growth regulators are lacking. These findings corroborate the hypothesis that the degree of tissue differentiation is one of decisive factors of flower regeneration in tissue cultures (Rajeevan, Lang 1993). It is reasonable to suggest, that at the beginning of flowering (gamete initiation), the complex modifying the photoperiodic reaction is labile; therefore, its manifestation does not depend on the photoperiod. The performed experiments showed that in tissues isolated from tobacco plants at a later stage of organogenesis the photoperiodic memory is a characteristic feature of the explant donor plant and is preserved in the first *in vitro* subculture.

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A transformation method for obtaining marker-free plants based on phosphomannose isomerase

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Abstract

A selectable marker system for plant transformation of rape plants that does not require the use of antibiotics or herbicides was developed. The selectable marker consists of the phosphomannose isomerase (PMI) gene from *E. coli* under the control of a plant promoter. The PMI gene was transferred to *Brassica napus* by *Agrobacterium*-mediated transformation, which allows the selection of transgenic plants with mannose and sucrose as selective agents. The highest transformation frequency of 7.9 % was obtained when a combination of 4.5 g l^{-1} mannose and 10 g l^{-1} sucrose was used. For early identification of transgenic events, histochemical staining with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) was used. Stable integration of the transgene was confirmed by PCR and Southern blot analysis. These preliminary results indicate that the mannose selection system can be used for *Agrobacterium*-mediated transformation of rape plants.

Key words: *Agrobacterium tumefaciens*, mannose selection marker, rape, transformation.

Introduction

The use of selection-marker genes in genetic transformation protocols has been developed to limit the regeneration of non-transgenic shoots after transformation. The majority of existing selection systems are based on antibiotic or herbicide resistance.

Regulatory committees and the public worldwide debate the safety of these markers in genetically modified food organisms. In order to turn the negative debate against Genetic Modified Organisms it is important to find alternatives to the use of antibiotics as selection agents.

One potential alternative is provided by the so-called positive selection systems, where the selective agent is converted into fully metabolised compounds (Joersbo 2001). One of these systems uses the phosphomannose isomerase (PMI), derived from *Escherichia coli* (Miles, Guest 1984), to convert mannose-6-phosphate to fructose-6-phosphate. Only transformed cells are capable of utilising mannose as a carbon source.

PMI has been used as a selectable marker for transformation of many plant species, such as sugar beet (Joersbo et al. 1998), maize (Negrotto et al. 2000; Wright et al. 2001), wheat (Reed et al. 2001), rice (Lucca et al. 2001) and pearl millet (O'Kennedey et al. 2004).

We report here on the use of mannose as a selectable agent for the recovery of transgenic rapeseed via *Agrobacterium tumefaciens*-mediated transformation. This is the first report of this system used in *B. napus* plants.

Materials and methods

Hypocotyl segments of *in vitro*-germinated plants of rape (*Brassica napus* Mill.) cv. 'Drakkar' were inoculated with the construct pNOV-GUS provided by Syngenta. The GUS intron gene (Vancanneyt et al. 1990) was cloned into the *Hind*III site of pNOV2819. The experimental design was completely randomized with two replications, each consisting of a minimum of 15 petri dishes (25 explants per dish). After three days of co-cultivation, the hypocotyl explants were subsequently selected on Murashige and Skoog (1962) medium supplemented with five combinations with different concentrations of mannose and sucrose (Table 1).

All selection media contained 500 mg l^{-1} carbenicillin in order to eliminate *Agrobacterium* contamination. Cultures were maintained under a 16/8-h photoperiod. The frequency of transformation was evaluated using a GUS assay. Leaf segments of regenerated plants were incubated in X-Gluc and GUS-extraction buffer at 36 °C overnight according to Jefferson (1987). The leaves was bleached with several washes of 70 % EtOH. Evaluation was based on the colour reaction. Transgenic plants were confirmed by PCR detection of the PMI gene. PMI-specific primers and the PCR protocol were described by Negrotto et al. (2000). For Southern blotting, about 2.5 μg of DNA were fragmented with *Eco*RV and an electrophoretically chemiluminescent detection system (CDP-Star) according to instruction of the manufacturer (Roche, Mannheim, Germany).

Results and discussion

Using traditional transformation protocols, plant cells or tissues are placed on culture media containing salts, hormones and carbon source (usually sucrose). For the PMI/mannose selection system plant explants are cultivated on a similar medium supplemented with both sucrose and mannose. This selection system is based on its ability to inhibit *in vitro* organogenesis when non-transformed explants are cultured in medium using mannose as a carbon source.

The transformation frequency that we obtained using the PMI/mannose system in rapeseed transformation is similar to that obtained by traditional selection systems.

Table 1. Combinations of mannose and sucrose in the Murashige and Skoog medium used in the experiments

Combinations	Mannose (M)/ Sucrose (S) ($g\ l^{-1}$) for the first 6 weeks	Mannose (M)/ Sucrose (S) ($g\ l^{-1}$) for the last 2 weeks
1	2 M / 20 S	4.5 M / 5 S
2	4.5 M / 5 S	4.5 M / 10 S
3	4.5 M / 10 S	4.5 M / 10 S
4	4.5 M / 20 S	4.5 M / 10 S
5	6 M / 10 S	6 M / 50 S

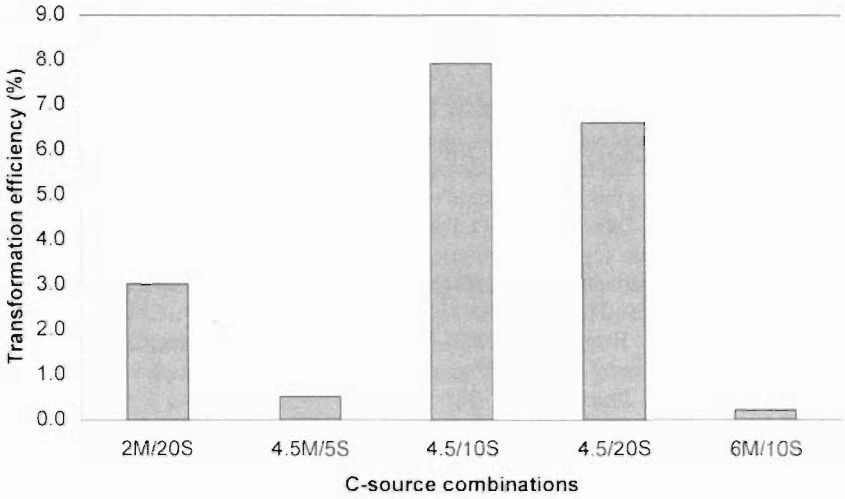

Fig. 1. Transformation frequencies (calculated as the number of GUS- and PCR-positive shoots relative to the number of inoculated hypocotyl explants) obtained at various combinations of mannose and sucrose in the MS medium. S, sucrose; M, mannose (g l^{-1}).

Screening of various combinations of mannose and sucrose indicated that 4.5 g l^{-1} mannose in combination with 10.0 g l^{-1} sucrose for the full period in selection medium attained the highest transformation frequency of 7.9 % (Fig. 1). The interaction of sucrose with mannose toxicity was studied at three levels of mannose: 2.0, 4.5, 6.0 g l^{-1}, respectively. Different levels of sucrose and mannose were used during selection and regeneration, but in the combination where the highest transformation frequency was obtained, the same level of mannose and sucrose was used in all stages. If the mannose level was higher than 5 g l^{-1}, the number of regenerated shoots was dramatically decreased. In this study, sucrose was found to strongly influence the transformation frequency, resulting in more than a 10-fold difference if 5 to 20 g l^{-1} sucrose was used. Similar observations were made with mannose selection for sugar beet transformation (Joersbo 1999) and with galactose selection for potato transformation (Joersbo et al. 2003).

PCR with *man*A-specific primers detected a fragment with a size of 550bp and Southern blot analysis showed at least one copy of the PMI gene in the transgenic plants.

These results demonstrate that rapeseed explants were able to utilize mannose as a carbon source via the integration of the PMI gene. The development of calli and shoots is comparable to using an antibiotic resistance marker. This first report on the use of the mannose selection system for the production of transgenic rapeseed plants indicates that PMI/mannose is an efficient selection system. Moreover, no potential for risk to animals, humans or environmental safety is known for this method of gene transformation. Further experiments should be carried out in combination with the gene of interest and to further increase the transformation frequency.

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Microspore mutagenesis in transgenic oilseed rape for the modification of fatty-acid composition

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Abstract

One of the first successful applications of the haploid embryogenic system in *Brassica* was aimed at mutagenesis and subsequent selection for desirable traits. A major advantage of the haploid embryogenic system for the selection of fatty-acid mutants is that analysis can be performed with only one of the two cotelydons while the remainder of the embryogenic tissue is available for plant regeneration. In the present study the applicability of the haploid technique of oilseed rape (*Brassica napus* L.) cv. 'Drakkar' transformed with the thioesterase gene *Cl*FatB4 from *Cuphea lanceolata* combined with *in vitro* mutagenesis for the selection of a high myristic-acid (C14:0) content and a lower ratio of palmitic acid/myristic acid (C16:0/C14:0) in the seed oil was tested. The embryogenic capability of isolated microspores varied between 0.03 and 2.6 embryos per bud. After ultraviolet-light (UV) treatment only 3 lines regenerated to plants. Applying an UV-dose of 0.005 J cm^{-2} resulted in 74 regenerants from which 23 % diploids were obtained. The fatty-acid composition of the different genotypes of the corresponding doubled-haploid lines displayed some changes but there was no clear evidence for their mutagenic nature. The offspring of nine additional mutagenised plants were studied and these results are presented.

Key words: *Brassica napus*, microspore, haploid production, fatty acid content, mutagenesis.

Introduction

The induction of haploid embryos from microspores in *Brassica* is recognised as the most rapid route to achieve homozygosity for the production of haploid and doubled haploid lines, which have considerable value in plant breeding. The doubled-haploid technique is an important tool for assembling desirable traits quickly into true breeding cultivars with minimal progeny numbers. For the efficient recovery of haploid embryos from *Brassica* one of the most important controlling factors is the genetic background of the donor plants (Ferrie, Keller 1995). This factor influences the frequency of embryogenesis, the quality of embryos and the way of plant regeneration (Kieffers et al. 1993; Ferrie et al. 1995).

One of the first attempts to exploit the haploid embryogenic system in *Brassica* was the selection for disease resistance (MacDonald et al. 1989; Ahmad et al. 1991). A major advantage of the haploid embryogenic system for the isolation of storage product mutants is that analysis can be performed using one of the two cotelydons, leaving the rest of the embryo for plant regeneration (Palmer, Keller 1999).

The aim of this project was to develop germplasm for the breeding of winter rapeseed with a high myristic-acid (C14:0) content and a lower palmitic-acid/myristic-acid (C16/C14) ratio in the seed oil by use of the haploid techniques combined with *in vitro* mutagenesis procedure and selection in the field.

