MAINTENANCE OF *VACCINIUM* GENE RESOURCES IN ACTIVELY GROWING *IN VITRO* CULTURES

UCHOVÁVÁNÍ GENETICKÝCH ZDROJŮ RODU *VACCINIUM* VE FORMĚ AKTIVNĚ ROSTOUCÍ *IN VITRO* KULTURY

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The security of genetic resource collections requires their conservation by several techniques. The possibility of establishment of duplicate collections of selected highbush blueberry (*Vaccinium corymbosum* L.) and lingonberry (*Vaccinium vitis-idaea* L.) cultivars in the form of actively growing shoot tip *in vitro* cultures is investigated in Research and Breeding Institute of Pomology Holovousy Ltd. Czech Republic. Three genotypes of highbush blueberry (Blueray, Bluecrop and Berkeley) and one genotype of lingonberry (Linnea) were successfully established *in vitro* using mercuric chloride in a concentration of 0.15% as a sterilization solution. Anderson's rhododendron medium (AN) and McCown woody plant medium (WPM) containing the cytokinin zeatin in concentrations 0.5, 1 or 2 $mg.l^{-1}$ were tested for repeated subcultures. WPM proved to be more suitable for cultivation of selected cultivars in *in vitro* cultures. Within the same range of zeatin concentration, the four genotypes gave higher multiplication rates on WPM medium. Across all experiments, the highest multiplication rate (4.8) was obtained for Berkeley on WPM medium with the highest concentration 2 $mg.l^{-1}$ of zeatin. On both tested media, Berkeley was the cultivar with the highest ability to produce new shoots. Micropropagation techniques increased multiplication mainly in highbush blueberry cultivar Berkeley and lingonberry cultivar Linnea on WPM medium. After three months of storage under normal growing conditions, the survival rate was very high (99–100%). After subsequent transfer to fresh WPM medium, *in vitro* shoots of three *Vaccinium* genotypes remained viable and resumed growth.

Key words: highbush blueberry, lingonberry, cultivars, sterilization, multiplication, germplasm

Highbush blueberry (*Vaccinium corymbosum* L.) and lingonberry (*Vaccinium vitis-idaea* L.), members of the family *Ericaceae*, are commercially important as fruit crops (Zmarlicki 2006). In the Czech Republic, highbush blueberry is grown mainly in smaller plantations and home gardens. Although plants of *Vaccinium* genus have not been cultivated on a large scale in the Czech Republic, there is potential for commercial blueberry and lingonberry production in some mountain regions (Paprštein et al. 2006).

Primary collections of *Vaccinium* genetic resources in the Czech Republic are held in field genebanks (Paprštein 1998; Paprštein et al. 2006). These field grown shrubs allow evaluation of vegetative characters and resistance to pests and diseases. Thus the response of plants to the particular environmental conditions and performance of the genotypes can be compared (Paprštein and Ludvíkova 2006). Unfortunately the conventional methods of maintaining fruit crops under field conditions require extensive space and labor. In addition, such germplasm collections are subject to

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environmental catastrophes, pests and disease outbreaks (Blažek 1999; Reed et al. 1998).

A safer conservation method for secure conservation of these collections must be developed. *In vitro* conservation has been under consideration in this role and a substantial progress has been made in its development over the last 20 years (Coman et al. 2004; Razdan and Cocking 2000; Reed et al. 1998; Withers 1992). Tissue culture techniques offer the opportunity for *in vitro* collecting, rapid propagation, medium and long term storage of germplasm and its distribution (Ashmore 1997).

The possibility of establishment of duplicate collections of selected highbush blueberry and lingonberry cultivars in the form of actively growing shoot tip *in vitro* cultures is also investigated in Research and Breeding Institute of Pomology (RBIP) Holovousy Ltd. Czech Republic. At the beginning, we did not use cold storage or cryopreservation in our conservation systems, instead we focused our studies on the normal *in vitro* culture and propagation using *in vitro* shoot tips. According to recent surveys (Watt et al. 1996), lack of cold hardiness and susceptibility to frosts were identified as the most important genetic limitations of current *Vaccinium* genotypes. In addition, stress caused by cold storage could select within an *in vitro* population and there is the risk of genetic instability/selection in slow growth conservation systems (Withers 1992). The presented study compares sterilization of initial explants, multiplication rates and short-term *in vitro* storage at normal cultivation temperature (22°C) of highbush blueberry genotypes (Blueray, Bluecrop and Berkeley) and lingonberry genotype (Linnea).