Materials and methods

Six transgenic lines of oilseed rape (*Brassica napus* L.) cv. 'Drakkar' which had been transformed with the thioesterase gene *Cl*FatB4 (Martini et al. 1999) were grown in a growth chamber at a day/night temperature of 14/8 °C to ensure induction of flowering. The floral buds, in which microspores were in late uniculate stage, were collected and microspores were isolated and cultured as described by Lichter (1982). The buds were sterilised in 3 % NaClO and homogenised in a modified Lichter medium. After filtration microspores were collected by centrifugation, washed, diluted to 60 000 spores ml^{-1}. Then microspores were plated in 35 mm petri dishes and subjected to a pulse in the range of 10 to 180 seconds of different UV intensity (0.005; 0.02; 0.03 J cm^{-2}) in 'Biometra' UV equipment to induce mutation in microspore DNA. Embryogenesis was induced at 32 °C for 3 days in dark, then cultures were transferred to 25 °C for embryo development. After 4 weeks, embryos were transferred to solid media. The ploidy level of the regenerated plants was analysed using a flow cytometer (Cell Analyser CA-II, Partec, Münster, Germany). *In vitro* grown plants were transferred to the greenhouse, where they were propagated by bagging after colchicine treatment of the haploid plants. Seeds of each plant were harvested separately and formed a doubled-haploid line. Fatty-acid composition was analysed using one cotelydon (half-seed technique), growing the remaining embryo for seed production according to Thies (1971). Both the mean myristic-acid (C14:0) content and the mean palmitic-acid/myristic-acid (C16/C14) ratio were compared among lines using the F-test and the Tukey-test, respectively, using the respective procedures of the SAS/STAT® software, release 6.12 (SAS Institute Inc.).

Results and discussion

The overall comparison of the six transgenic lines of cv. 'Drakkar' reflected great variation of lines in embryogenic capability from 0.03 to 2.6 embryos per bud. Different effects on embryogenic response have been observed in many species of *Brassicaceae* (Phippen, Ockendon 1990; Ferrie et al. 1995). After UV treatment, only three of the six transgenic lines of cv. 'Drakkar' regenerated to homozygous plants for use in breeding programs. Altogether, after UV irradiation (0.005 J cm^{-2}) 74 regenerants developed, from which 23 % spontaneous diploids were obtained. Similar results were obtained with spontaneous chromosome doubling by Hansen and Anderson (1996) for the cv. 'Topas'.

In the first step, the fatty-acid composition of different genotypes of these doubled haploid lines displayed some changes, but there was no clear evidence of their mutagenic nature.

The offspring of nine plants were studied, which originated from two different transgenic lines #6 and #39. Seven plants from #6 were identified in the *in vitro* phase as haploid (1n) and one (6E) as diploid (2n), respectively. The two plants of #39 (39A, 39B) were haploid (1n). The haploid plants were doubled by colchicine treatment and all plants

Table 1. Fatty acid composition in the offspring of regenerated oilseed rape plants resulting from UV mutagenesis of microspores from transgenic lines. *, F-test between #39 A, B: F_{obs} = 1.48 (C14:0%), 1.49 (C16/C14); $F_{0.05\ (P=0.95;\ 26/37\ FG)}$ = 1.86. **, Tukey-test of # 6 A-G. Means followed by an identical letter are not significantly different (P = 0.95). [a], *in vitro* plants; [b], number of analysed seeds. C.V., coefficient of variation

Offspring	Ploidy[a]	N[b]	C14:0 (%)			C16:0/C14:0 ratio		
			Mean	Significance	C.V. (%)	Mean	Significance	C.V. (%)
39A	1n	27	19.9	n.s.*	23.0	1.15	n.s.*	16.6
39B	1n	38	19.6	n.s.*	19.1	1.12	n.s.*	20.8
6A	1n	25	21.6	a**	17.9	1.04	be**	15.4
6B	1n	5	17.5	ac**	23.1	1.27	ade**	16.3
6C	1n	9	20.5	a**	16.3	1.47	ac**	14.8
6D	1n	9	23.8	a**	16.7	1.12	bd**	15.2
6E	2n	10	19.3	ac**	18.1	1.35	ad**	14.0
6F	1n	16	20.1	a**	17.5	1.13	bd**	19.4
6G	1n	6	14.5	bc**	29.2	1.23	ade**	22.6

were propagated in the greenhouse by bagging. The C14:0 and the C16:0 contents of the seeds were analysed by half-seed technique. The 80 seeds of #6 varied between 9.7 and 33.3 % C14:0 and the C16/C14 ratio ranged from 0.83 to 1.78. The mean C14:0 content of the seven lines of #6 varied between 14.5 and 23.8 %. There were significant differences between the lines. The 65 seeds of #39 displayed a C14:0 content between 7.3 and 29.6 % and a C16/C14 ratio ranging from 0.84 to 1.96. The two lines of #39 did not show any significant differences in the C14:0 content (Table 1). The number of seeds displaying a C16/C14 quotient < 1.0 was 18 (22 %) in #6, and 19 (29 %) in #39, respectively. The coefficient of variability between the seeds of the same plant was in all cases relatively high. Particularly considering that all but one regenerated *in vitro* plants displayed a haploid DNA content, the colchicine-induced diploids should be homogeneous in their characters. The variability may be due to modifying influences of the culture conditions. The first generation after *in vitro* regeneration and colchicine treatment often shows delayed flowering and seed ripening. Fifty-two half-seed individuals of the 145 analysed seeds were selected on the basis of a high C14:0 content and low C16/C14 ratio, respectively. Grown in a greenhouse, they showed a normal and homogenous development. The fatty-acid analysis of their offspring will show whether the effect of environmental influences on plant development is an appropriate explanation for the observed variability.

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Plant regeneration from leaves of *Cydonia oblonga* cultivars

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Abstract

Regeneration experiments *in vitro* were started with the aim to create a transformation system for *Cydonia oblonga* Mill. The optimal conditions for microplant regeneration of cvs. K.11, K.16, K.19 of *Cydonia oblonga* were determined. Leaves were isolated from microshoots from *in vitro* culture and grown on Murashige and Skoog nutrient medium supplemented with inosite, vitamins, naphtilacetic acid, sucrose and various amounts of thidiazuron. It was determined that by modifying the thidiazuron concentration in nutrient medium was possible to induce morphogenesis in a sufficient frequency for transformation. The frequency of morphogenesis depended on the plant genotype. The highest output of regenerants was obtained by using *Cydonia oblonga* cv. K.11. The various plant genotypes required different thidiazuron concentrations in nutrient medium. A higher thidiazuron concentration was necessary (32 μM) to produce a given number of regenerants by leaves of cv. K.19 in comparison with cv. K.16. In contrast with plum trees, the morphogenesis of *Cydonia oblonga in vitro* was associated with callus induction.

Key words: *Cydonia oblonga*, plant regeneration, tissue culture.

Introduction

Genetic engineering is a modern tool for plant breeding, especially for vegetatively propagated fruit plants. Successful genetic transformation has been reported for apple (Zhu et al. 2000) and pear (Mourgues et al. 1996). A prerequisite for the transformation is adventitious shoot regeneration in tissue culture from plant organs. The *in vitro* regeneration of shoots from leaf discs of *Cydonia oblonga* Mill. (quince), genotype 'Quince A', was investigated by Dolcet-Sanjuan and co-authors (1991), but the dependence of the morfogenetic reaction of leaf discs on the quince genotype under *in vitro* conditions has not been accessed. The aim of the present work was to investigate the morphogenetic characteristics of *Cydonia oblonga* Lithuanian cultivars in leaf culture and to develop a protocol of shoots regeneration *in vitro* for further genetic transformation.

Materials and methods

The quince (*Cydonia oblonga*) cultivars of Lithuanian origin K.11, K.16, K.19 were used for the investigations. The cultivars were selected from open pollinated seedlings in the middle of 20th century. In total 96 explants were isolated from microshoots developed in *in vitro* culture for each variant of the investigation. The experiment was repeated

three times. The explants were grown on Murashige and Skoog (1962) nutrient medium, supplemented with 100 mg l^{-1} inosite; 0.5 mg l^{-1} thiamine; 0.5 mg l^{-1} pyridoxine; 0.5 mg l^{-1} nicotinic acid; 1 mg l^{-1} ascorbic acid; 0.056 mg l^{-1} naphthylacetic acid and different concentrations (0.2; 2.2; 7.05; 14.1 mg l^{-1}) of thidiazuron (TDZ). The explants were grown in a growth chamber at a temperature 21 to 25 °C and 50 mmol m^{-2} s^{-1} photon flux density illumination for 16 h with luminescent lamps. Morphogenesis was evaluated after 60 days. Significant differences of treatment means were determined by the Duncan's multiple range test.

Results

The investigations showed that by modifying TDZ concentration in the nutrient medium it was possible to induce morphogenesis in quince leaves at the frequency sufficient for transformation experiments. The regeneration frequency depended on the plant genotype and TDZ concentration in the nutrient medium (Table 1). Various plant genotypes required different optimum TDZ concentrations for morphogenesis. The highest number of shoots per explant were regenerated on nutrient medium with 2.2 to 7.05 mg l^{-1} of TDZ. Callus formation occurred at the wound surfaces of leaf explants (Table 1). The frequency of callusogenesis depended on the TDZ concentration. Depending on the genotype of quince, 33.3 to 43.7 % of explants regenerated shoots on the optimal nutrient medium (Table 1).

Discussion

TDZ was the limiting factor for *Cydonia oblonga* shoot regeneration from leaves *in vitro*. The efficiency of TDZ in regeneration of quince may be due to the particularly high cytokinin requirement of this species. The optimal concentration was 2.2 mg l^{-1} TDZ for two *C. oblonga* cultivars of Lithuanian origin used in our investigations, compared to a three times higher (7.05 mg l^{-1}) optimal TDZ concentration for 'Quince A' (Dolcet-

Table 1. Effect of thidiazuron (TDZ) on regeneration of shoots from leaves of *Cydonia oblonga* cultivars *in vitro* (96 explants from four weeks old microshoots were used in three replications). Means followed by the same letter are not significantly different (p=0.05). +, weak callusogenesis; ++, medium callusogenesis; +++, intense callusogenesis

Medium	*Cydonia oblonga* cultivars					
	K.11		K.16		K.19	
	Callus formation	Shoot regeneration (%)	Callus formation	Shoot regeneration (%)	Callus formation	Shoot regeneration (%)
MS 0 mg l^{-1} TDZ	–	0^{e}	–	0^{e}	–	0^{e}
MS 0.2 mg l^{-1} TDZ	+	2.9^{e}	+	12.5^{bc}	+	4.2^{e}
MS 2.2 mg l^{-1} TDZ	+++	43.8^{a}	+++	35.0^{a}	+++	20.8^{b}
MS 7.05 mg l^{-1} TDZ	+++	34.4^{b}	+++	27.8^{a}	+++	33.3^{a}
MS 14.1 mg l^{-1} TDZ	++	29.2^{b}	++	21.9^{ab}	++	4.2^{c}

Sanjuan et al. 1991). The optimal TDZ concentration for K.19 and 'Quince A' resulted in a decreased output of regenerants when used for the cvs. K.11 and K.16. Genotypic differences should be taken into account when developing protocols for shoot regeneration from leaves *in vitro*. A 14.1 mg l^{-1} TDZ concentration decreased the output of regenerants in all the investigated genotypes. In contrast to plum trees (Bassi, Cossio 1994), the regeneration of quince micro shoots from leaves was associated with callus induction.

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In vitro induction of polyploidy in *Ribes*

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Abstract

To restore the fertility of interspecific currant hybrids and to obtain tetraploidic varieties, we investigated currant polyploidization *in vitro* using microshoots and isolated embryos. Explants of *Ribes nigrum*, *R. hudzonianum*, *R. aureum*, *R. americanum*, *R. uva-crispa* and their hybrids were used for the studies. Explants were treated with colchicine and orysalin solutions in various concentrations. Colchicine (0.25 %), to a lesser extent than orysalin (from 20 to 40 μM), decreased the vitality of isolated microshoots of currants and the output of rooted plantlets. Systems of isolated embryos and microshoots are equally effective for creating polyploids currant. Chimeric plants were obtained during polyploidization *in vitro*. The total number of regenerants decreased with polyploidogen treatment, but the total number of regenerants with a higher number of chromosomes did not depend on the kind of polyploidogen and its concentration. The biggest output of regenerants was obtained by treating isolated explants with 20 μM oryzalin. The frequency of regeneration after polyploidogen treatment reached 19.3 % from embryos and 32.3 % from microshoots.

Key words: colchicine, *in vitro*, oryzalin, polyploidisation, *Ribes* sp.

Introduction

The induction of chromosome doubling allows to restore the fertility of the distant hybrids, to strengthen the heterosis effect, and to create polyploidic plant cultivars (Keep 1981). Polyploidic rows have not been determined in *Ribes* sp. All species have a diploidic (2n = 16) chromosome number (Brezhnev, Korovina 1981). Polyploidy has been used episodically in the breeding of currants (Kursakov 1986). The first alotetraploidic cultivars were created by the method of seed and bud colchicination (Bauer 1978; Nilsson 1978). The investigations of currant polyploidization *in vitro* have not been carried out.

The aim of the present investigations was to test methods of currant polyploidization *in vitro* using microshoots and isolated embryos, and to evaluate the efficiency of polyploidization.

Materials and methods

Isolated embryos of currant species *R. nigrum* (cvs. 'Titania', 'Vakariai', 'Ben Alder', 'Ojebyn', 'Saniuta'), interspecific hybrids *R. nigrum* × *R. uvae-crispa* (cvs. 'Kaptivator', 'Beloruskij', 'Kuršu dzintars'); *R. nigrum* × *R. aureum* (cv. 'Corona'); *R. nigrum* × *R. petraeum* (cvs. 'Jonkheer van Tets', 'Random'); *R. nigrum* × *R. americanum* and microshoots of *R. hudzonianum* grown *in vitro* were investigated. In the different variants, from 22 to

693 embryos and from 20 to 73 microshoots in three replications were used.

The explants were treated for four days with 0.25 % colchicine or for one day by 20 μM or 30 μM orysalin. Microshoots were grown in Murashige and Skoog (1962) nutrient medium supplemented with 1 mg l^{-1} benzylaminopurine. Isolated embryos were grown in White (1943) nutrient medium adapted for currant embryos (Stanys 1997). The ploidy level of the rooted regenerants was evaluated by counting the chromosome numbers in the cells of root meristems. Significant differences between the treatment means were determined by the Duncan's multiple range test.

Results

The polyploidogens colchicine and orysalin inhibited the regeneration of currant embryos. In the control variant, 50 % of explants regenerated (Table 1). With colchicine treatment 0.8 to 8.1 % embryos regenerated, depending on the variant of the experiment. Orysalin inhibited the regeneration to the lower extent. Under orysalin treatment 15.6 to 19.3 % of the embryos regenerated.

The frequency of regeneration reached 32.3 % when microshoots of *R. hudzonianum* were treated with orysalin. The highest number (from 12 to 16 of polyploidic plants) was obtained when embryos were treated with orysalin solution and from 0 to 3 of polyploidic shoots when colchicine was used.

Table 1. The effect of colchicine and orysalin on plant regeneration *in vitro* of different *Ribes* sp. and hybrids and on the output of polyploids (explants were treated for four days with colchicine or for one day by orysalin when embryos were 45 days and microshoots 28 days old). Means followed by the same letter are not significantly different (p = 0.05)

Species or hybrid	Polyploidogen, concentration	No. of affected explants	Regenerants (%)	No. of polyploids
		Embryos		
R. nigrum	Colchicine 0 %	30	50.0^{a}	0
R. nigrum	Colchicine 0.25 %	295	6.1^{e}	3
R. nigrum × *R. uvae–crispa*	Colchicine 0.25 %	693	4.5^{e}	1
R. nigrum × *R. aureum*	Colchicine 0.25 %	172	8.1^{d}	0
R. nigrum × *R. petraeum*	Colchicine 0.25 %	358	2.5^{f}	0
R. nigrum × *R. americanum*	Colchicine 0.25 %	263	0.8^{f}	0
R. nigrum	Orysalin 0 μM	22	50.0^{a}	0
R. nigrum	Orysalin 20 μM	202	19.3^{b}	16
R. nigrum	Orysalin 30 μM	147	15.6^{c}	12
LSD (1 %)			1.9	
		Microshoots		
R. hudzonianum	Orysalin 0 μM	20	40.0^{a}	0
R. hudzonianum	Orysalin 20 μM	73	32.3^{a}	4
LSD (1 %)			14.1	

Discussion

The polyploidogens colchicine and orysalin can be used to induce polyploids *in vitro*. In our treatments as in experiments with rhododendrons (Väinölä 2000) oryzalin was more efficient for the induction of chromosome doubling. The highest number of polyploidic plants was obtained by orysalin treatment. The number of regenerants with an increased chromosome number was directly correlated with their output. Systems of isolated embryos and microshoots *in vitro* can be used to create currant polyploids.

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The influence of thidiazuron on shoot regeneration and proliferation of rhododendrons *in vitro*

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Abstract

To improve shoot regeneration and proliferation methods for *Rhododendron* L., the present study was initiated to test the effect of thidiazuron (TDZ) on (i) adventitious shoot regeneration from flower explants (each consisting of an ovary with pedicel) and (ii) shoot proliferation. For regeneration, flower explants of evergreen rhododendron cv. 'Irina' were placed on Anderson's medium supplemented with various combinations of TDZ (0 - 2 mg l^{-1}), N^6-Δ^2-isopentenyl adenine (15 mg l^{-1}) and indole-3-butyric acid (3 mg l^{-1}). For proliferation two-node microshoot cuttings were placed on Anderson's medium supplemented with various concentrations of TDZ (0 - 2 mg l^{-1}). Application of TDZ induced direct adventitious shoot regeneration from flower explants. Concentrations of 0.05 to 1 mg l^{-1} TDZ were found to be efficient. A higher TDZ concentration irreversible suppressed shoot elongation. As TDZ treatment inhibited further development of axillary buds, it could not be used for promotion of rhododendrons shoot proliferation *in vitro*.

Key words: proliferation, rhododendron, shoot regeneration, thidiazuron.

Introduction

The improvement of the adventitious shoot regeneration system using tissue culture methods of *Rhododendron* L. genus plants is still important due to the diverse morphogenic potential of different genotypes. A good shoot regeneration system is a prerequisite for the development of a successful micropropagation protocol and the evolution of genetic transformation techniques. The shoot regeneration of rhododendrons has been stimulated *in vitro* from different types of explants by using various combinations of auxins and cytokinins (for summary see Tomsone, Gertnere 2003). Explants isolated from rhododendron flowers have several advantages, compared to other explants, the main being easier control of contamination and a long time interval for explant harvest (Mayer 1982; Dai et al. 1987). Therefore, attention has been focused on the improvement of direct shoot regeneration from flower explants. There are many reports showing that the application of thidiazuron (TDZ; N-phenyl-N'-1,2,3-thiadiazol-5-ylurea) results in a better shoot regeneration capacity in comparison with other cytokinins (Babaoglu, Yorgancilar 2000; Srikandarajah et al. 2001; Zhang et al. 2001). Concerning TDZ application to rhododendrons, some attempts have been made using leaf explants (Preece, Immel 1991) and stamens (Shevade, Preece 1993). The results of our previous investigation indicated that adding TDZ together with N^6-Δ^2-isopentenyl adenine (2iP) and indole-3-butyric acid (IBA) to the medium improved adventitious shoot regeneration from flower explants and

resulted in a higher explants survival rate (Tomsone, Gertnere 2003).

The objective of the present study was to determine the optimal concentration of TDZ for adventitious shoot regeneration. In addition to that, the effect of TDZ on the development of axillary buds on microshoots was investigated.

Materials and methods

For shoot regeneration tests, evergreen rhododendron (*Rhododendron* L.) cv. 'Irina' flower buds were sampled in October from open fields of the Botanical Garden of the University of Latvia. Raceme buds were washed with an antibacterial soap and rinsed in running tap water. Subsequently, buds were surface disinfected for 15 min in 17 % of a commercial bleach (Domestos, Hungary) solution containing 5 % sodium hypochlorite and rinsed with sterile distilled water. The outer bud scales were removed, florets excised, ovary with pedicel isolated (named "flower explants") and then explants, one per tube (0.15 × 15 cm), were placed on the surface of the medium. Anderson's medium (1984) containing 20 g l^{-1} sucrose, 10 g l^{-1} glucose, 6.1 g l^{-1} agar, supplemented with 15 mg l^{-1} 2iP, 3 mg l^{-1} IBA and 0 to 2 mg l^{-1} TDZ was used for shoot regeneration for 12 weeks. Subsequent explants were transferred for shoot elongation to Anderson's medium with 3 mg l^{-1} 2iP and cultivated for 8 weeks.

For proliferation tests, two node microshoot cuttings of cv. 'Irina', one per tube (0.15 × 15 cm), were placed on Anderson's medium supplemented with TDZ (0 to 2 mg l^{-1}) and cultivated for 12 weeks.

The pH of the medium was adjusted to 5.5 prior to autoclaving. The cultures were incubated under cool white fluorescent light (35 - 50 mmol m^{-2} s^{-1}) with a 16-h photoperiod at 25 ± 2 °C. For each treatment 20 explants were used.

Results and discussion

Without TDZ, the shoot regeneration capacity from rhododendron flower explants was extremely low. TDZ promoted production of granular masses of tissues and numerous shoot primordia directly on the surface flower explants. During a 12-week period, the highest shoot tips reached 3 to 5 mm in height and then stunted. As the minimal height of shoots suitable for further subcultivation was 5 mm, the number of shoots reaching at least 5 mm in height within 8 weeks of cultivation on shoot elongation medium was used as the criterion of evaluation of the effect of TDZ on regeneration. The results showed that TDZ stimulated shoot regeneration in a concentration-dependent manner (Fig. 1A). However, higher TDZ concentrations significantly reduced the height of newly formed shoots (Fig. 1B). Considering the positive effect of TDZ on shoot induction and the negative effect on shoot height, the optimal TDZ concentration for shoot regeneration from flower explants was in a range between 0.05 to 0.5 mg l^{-1}. The obtained results confirm that TDZ stimulated adventitious organogenesis similarly as shown for stamen explants of rhododendrons (Shevade 1993) and leaf explants (Preece 1991). The addition of TDZ to the medium significantly improved shoot regeneration from flower explants, compared to traditionally used 2iP together with indole-3-acetic acid (Mayer 1982; Dai et al. 1987; Gertnere, Tomsone 1996) or 2iP together with IBA (Fig. 1).

When used on two-node microshoot cuttings, TDZ treatment resulted in the

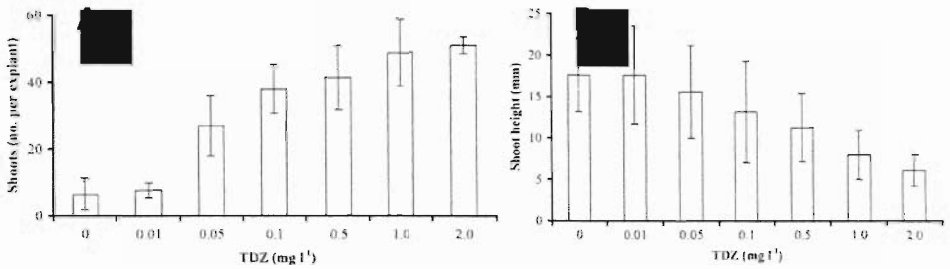

Fig. 1. The effect of thidiazuron (TDZ) on shoot regeneration from flower explants of rhododendron cv. 'Irina'. A, shoot number; B, shoot height. Measurements were made after 12 weeks on regeneration medium with TDZ and a subsequent 8 weeks of cultivation on elongation medium. Data presented are means ± SD from 20 explants. Only shoots at least 5 mm in length were measured.

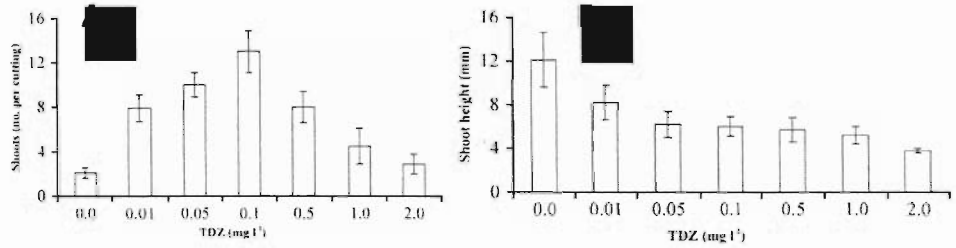

Fig. 2. The effect of thidiazuron (TDZ) on shoot proliferation from two-node microshoot cuttings of rhododendron cv. 'Irina'. A, shoot number; B, shoot height. Measurements were made after 12 weeks of cultivation.

development of a number of short, deformed shoots (Fig. 2). The shoots developed on the surfaces of swollen axillary buds. It is obvious that TDZ efficiently stimulated direct adventitive shoot regeneration from flower explants of rhododendrons, but inhibited shoot elongation. As TDZ suppressed development of axillary shoots, it could not be used for the promotion of microshoot proliferation.

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Preservation of the rare terrestrial orchids *in vitro*

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Abstract

The aim of this study was to establish viable tissue culture of Estonian terrestrial orchids for *in vitro* conservation and for final reindroduction of propagated plants to their areas. In total 28 meristems of *Dactylorhiza baltica* (Klinge) Orlova were excised from protocorm-like bodies and planted to modified Murashige and Skoog medium supplemented with 0.5 mg l^{-1} naphthaleneacetic acid and 2.0 mg l^{-1} N^6-Δ^2-isopentenyl adenine. After four weeks of culture, 60 % of excised apical meristems of *D. baltica* had started to grow while none of the explants originating from the lateral meristems expressed any tissue development. Semi-ripened seeds of *D. ruthei* (M. Schulze ex Ruthe) Soo and *D. praetermissa* (Druce) Soo. were sown to modified Heller, Lindemann, Norstog or Murashige and Skoog (control) medium. The current studies showed that high concentrations of microelements are necessary for germination of orchid seeds. Depending on species, germination of orchid seeds started two months after initiation. Preliminary results have been obtained regarding in vitro culture of *D. baltica*, *D. ruthei* and *D. praetermissa*.

Key words: orchids, preservation, regeneration, seeds, tissue culture.

Introduction

In vitro germination of seeds of north temperate terrestrial orchids species has been more difficult than with tropical epiphytic orchids. Terrestrial species have more stringent requirements for germination but little is known about their specific requirements (De Pauw et al. 1995). In Estonia there are 36 species of terrestrial orchids, only three of them have been taken under protection. Declining numbers of plants in the populations are caused by climatic changes, human activities, increased overgrowth of growing areas with bushes etc.

The present investigation examined the specific requirements for the germination and protocorm growth of *Dactylorhiza ruthei*, *D. praetermissa* and of meristem initiation of *D. baltica*.

Materials and methods

The protocorm-like bodies of *Dactylorhiza baltica* (Klinge) Orlova and semi-ripened seeds of *D. ruthei* (M. Schulze ex Ruthe) Soo and *D. praetermissa* (Druce) Soo were used as explants. The seed capsules of *D. ruthei* and *D. praetermissa* were collected seven and ten weeks after pollination. The protocorm-like bodies and semi-ripened

capsules were dipped in 75 % ethyl alcohol and rinsed with distilled autoclaved water. This was followed by sterilization in sodium hypochlorite solution for 20 min. In total 28 meristems of *D. baltica* were excised from buds from protocorm-like bodies and planted to modified Murashige–Skoog medium supplemented with 0.5 mg l^{-1} 1-naphthaleneacetic acid (NAA) and 2.0 mg l^{-1} N^6-Δ^2-isopentenyl adenine (2iP). The size of meristems was measured every tenth day.

Semi-ripened seeds were sown to modified Heller (1953), Lindemann (1970), Norstog (1973) or Murashige and Skoog (1962) (control) medium. Seeds from each individual capsule were assigned to all treatments: approximately 100 seeds per plate and two replications of each treatment per capsule. The size of seeds was measured after sowing every ten days. Germination was considered to have occurred when the embryo emerged from testa.

Results

After four weeks of culture, 60 % of excised apical meristems of *D. baltica* had started to grow while none of the explants orginated from the lateral meristems expressed any tissue development (Table 1). Protocorm-like bodies of *D. baltica* formed after four weeks on meristems taken from the apical section of growth buds form the protocorm- like bodies. Root formation of *D. baltica* occurred two months after planting of meristems to media.

The composition of culture medium and the stage of ripeness of seeds had a strong influence on the development of orchids seeds *in vitro*. Depending on species, germination was initiated approximately two months after inoculation in most media variants (Table 2, 3). After four and six months in culture, a higher percentage of the final germination occurred on modified Norstog (1973) medium compared to the control Murashige and Skoog (1962) medium. The germination percentage of seeds of *D. ruthei* (M. Schulze ex Ruthe) Soo was 14 % higher on modified Norstog (1973) than control Murashige-Skoog (1962) medium *D. praetermissa* (Druce) Soo.

Discussion

The present investigation examined specific requirements for *D. baltica* meristem initiation. Protocorm-like bodies of *D. baltica* formed after four weeks on in vitro culture. Explants of *Diuris longifolia* R.Br. formed protocorm-like bodies after forty nine days on the in vitro culture (Collins et al 1992). No tissue development was expressed on meristems of *D. baltica* which orginated from the lateral buds.

Research on *in vitro* germination of terrestrial orchid seeds is often a long, slow process due to the considerable periods of time required for germination to occur (De

Table 1. Regeneration of *Dactylorhiza baltica* meristems depending on position of buds on protocorm-like bodies

Position of buds	Number of excised explants	Number of live explants		
		4 weeeks	6 weeks	8 weeks
Apical	14	8	8	8
Lateral	14	0	0	0

Table 2. Time-course of germination percentage of *Dactylorhiza ruthei* seeds on different cultivation media

Medium	Time of cultivation (months)			
	2	4	6	8
Heller	0	5	7	8
Lindemann	0	6	9	10
Norstog	0	10	20	25
Murashige and Skoog (control)	0	6	10	11

Table 3. Time-course of germination percentage of *Dactylorhiza praetermissa* seeds on different cultivation media

Medium	Time of cultivation (months)			
	2	4	6	8
Heller	0	0	4	8
Lindemann	0	2	5	5
Norstog	0	7	15	20
Murashige and Skoog (control)	0	2	6	9

Pauw et al. 1993). *D. ruthei* and *D. praetermissa* seeds started to germinate after four mounth of culture. Germination of *D. ruthei* and *D. praetermissa* seeds depended on the concentration of $MnSO_4$ in the media. Stancato (1996) showed that micronutrients play a fundamental role in the growth of seedlings of Laelia cinnabarina Batem. Seeds of *Dactylorhiza maculata* started to germinate after 3.5 months of incubation in soil (Kinderen 1995). Our study showed that a high precentage of seeds germinate within three months of culture, and after this time, the increase in germination is very small.

Modified Norstog (1973) medium is characterised by a high concentration $MnSO_4$ (4 g l^{-1}) compared to other media tested. It can be assumed that $MnSO_4$ is an important component in germination of orchids seeds and that it significantly enhances the development of protocorm-like bodies.

Acknowledgements

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Agrobacterium rhizogenes-mediated transformation and regeneration of bisexual *Actinidia kolomikta*

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Abstract

The aim of the work was to produce *Actinidia kolomikta* plants with modified growth and root capacity. Four highly branched root clones were obtained from leaf explants of bisexual *A. kolomikta* plants by *Agrobacterium rhizogenes* transformation. Two transformed shoot clones were regenerated from hairy roots on solid half-strength Woody Plant Medium with varying concentrations of benzyladenine (0.5 to 5.0 mg l^{-1}). Shoot cultures of transformed plants required culture media for proliferation and rooting different from those suitable for the control. The response to exogenous hormones was modified in the transformants. Shoot proliferation required benzyladenine as the only hormone. Microcuttings from transformed in vitro plants formed dense root systems on auxin-free Murashige and Skoog medium. Leaf explants from transformed plants produced roots on hormone-free Murashige and Skoog medium, a typical response of plants regenerated from hairy roots. No rooting was observed on untransformed actinidia leaf explants under the same conditions. Transformants that had grown in the unheated greenhouse suffered from pests, and they tended to be sensitive to frost in winter.

Key words: *Agrobacterium rhizogenes*, bisexual *Actinidia kolomikta*, hairy roots, plant regeneration.

Introduction

In Estonia *Actinidia kolomikta* has been cultivated mainly as an ornamnetal plant. *A. kolomikta* deserves attention as a berry plant as well. Its fruits are tasty and contain a large amount of vitamin C (Bojarkova 1949). *A. kolomikt*a is usually unisexual (Kachalov 1970). A bisexual plant of *A. kolomikta* was found among the plants introduced to Estonia from the Far East (vicinity of Vladivostok). This plant was micpropagated in order to preserve its genetic potential (Vardja, Vardja 1998). The growth of *A. kolomikta* vines is about one meter during the growing season. The *Agrobacterium rhizogenes* transformation system has been used previously to modify plant growth and development (van der Salm et al. 1996). Plants regenerated from transformed roots had a "hairy root" phenotype – altered growth and morphology, a very dense root system, reduced apical dominance, short internodes, the ability to profusely regenerate roots from leaf explants, and some other features. In this work, we report on the genetic transformation of bisexual *A. kolomikta* using *A. rhizogenes* as a vector.

Materials and methods

The plant material for *A. rhizogenes*-mediated transformation was derived from intensively growing *in vitro* plants (Vardja, Vardja 1998).

The Agropine strain of *A. rhizogenes* LBA 9402 (gift from Norwich Laboratory, England) was used in the present study. For transformation experiments, a 24-h-old bacterial suspension grown in liquid yeast-mannitol (0.5 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4$, 0.1 g l^{-1} NaCl, 10 g l^{-1} mannitol, 0.4 g l^{-1} yeast extract, 50 mg l^{-1} kanamycin, pH 7.0) medium at 25 °C on a rotatory shaker (90 rpm) was used.

Plant explants (whole leaves with petioles, leaf petioles and pieces of leaves) were co-cultured with *A. rhizogenes* suspension diluted with Murashige, Skoog (1962; MS) liquid medium. The explants were immersed in the bacterial suspension for 50 min. After two days of co-culture on MS solid medium, the explants were rinsed in liquid MS medium containing cefotaxime (0.5 g l^{-1}). The explants were then transferred to solid MS medium without growth regulators and supplemented with both cefotaxime (500 mg l^{-1}) and kanamycin (50 mg l^{-1}) to eliminate bacteria and to select kanamycin-resistant primary hairy roots. To confirm the absence of bacteria in root cultures, root pieces were cultured on solid yeast-mannitol medium (with 10 g l^{-1} agar at 25 °C in the dark conditions). After purification from bacteria the root culture was maintained and propagated for several subcultures in liquid hormone-free ½ Woody Plant Medium (Lloyd, McCown 1981; WPM) with 20 g l^{-1} sucrose.

To induce shoot regeneration, segments of each root clone with a length of about 2 cm were cultured on solid ½ WPM media with varying benzyladenine (BA) concentration (0.5 to 5.0 mg l^{-1}). This experiment was repeated three times with 30 root explants per treatment. The shoots regenerated from hairy roots were micropropagated and maintained on standard MS medium with BA as the only hormone or without any hormone.

Results and discussion

Hairy root formation on inoculated whole leaf explants was low (6.6 %). The other explants, both non-inoculated and inoculated, did not produce any roots.

Four of ten kanamycin-resistant root clones were highly branched and grew rapidly in hormone-free liquid medium. In three months shoot regeneration from roots had occurred only in the case of two hairy root clones on solid half-strength WPM medium. The clone 4 regenerated with 0.5 mg l^{-1} BA and the clone 3 only with 5 mg l^{-1} BA. No shoots from the other two clones developed, although calli were formed on root explants (data not shown).

The transformants grew and proliferated poorly on the medium used for propagation of wild-type actinidia (Vardja, Vardja 1998). Therefore, MS media supplemented with different hormones were tested for propagation (Table 1). The final medium adopted for propagation was the standard MS with BA as the only hormone. The observation that shoot cultures of transformed plants require proliferation and rooting media different from those suitable for the control has been previously reported (Lambert 1992).

Abundant callus (to 1.5 cm diameter) formation was observed at the base of microcuttings (C3, C4) when cultured on MS medium with indolebutyric acid (0.1 to 1.0 mg l^{-1}). Numerous roots and also buds developed from this callus (data not shown).

Table 1. Proliferation, growth and root regeneration of transformed clone 4 of *A. kolomikta* (growth period 6 weeks) on Murashige and Skoog medium without growth regulators or supplemented with different growth regulators (BA, benzyladenine; GA, gibberellic acid; Z, zeatin, and ABA, abscisic acid) or their combinations. Means within columns with different letters are significantly different ($P < 0.05$) according to the Duncan's test. [1], new buds from callus; [2], most roots derived from callus; [3], most shoots were vitrified

Growth regulator (mg l^{-1})	Type of explant	Diameter of callus (cm)	Roots per microcutting	Axillary shoots per explant	Length of the longest shoot (cm)	Number of nodes of the longest shoot
Control	Shoot tip	0.3 - 0.5[e]	20 - 30[2]	1.4[b]	3.6[c]	3.1[b]
	One-node	0.1 - 0.2[e]	20 - 25[2]	1.2[c]	3.2[b]	3.8[a]
BA 0.5	Shoot-tip	0.3 - 0.5[e]	20 - 30[2]	1.5[b]	3.5[c]	3.3[b]
	Two-node	0.3 - 0.5[e]	20 - 30[d]	1.6[b]	3.5[c]	3.0[b]
BA 1.0	Two-node	0.1 - 0.2[e]	4.1 ± 1.3	2.0[a]	2.9[b]	3.0[b]
BA 1.0 + GA 1.0	Shoot tip	0.6 - 0.8[1c]	3.0 ± 0.8	1.1[d]	2.0[c]	2.0
	One-node	0.1 - 0.2[e]	10 - 15	1.0[d]	3.2[b]	3.0[b]
	Two-node	0.1 - 0.2[e]	5.0 ± 1.4	2.0[a]	2.1[c]	3.1[b]
Z 1.0	Shoot-tip	0.1 - 0.2[1e]	4.1[2]	2.2[a]	1.8[c]	2.8[b]
	One-node	0.3 - 0.5[1d]	0	1.0[3]	1.6[c]	2.8[b]
	Two-node	0.3 - 0.5[e]	1.5 ± 0.5	2.0[a]	1.8[c]	2.8[b]
Z 1.0 + GA 1.0	Shoot tip	1.5 - 1.7[c]	2.0 ± 1.2	1.1[c]	1.5[c]	2.0[c]
	One-node	1.0 - 1.3[b]	2.0 ± 1.3	1.1[d]	1.5[5c]	2.2[c]
	Two-node	1.0 - 1.3[b]	2.0 ± 1.1	1.1[3]	1.6[5c]	2.0[c]
ABA 2.6	One-node	1.5 - 1.7[c]	5.1 ± 1.5	1.0[3]	1.1[d]	2.0[c]

Microcuttings from transgenic plants showed an increased ability to produse roots on auxin-free MS medium (Table 2). Leaf explants from transformants (C4 and C3) produced roots on hormone-free MS medium, a typical response of plants regenerated from hairy roots. No rooting was observed on untransformed *A. kolomikta* leaf explants under the same conditions. Transgenic plants of clone 4 exhibited enhanced growth in vitro and in greenhouse conditions as compared to clone 3 and control plants. An increased growth

Table 1. The growth and rooting of shoot explants of bisexual *A. kolomikta* subcultured on hormone-free MS medium (four weeks). Transformed clones (C3; C4) and wild type. Means within columns with different letters are significantly different ($P < 0.05$) according to the Duncan's test

Plant	Axillary shoots per explant	Length of shoots (cm)	Number of nodes	Roots per microcuttings	Length of leaves (cm)
Wild type	1.0[b]	1.7[c]	3.6[b]	1.0	2.1[c]
Clone 3	1.0[b]	1.2[c]	2.7[c]	>30	1.7[b]
Clone 4	1.4[a]	3.2[a]	4.3[a]	>20	1.3[c]

rate of transgenic plants was previously reported in work on aspen (Tzfira et al. 1999).

Transformants that had grown in the unheated greenhouse suffered from pests, and they tended to be sensitive to frost in winter.

The results from this study demonstrate that *A. rhizogenes*-mediated transformation is an effective method for producing bisexual *A. kolomikta* plants with altered growth and increased rooting capacity.

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Application of antioxidants in rooting of *Prunus avium* L. microshoots

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Abstract

The influence of antioxidants on *in vitro* rooting, acclimatization and *ex vitro* growth of sweet cherry (*Prunus avium* L.) microshoots was studied. Trials were conducted with Estonian sweet cherry cultivar 'Kristiina'. Four antioxidants were tested: glutathione (reduced form), dithiothreitol, propyl gallate and ascorbic acid. The *in vitro* rooting and subsequent growth of plants in greenhouse was promoted by antioxidants, but the influence on acclimatization was not always significant. The *in vitro* rooting of microshoots was most improved by 0.25 mM dithiothreitol: 27 % more shoots formed roots compared to untreated shoots. Dithiothreitol 0.25 mM improved the acclimatization of the plants in the greenhouse. The shoot and root growth of cv. 'Kristiina' was most vigorous also on plants that were rooted *in vitro* in the presence of 0.25 mM dithiothreitol.

Key words: acclimatization, antioxidants, micropropagation, *Prunus avium*, rooting, sweet cherry.

Introduction

Micropropagated plants are grown *in vitro* under low levels of light, aseptic conditions, on a medium containing sugar and nutrients to allow heterotrophic growth and at a high relative humidity. Therefore the leaves that develop *in vitro* generally lack well developed epicuticular waxes (Sutter, Langhans 1982), they have malfunctioning stomata (Brainerd, Fuchigami 1981; Marin et al. 1988), and a poorly structured internal anatomy (Marin et al. 1988; Noé, Bonini 1996), and they may not be photosynthetically efficient (Donnelli, Vidaver 1984; Grout 1988). This increases the susceptibility of plantlets to stress under the unstable climatic conditions in greenhouse. Some scientists suggest that rooting of micropropagated plants can be improved by treatment with antioxidants (Stonier 1971). Antioxidants can potentially protect the natural plant rooting hormones from oxidation, enhancing rooting and increasing the tolerance of plants to greenhouse conditions (Lis-Balchin 1989). The aim of the current research was to study the influence of antioxidants on *in vitro* rooting, acclimatization and *ex vitro* growth of sweet cherry microshoots.

Materials and methods

Trials were conducted with sweet cherry cultivar of Estonian origin – 'Kristiina'. In all trials one- or two-years-old tissue culture material was used. Shoots 2.0 to 3.0 cm in

length were rooted on modified Murashige and Skoog (1962; MS) medium containing ½ MS macroelements, full concentration of MS microelements and vitamins, 30 g l^{-1} sucrose, and 2 mg l^{-1} IBA. The pH of media was adjusted to 5.7 - 5.8.

The solutions of antioxidants were sterile-filtrated and added after autoclaving of media. Four antioxidants – glutathione (reduced form, GSH), dithiothreitol (DTT), propyl gallate (PrGl) and ascorbic acid (AscA) were used. Antioxidant concentrations tested were 0, 0.0025 mM, 0.025 mM, and 0.25 mM.

Temperature in the growth room was 22 to 24 °C, photoperiod 16 h and light intensity 35 to 40 μmol m^{-2} s^{-1}. After two weeks of *in vitro* rooting the microshoots were planted in peat substrate in seed trays (VP-96 type) and transferred to greenhouse for acclimatization and *ex vitro* growth. The growth conditions in the greenhouse were set at temperature 20 to 25 °C during the day and 16 to 18 °C at night. After four week acclimatization the survived plants were counted, removed from the substrate and dry massess of shoots and roots were measured.

All trials were conducted in one replication but were repeated three times. For each experiment 100 plants per treatment were planted *in vitro* onto rooting media, and 96 plants were planted *ex vitro* into trays. The results were analyzed statistically using computer software SAS for Windows 8.2 (http://www.sas.com) by single-factor Student's *t*-test. The 95 % least significant difference $LSD_{0.05}$ was determined.

Results

The *in vitro* rooting of cv. 'Kristiina' microshoots varied between treatments, ranging from 49 to 89 % of all planted shoots (Table 1). The highest increase in the number of rooted shoots was achieved by adding DTT to the rooting medium. In the presence of 0.025 mM and 0.25 mM DTT, 27 % more shoots formed roots compared to untreated shoots. Antioxidant treatment affected also the number of roots formed on the shoot. DTT, PrgGl and AscA increased the number of roots per shoot significantly at a 0.25 mM concentration.

The subsequent influence of *in vitro* rooting conditions on the survival of microplants in the greenhouse varied between treatments (Fig. 1). Among the tested antioxidants, only DTT and AscA had improved the survival of microplants. The survival in greenhouse was

Table 1. The influence of antioxidants on the *in vitro* rooting of *Prunus avium* L. microshoots of cv. 'Kristiina'. Evaluation after 2 weeks of *in vitro* rooting. Values represent the mean of 100 explants per treatment of three repeated tests. GSH, glutathione (reduced form); DTT, dithiothreitol; PrGl, propyl gallate; AscA, ascorbic acid. *, values significantly different from untreated control

Concentration	Root-forming shoots *in vitro* (%)				No. of roots per shoot			
(mM)	GSH	DTT	PrGl	AscA	GSH	DTT	PrGl	AscA
0	73	49	64	58	5.3	5.1	5.4	5.4
0.0025	64	68*	78*	61	5.3	5.1	6.5	5.9
0.025	79	76*	72	67	6.8*	5.1	5.5	5.8
0.25	65	76*	89*	65	5.9	6.4*	8.7*	7.3*
$LSD_{0.05}$	13	13	12	14	1.4	1.3	1.6	1.6

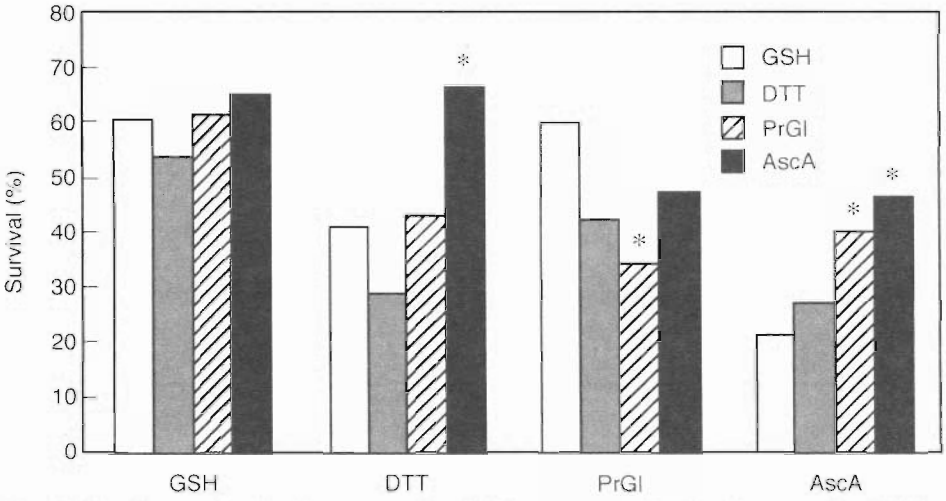

Fig. 1. The influence of antioxidants on survival of *Prunus avium* L. microshoots *ex vitro*. $LSD_{0.05}$ GSH = 14; DTT = 14; PrGl = 15; AscA = 14. Evaluation after 4 weeks of *ex vitro* growth. Values represent the mean of 96 explants per treatment (three repeated tests). GSH, glutathione (reduced form); DTT, dithiothreitol; PrGl, propyl gallate; AscA, ascorbic acid. *, values significantly different from untreated control.

improved 1.6 fold when shoots were rooted on the medium containing 0.25 mM DTT, and 2.1 fold when they were rooted at 0.25 mM AscA as compared to untreated shoots.

There was no clear relationship between survival percentage and dry mass ofshoots and roots of plants acclimatized in the greenhouse. The dry mass of shoots and roots was raised by treatment with DTT at the concentration 0.25 mM (Fig. 2). The other antioxidants demonstrated a slight positive effect on the growth of shoots and/or roots but the difference was insignificant compared to the untreated plants (data not shown).

Discussion

The rooting intensity of sweet cherry *in vitro* varied in relatively large scale between untreated microshoots. The rooting capacity of microshoots depends on many factors – the age of explants (number of subcultures); amount of BA in culture medium and the amount of BA taken up by the tissues during *in vitro* cultivation; the ratio between cytokinins and auxins in culture medium etc. (George 1993). It has been found that the rooting capacity of apple microshoots varied not only within the cultivar but even within the same shoot cluster (Hicks 1987; Welander, Pawlicki 1993). In the present study it was rather difficult to select microshoots by their rooting ability. There were no visual symptoms referring to low rooting capacity. All malformed shoots (e.g. vitrified or presenting other epigenetic changes) were immediately discarded.

Our trials were conducted during three years. The initial test material was renewed periodically in order to use shoots of the same age in all the trials. Because of persistent renewal, the microshoots used in different trials originated from different mother plants which may have caused the large variation in rooting capacity. Nevertheless, it was evident that in the case of low rooting capacity of microshoots the formation of roots

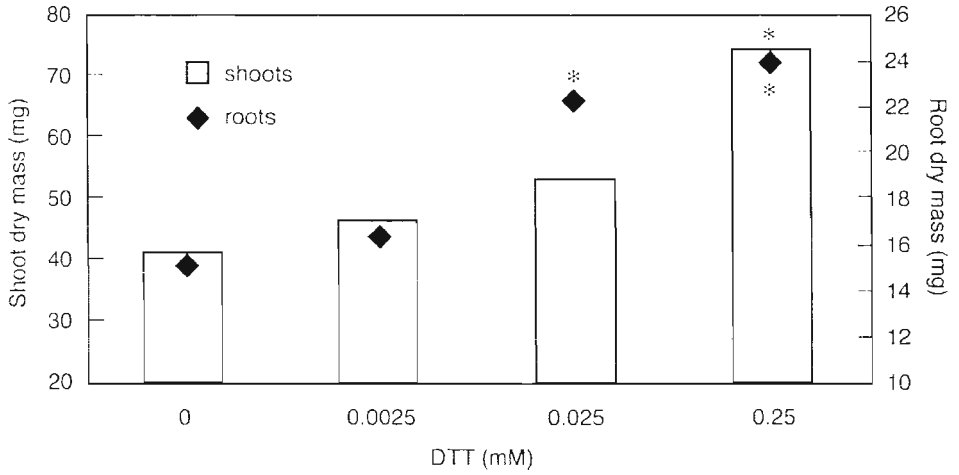

Fig. 2. The influence of dithiothreitol (DTT) on dry mass of shoots and roots of micropropagated *Prunus avium* L. *ex vitro*. $LSD_{0.05}$ shoots = 15; roots = 5. Evaluation was performed after 4 weeks of *ex vitro* growth. Values represent the mean of 96 explants per treatment (three repeated tests). *, values significantly different from untreated control.

in vitro was enhanced by supplementing the rooting medium with a certain amount of antioxidant. Similar to that, the variation in survival percentage of untreated microplants can be affected by the time of the year the tests were performed.

The effect of antioxidants depends on their concentration in the medium as well as on the plant genotype. Bonner and Axtmann (1937) demonstrated that the rooting of pea microshoots was promoted by 50 mg l^{-1} (0.25 mM) ascorbic acid, while 25 mg l^{-1} (0.126 mM) and 100 mg l^{-1} (50 mM) were detrimental. The best *in vitro* rooting of soybean was achieved with 0.1 mM GSH and DTT, and the best rooting of apple with 0.25 mM DTT or 0.075 mM GSH (Auderset et al. 1996). In our trials rooting was improved by antioxidants at a 0.25 mM concentration. Although the rooting percentage was increased already by 0.0025 mM DTT, a 0.25 mM concentration was needed to increase also the number of roots.

During the acclimatization process plants have to adapt to the new environmental conditions such as lower relative humidity, higher light density, fluctuating temperatures and constant disease stress (Preece, Sutter 1991). A prerequisite for the survival of rooted microcuttings is that the roots support the plant while new leaves and stems are produced during acclimatization (Nemeth 1986). It has been observed that *ex vitro* rooting and simultaneous acclimatization for different crops can save time and other resources (Maene, Debergh 1983; Preece, Sutter 1991). In our trials the survival of shoots that had not formed roots *in vitro* varied within the same range as that of rooted microplants. The positive effect of antioxidants was more pronounced in the trials where the overall survival of microplants was low.

In our trials the survival of shoots that had not formed roots *in vitro* varied at the same range as that of rooted microplants. However, the survival of untreated shoots was correlated to the formation of roots *in vitro* of the same treatment and depended on the physiological conditions of microplants at the moment of planting *ex vitro*. The

rooted microshoots were apparently stronger and therefore better adapted to greenhouse conditions than unrooted shoots. Similarly to *in vitro* rooting results, the positive effect of antioxidants was more expressed in the trials where the overall survival of microplants was low.

There was no relationship between the *in vitro* rooting of microshoots and *ex vitro* growth of plants. This is consistent with the results of micropropagated grapes where the number new roots produced *ex vitro* was independent from roots formed *in vitro* (Thomas, Ravindra 1997).

It has been demonstrated that a mixture of two antioxidants can affect the rooting of cuttings synergistically. There was a considerable enhancement in rooting of *Geraniceae* cuttings after treatment with a mixture of vitamins C and E or propyl gallate and butyl-hydroxyanisole than when these components were used alone (Lis-Balchin 1989). In our trials all antioxidants were used alone, not in mixtures, which can may explain why the effect of some of the compounds remained relatively low. In future studies we are planning to test the efficacy of combinations of different antioxidants on the *in vitro* rooting, subsequent acclimatization and *ex vitro* growth of micropropagated plants.

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Propagation of some *Abies* species by somatic embryogenesis

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Abstract

Somatic embryogenesis was used for propagation of *Abies cilicica*, *Abies numidica* and *Abies concolor*. The same experimental conditions were suitable for plantlets regeneration of all tested species. Initiation of embryogenic tissue was obtained from immature embryos on Schenk and Hildebrandt (SH) initiation medium with 1 mg l^{-1} benzylaminopurine and 20 g l^{-1} sucrose. The best maturation of somatic embryos was achieved on modified Murashige and Skoog medium supplemented with 40 g l^{-1} maltose, 100 g l^{-1} polyethylene glycol-4000, 10 mg l^{-1} abscisic acid and 500 mg l^{-1} L-glutamine and casein hydrolysate. After three weeks of partial drying, mature embryo germination occurred on medium containing ½-SH medium salts, 10 g l^{-1} sucrose, 10 g l^{-1} charcoal and 3 g l^{-1} Phytagel. Plantlets with cotyledons, hypocotyl and radicula were obtained and transferred to small pots containing autoclaved peat / vermiculite / perlite mixture. Plantlet regeneration of other *Abies* species can be achieved under the same experimental conditions, but with optimizing for individual species to affect the yield of regenerants.

Key words: *Abies*, embryogenic tissue, firs, induction, germination, maturation.

Introduction

Somatic embryogenesis is a very convenient method for mass multiplication of conifers including of the genus *Abies*. The technology to initiate, maintain, and develop somatic embryos and emblings of conifer species via somatic embryogenesis is now well established. Somatic embryogenesis in *Abies* with limited success or succesfull regeneration has been reported for some species (for review see Vooková, Kormuťák 2001). *A. cilicica* Carr. is a fast growing species whose natural distribution is in Asia Minor (Bozkus 1987). *A. numidica* De Lann. is a North African species, and the *A. concolor* (Gord. et Glend) distribution is North America (Liu 1971).

The objective of the present study was to apply somatic embryogenesis technology and try to generalize micropropagation conditions of three *Abies* species growing in Arboretum Mlyňany, Slovakia.

Materials and methods

Cones containing immature seeds of *A. cilicica* Carr., *A. numidica* De Lann. and *A. concolor* (Gord. et Glend) was colected in June 8th, 1997 from the Arboretum Mlyňany.

Slovakia. Imature seeds were surface-sterilized for 10 min in 10 % H_2O_2. Megametophytes containing embryos were cultured on Schenk, Hildebrandt (1972; SH) medium with 1 mg l^{-1} 6-benzylaminopurine (BAP). Induced embryogenic tissue was maintained at 24 °C in the dark on proliferation medium supplemented with 1 mg l^{-1} BAP, 1 g l^{-1} casein hydrolysate, 500 mg l^{-1} L-glutamine and 3 g l^{-1} Phytagel.

To assess the most beneficial medium for somatic embryo maturation, embryogenic tissue of one cell line per species was cultured on SH, Gresshoff, Doy (1972; GD) and modified Murashige and Skoog (1962; MS) media. SH and GD media contained the original macro, micro-elements, FeEDTA and vitamins; MS medium contained ½ strength MS macro elements, original micro elements and FeEDTA, and modified vitamins: 1 mg l^{-1} nicotinic acid, 1 mg l^{-1} thiamine HCl, 1 mg l^{-1} pyridoxin HCl, 2 mg l^{-1} glycine, 100 mg l^{-1} *myo*-inositol. All media contained 10 mg l^{-1} abscisic acid (ABA), 40 g l^{-1} maltose, 100 g l^{-1} polyethylene glycol, 500 mg l^{-1} casein hydrolysate and 500 mg l^{-1} L-glutamine. The maturation medium was gelled with 3 g l^{-1} Phytagel.

Pieces of embryogenic tissue with approximate weight of 350 mg were cultured in 60 mm plastic Petri dishes on maturation medium in the dark at 21 to 23 °C. Mature somatic embryos were subjected to partial desiccation during three weeks at 24 °C in the dark (Vooková et al. 1997/1998). The embryos were allowed to germinate on SH medium containing ½-SH medium salts, 10 g l^{-1} sucrose, 10 g l^{-1} charcoal and 3 g l^{-1} Phytagel. Six replications of ten embryos were cultivated in Erlenmayer flask with 50 ml media per treatment. Germination percentages were evaluated after 40 d of cultivation. Plantlets with a root were transferred to small pots containing autoclaved peat / vermiculite / perlite mixture.

Results and discussion

Embryogenic tissue was induced in all of the studied species of *Abies*. After four to eight weeks of cultivation on SH induction medium with BAP, white, mucilaginous extrusions were observed (Fig. 1A). This embryogenic tissue consisted of single elongated highly vacuolated cells, clumps of small and densely cytoplasmic cells and somatic embryos in an early stage of development (Fig. 1B). The initiation percentage of embryogenic tissue is shown in the Table 1. We obtained 26 cell lines of *A. cilicica*, three lines of *A. numidica* and two cell lines of *A. concolor* with different growth characteristics. The same SH induction medium was suitable also for initiation of embryogenic tissue in some hybrids (Gajdošová et al. 1995). Unlike other genera in the *Pinaceae*, *Abies* requires only cytokinin for induction of embryogenic tissue from zygotic embryos (Salajová et al. 1996).

The duration of maturation treatment was eight to ten weeks. The cell lines differed in their response to the three maturation media. Somatic embryos reached the cotyledonary

Table 1. Percentage of embryogenic tissue from immature zygotic embryos of *Abies* species

Species	Number of explants	Initiation (%)	Number of cell lines
Abies cilicica	63	65.5	26
Abies numidica	44	6.8	3
Abies concolor	64	5.6	2

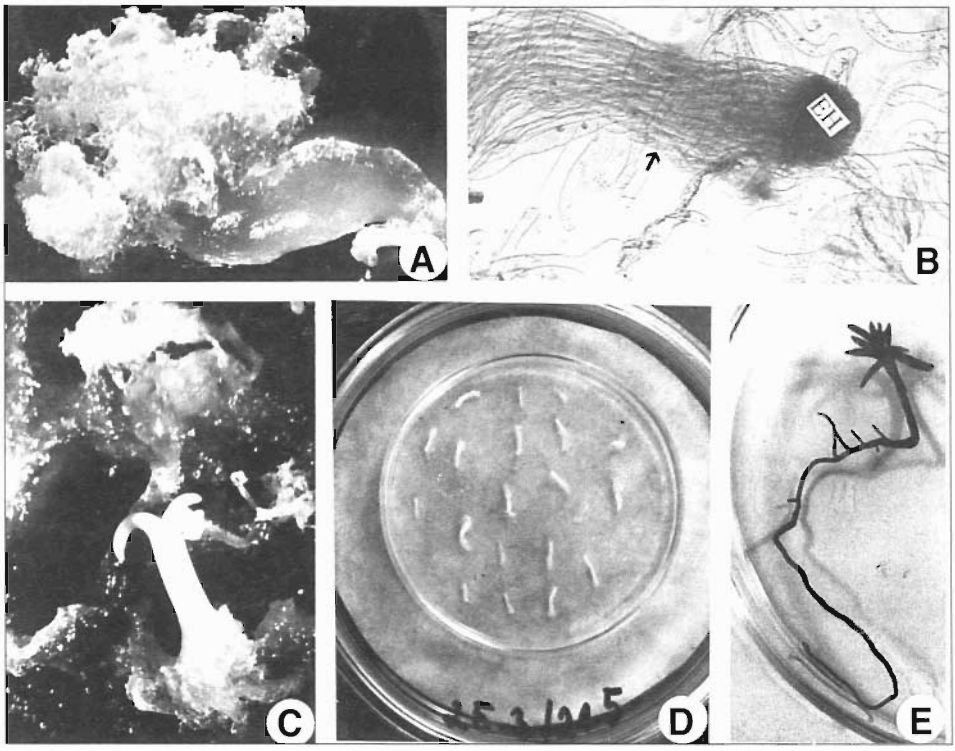

Fig. 1. Embryogenic tissue induction and plantlet regeneration of *Abies species*. A. Initiation of embryogenic tissue from immature seeds of *Abies cilicica*. B. Early somatic embryo of *Abies numidica* with embryonal head (EH) and long suspensor cells (arrow). C. Developing cotyledonary somatic embryo of *Abies concolor* after eight weeks on MS maturation medium with ABA. D. Partial desiccation of mature somatic embryos placed in Petri dishes (ø 60 mm). Petri dish was open and placed on moist filter paper in a Petri dish (ø 90 mm), which was sealed with parafilm. E. *Abies numidica* plantlet with developed root system and primary shoot five months after transfer into the soil.

stage of development on MS and SH medium (Fig. 1C). The number of mature cotyledonary embryos per g of embryogenic tissue was different in individual species (Table 2). The tendency for better maturation on MS medium was typical for all tested species. GD medium was not suitable because maturation was slow and only the precotyledonary stage of development was achieved.

Table 2. The number (± SE) of cotyledonary somatic embryos of *Abies* species (per g of embryogenic tissue) matured on Shenk and Hildebrandt (SH), Murashige and Skoog (MS) and Gresshoff and Doy (GD) media

Species	SH medium	MS medium	GD medium
Abies cilicica	6 ± 2	16 ± 2	0
Abies numidica	16 ± 5	26 ± 3	1 ± 1
Abies concolor	-	61 ± 8	0

Table 3. Germination of somatic embryos of *Abies* species on germination medium. Means ± SE, n = 6

Species	Embryos forming roots (%)
Abies cilicica	75.0 ± 6.8
Abies numidica	85.5 ± 4.1
Abies concolor	77.1 ± 5.2

After partial desiccation (Fig. 1D) mature embryos underwent germination on medium with charcoal. They developed into plantlets with green cotyledons, red hypocotyl and white radicula. A high rooting percentage was achieved for all tested species (Table 3). In previous work (Vooková, Kormuťák 2001) the efficacy of the same germination medium was tested also for some Abies hybrids and its general application was confirmed. Plantlets that had formed a radicle were transferred to a soil / peat / perlite mixture. During the seven-months-period after transfer to the soil some of them had survived (Fig. 1E).

Our results showed that the same experimental conditions were suitable for plantlet regeneration of the all tested species. It seems that plantlet regeneration of more *Abies* species can be achieved using the same experimental conditions, but probably optimizing for individual species can significantly affect the yield of regenerants.

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Some evidence regarding chloroplast proteins of frost resistant hybrids of potato

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Abstract

Frost resistance is an important trait in plant breeding. The cultivated potato *Solanum tuberosum* L. is reputed to be a frost-sensitive crop. Frost resistance was incorporated into cultivated potato by protoplast fusion from a wild species *S. commersonii* Dun. The soluble proteins in leaves of asymmetric somatic hybrids of *Solanum tuberosum* and frost resistant *S. commersonii* were analysed. Differences of total soluble protein and chloroplast contents in frost resistant hybrids of potato were detected. These proteins were responsive to low temperature and may be play a role in frost resistance as representatives of the family of cold regulated proteins.

Key words: chloroplast, frost resistance, *Solanum commersonii* Dun., *Solanum tuberosum* L., soluble proteins.

Introduction

Plant resistance to environmental factors is an important property for consideration during the creation of new cultivars. It is possible to introduce valuable adaptive features, such as resistance to drought, temperature variations, ultraviolet radiation from wild plant species into cultivated species (Vayda 1994; Bujauskas 2001). The acquired tolerance to stress factors equips plants with a better degree of adaptation to varying environmental factors. Somatic hybridization *in vitro* is one of the ways to restore part of the genetic information in cultivated plants lost during selection, during cultivation of wild plants.

Solanum tuberosum plants grown in Lithuania are frost sensitive, as are the majority of potato cultivars. Cultivars of *S. tuberosum* can withstand a temperature of -3 °C, but the wild species *S. commersonii* and *S. acaule* in South America can survive at -4 °C, even to -11 °C after acclimation (Vayda 1994). Cold acclimation is associated with numerous biochemical changes including accumulation of special cryoprotective solutes, changes of pigment composition and the level of ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco), as well as synthesis of cold-regulated (COR) proteins (Zhang et al. 2002; Spetea et al. 2004). The COR-15 protein induced in *Arabidopsis thaliana* participates in the mechanism of freezing tolerance (Steponkus et al.1998). The Rubisco protein and cold induced proteins are coded by the nucleus and chloroplast genomes. Both these proteins are related with chloroplasts and cold resistance (Zhang et al.2002). The protein COR-15 is encoded by the nucleus genome, synthesized in the cytoplasm and later localized in chloroplasts during cold acclimation (Steponkus et al. 1998). The subunits of Rubisco protein, coded by the nucleus and chloroplast genomes, can be used as specific markers

for determination of the origin of genetic information in hybrids, mutants and transgenic plants.

Frost resistant hybrids of potato have been created by incorporating the resistance into cultivated potato from wild species *S. commersonii*. The hybrids were created applying protoplast fusion by Dr. J. Proscevičius et al. (1998) of a wild potato species *S. commersonii* (donor) and cultivated species *S. tuberosum* 'Matilda', 'Venta' (recipient). The irradiated *S. commersonii* (donor) genome is fragmented and asymmetric hybrids contain the complete genome of the recipient and a part of the donor genome. Phenotypes of frost resistant hybrids do not differ from the cultivar phenotype and they have acquired valuable characteristic from the donor i.e., frost resistance.

Our previous studies detected that species *S. commersonii* and *S. tuberosum* differed in four chloroplasts proteins, which were discriminated after fractionation by electrophoresis in denatured condition (Vyšniauskienė et al. 2003).

The goal of this study was to investigate the component spectrum of total protein and soluble chloroplast proteins of frost resistant hybrids, parental plants and to determine whether these changes in frost resistant hybrids were inherited from the wild species *S. commersonii*.

Materials and methods

Plants of wild species of potato *Solanum commersonii* Dun., cultivars of *S. tuberosum* L. 'Matilda' and frost resistant hybrids: H269, H515, H545, H188, H323, H487 were grown *in vitro* at 20 - 25 °C and under a 16-h photoperiod. The chloroplasts from leaves (1 g) were homogenized with 2 ml of cold buffer (0.35 M sucrose, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM $MgCl_2$, 5 mM DDT) with mortar and pestle at 4 °C (Ishida et al. 2002). Following extraction, the samples were centrifuged at 1000 g to remove insoluble debris. The supernatants were centrifuged at 4000 g and the debris was homogenized with buffer without sucrose. The soluble proteins from chloroplasts were separated on 10 % polyacrilamide gels (Laemmli 1970).

Results

Applying the Rubisco methods of protein extraction, the protein content of two frost resistant potato hybrids (H188 and H323) and parental forms – *S. tuberosum* (recipient) and *S. commersonii* (donor) – was analyzed by PAGE. It was found that the protein content of parental protein forms differ among themselves (Fig. 1A; 1, 2), but the profiles of the hybrids were the same as of the recipient *S. tuberosum*, but with changes in the amounts of some proteins (Fig. 1A; 2, 3, 4). SDS-PAGE showed that protein spectra of denatured proteins from the leaf extracts of hybrid H188 were discriminated from those of hybrid H323 and *S. tuberosum* 'Matilda'. The hybrid H188 had new band at 20 kDa. The protein spectra of *S. commersonii* differed from that of cultivated *S. tuberosum* 'Matilda' (Fig. 1B). It is difficult to detect whether the 20 kDa protein was inherited from *S. commersonii* as this protein was not found in the *S. commersonii* spectrum.

Employing PAGE, we further examined the component spectrum of the soluble chloroplast proteins of the frost resistant hybrids: H269, H515, H545, H188, H323. It was revealed that the wild species *S. commersonii* had four specific protein fractions: 1, 2, 3, 4

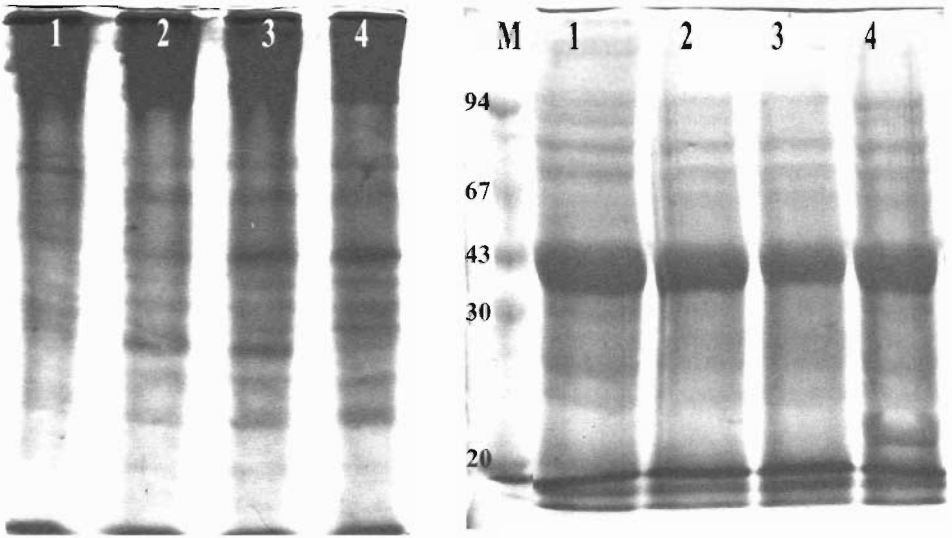

Fig. 1. The protein spectrum of frost resistant hybrids in 10 %-PAG. A, without 0.1 % SDS; B, with 0.1 % SDS. 1, *S. commersonii*, 2, *S. tuberosum* cultivar 'Matilda'; 3, frost resistant H323; 4, frost resistant H188.

not found in cultivated frost sensitive *S. tuberosum* 'Matilda'. Electrophoretical mobilities (Rf) of the fractions were 0.17, 0.20, 0.46, 0.62, respectively (Fig 2). The chloroplast protein spectrum of the hybrids H269, H188, H323 contained three proteins: 1, 2, 3, with Rf 0.17, 0.20, 0.46, also found in the *S. commersonii* spectrum. The hybrid H269 had one more protein (band 4) from the chloroplast protein spectrum of *S. commersonii*. Consequently, H269 had four additional protein fractions inherited from the wild species.

Thus, all frost resistant hybrids of the investigated potato inherited the chloroplast protein spectrum of *S. tuberosum* cultivars, but the hybrids H269, H188, and H323 together with the *S. tuberosum* protein spectrum also inherited some specific proteins from the parental wild *S. commersonii* species. The other frost resistant hybrids H515 and H545 had an identical chloroplast protein spectrum to *S. tuberosum* cultivar 'Matilda' and lacked a single protein from the wild species.

Discussion

The 20 kDa protein recorded in the hybrid H188 was not found in the protein spectra of *S. commersonii*, *S. tuberosum*, or hybrid H323. Supposedly, it is one of the Rubisco protein subunits coded by the nucleus genome (Zhang et al. 2002). Presently, the cold induced genes and Rubisco proteins of transgenic plants are being intensively studied in order to determine the gene expression in transgenic plants i.e., large subunit 55 kDa, coded by the genome of chloroplasts, and small subunit 14 kDa, coded by the nucleus genome. Each species is characterised by specificity of relative molecular mass of Rubisco subunits: *Coffea arabica* – 15 kDa, *Pisum sativum* L. – 14 kDa (Zhang et al. 2002). Both these subunits in the stroma of chloroplasts form an active functional enzymatic complex.

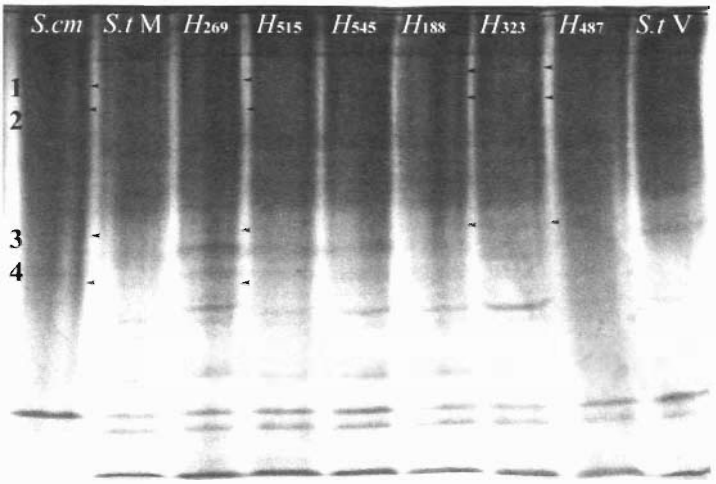

Fig. 2. The chloroplast protein spectrum in potatoe cultivars *S. tuberosum* and *S. commersonii* Dun. and their frost resistant hybrids. S.cm, *S. commersonii*; S.t.M, *S. tuberosum* cv. 'Matilda'; S.tV S. tuberosum cv. 'Venta'. Frost resistant hybrids: H269, H515, H545, H188, H323, H487.

Further investigations will be aimed at identification of this 20 kDa protein detected in the hybrid H188.

Determination of donor-specific *S. commersonii* proteins in the chloroplast protein spectrum of the hybrids H188 and H323 show that the hybrids inherited specific chloroplast proteins from the wild species. However, in the chloroplast protein spectrum of the hybrids H515 and H545, not a single specific protein characteristic of the wild donor was found, while their frost resistance had been confirmed. Therefore, specific proteins of the wild donor cannot be fully related with their participation in the processes of frost resistance. Still, the results of earlier investigations revealed that only hybrids H188 and H323 inherited two systems of the resistance to freezing from the wild species *S. commersonii*: permanent and inducible, while the other hybrids inherited only one of them (Prosevičius et al. 1999). Perhaps the inheritance of two systems predetermines a higher degree of resistance. However, the difference in chloroplast proteins between cultivars of *S. tuberosum* 'Matilda', 'Venta' and species *S. commersonii* Dun. can be used as a sampling method of hybrids after protoplast fusion. Resistance is a polygenic feature determined by the expression of many genes and the interaction of resistance systems (Vayda 1994). Further experiments should be performed to identify other defense mechanisms participating in frost resistance. These proteins are responsive to low temperature, and, may play a role in frost resistance as representatives of the family of COR (cold regulated) proteins. The analysis and comparison of new proteins by acclimation with low temperature should allow to understand better the frost resistance mechanism.

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Tables and figures should be self-explanatory without reference to text. Do not duplicate any data both in the form of tables and figures. Large bodies of primary data should not be presented.

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Figures. Number of figures should be kept to the minimum. All figures should be numbered with Arabic numerals and must be metioned sequentially in the text. Provide a caption in a form of a complete sentence followed by an expianatory legend. If a figure is composed of several parts (diagrams, graphs, pictures), label them as A, B, C etc. Place each figure on a separate A4 sheat considering reduction in printed version. Keep in mind that final dimensions of figures in the article should be no larger than 12.5 cm in width and 17 cm in height. Type size should not be less than 2 mm after reduction. For graphs prefferred symbols are closed or open circle, square, and triangle. Avoid using +, × or *. In histograms use closed, open, or striped bars. Do not use 3-D columns or different intensities of gray. Every figure should be marked with authors' names and figure number. Captions and legends should be included with the manuscript.

Style. Manuscript should be written in simple declarative sentences and must conform to accepted standards of English style and usage.

Literature citations. References should be cited in text by last names and year of publication. Cite references chronologically. When the given publication has two authors, name both authors. With more than two authors per publication, name only a first author followed by 'et al.'. When a particular author or a group of authors has several publications for the given year, give letters 'a', 'b', 'c' etc. after the year of publication. Separate individual references by a semicolon. As an example, (Collins 1999) or (Collins, Chapper 1999) or (Collins et al. 1999) or (Collins 1999a; Collins 1999b) or (Collins 1999a; Chapper 1999b). It is possible to use also the following format 'It is important to note, that Collins (1999) already described...' As an exception, the reference to

Internet may be given without including it in the list of references. Unpublished results should be cited by the name of the author only in the text, e.a., (Collins, unpublished data) or (Chapper, personal communication).

Units of measure. Temperature is expressed in degrees Celsius (°C), time in seconds (s), minutes (min), hours (h), days etc. It is appropriate to use litre (l) as a special designation for 1 dm^3. Consequently, mililitre (ml) and microlitre (μl) also can be used. In all other cases SI units must be used as much as possible. Use negative exponents to indicate units in the denominator, e.g. kg m^{-2}; m m^{-2} s^{-1}. Write out numerals one through nine, except when used with units of measure. Write out all numbers or fractions that begin a sentence, or rephrase the sentence to avoid beginning with a numeral. Use the preposition 'to' between numerals (avoid using a dash): e.g. '8 to 12 h'.

Nomenclature. In the abstract, at first mention in the text, and in *Materials and methods*, include complete biological names for all experimental organisms. Following first mentions, generic names should be abbreviated to the initial, except when confusion could arise by reference to genera with the same initial. Exceptions may be accepted in purely taxonomic papers.

Abbreviations. Use only standard scientific abbreviations accepted internationally. Abbreviations can be used if the term appears at least three times in the text. Do not use abbreviations in the abstract except if used at least three times. At first use of the term in the text or in the abstract spell it out and introduce the abbreviation parenthetically.

Discussion. Describe importance of acquired results, analysing discovered relationships in a logical sequence. References to figures and tables as well as literature sources should be given. As an exception, due to a logical sequence of performed experiments, it is possible to form a joint section *Results and discussion*).

Acknowledgements. First, provide any details on financial support received. Second, acknowledge any person you need to thank for essential help during experiments or writing your work.

References. Include only publications cited in the text. Place references in strict alphabetic order, i.e., firstly by the name of the first author, then by the name of the second author if the first author is identical for more than one reference, then by the name of the third author if the first two authors are identical, and so on. Include the names of all authors and a full title of each paper or a book. Publications by the same atuthor or the group of authors place in a chronological order. Abbreviate journal names according to ISI standards. Provide English translations for titles of all publications other than English, German, or French. The corresponding author has a full responsibility for an accuracy in citations. Citations will be edited for format only.

The following standard form of citation should be used:

Journal articles

Enkerli J., Felix G., Boller T. 1999. The enzymatic activity of fungal xylanase is not necessary for its elicitor activity. *Plant Physiol.* 121: 391–397.

Book articles

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Monographs

Fahn A. 1979. *Secretory Tissues in Plants.* Academic Press, London. 250 p.