MATERIAL AND METHODS

In vitro culture initiation

For the *in vitro* culture establishment, twenty actively growing shoot tips (5 to 15 mm in length) were cut from shoots of blueberry cultivars (Blueray, Bluecrop, Berkeley) and lingonberry cultivar (Linnea) sprouting in laboratory conditions. The donor shoots were collected in March from mature shrubs growing in field germplasm collection of RBIP Holovousy. Excised shoot tips were disinfected with a 0.15% solution of $HgCl_2$ for 1 min followed by wash in sterile distilled water. This was carried out under sterile conditions. All sterile manipulations were in a laminar flow hood. The sterilized shoot tips were placed on 35 ml of culture medium contained in 200 ml glass culture flasks (7 shoots per flask). The initial culture medium was based on WPM (woody plant medium) according to Lloyd and McCown (1981) with 1 $mg.l^{-1}$ zeatin. Culture flasks were capped with clear permeable polypropylene caps. Contamination rate, survival and development of shoots from excised shoot tips were analyzed after sterilization. Uncontaminated shoots established on WPM medium were transferred after one month to fresh proliferation medium.

All initiation and multiplication media contained 7.0 $g.l^{-1}$ Difco agar. The pH of the media was adjusted to 5.2 before autoclaving at 120 °C at 100 kPa for 15 minutes. Cultures were incubated at 22 ± 1 °C under cool-white fluorescent tubular lamps at 60 $\mu mol.m^{-2}.s^{-1}$ (16-hour photoperiod).

Shoot multiplication

All shoot cultures were serially subcultured for at least nine months on a WPM medium supplemented with 1 $mg.l^{-1}$ zeatin. This provided a stock collection of shoots for multiplication studies.

To determine favorable conditions for shoot initiation and multiplication, two basal nutrient media WPM and AN (Anderson's rhododendron medium) according to Anderson (1980) supplemented with three different concentrations 0.5, 1 or 2 $mg.l^{-1}$ of the cytokinin zeatin were tested. Zeatin was filter sterilized (25 mm, Acrodisc Syringe Filter 0.2 μm, Pall Gelman, USA) and added to media after autoclaving. Uniform shoot tips (5 to 10 mm in length) excised from apical parts of established proliferating cultures were used in all multiplication experiments.

To evaluate the effect of basal nutrient media and cytokinin zeatin in different concentrations on multiplication of selected cultivars, newly formed shoots were counted and multiplication rate was determined. Multiplication rate was defined as the number of newly formed shoots (>10 mm) per initial shoot tip after four weeks of culture. The shoot formation was

recorded between the tenth and fifteenth subcultures. In all experiments, 25 initial shoot tips were used. All experiments were repeated four times. Data from four independent experiments were pooled and expressed as the mean. Treatment means were compared with the standard error (SE) of the mean. The statistical analysis was done using program STATISTICA 7.0 (StatSoft CR).

In vitro storage

Highbush blueberry (Bluecrop, Berkeley) and lingonberry (Linnea) cultivars were used as model genotypes in our short-term *in vitro* storage studies. *In vitro* cultures of these four cultivars on multiplication WPM medium with 1 $mg.l^{-1}$ zeatin were kept without subculture on the shelf of growth room at normal cultivation temperature 22 ± 1 °C under cool-white fluorescent tubular lamps (16-hour photoperiod) for a period of three months.

The number of surviving *in vitro* cold-stored plants was visually evaluated and counted at monthly intervals for the period of three months. Brown and necrotic shoots were considered to be dead. After retrieval from storage conditions, shoots were transferred to fresh WPM medium with 1 $mg.l^{-1}$ zeatin for viability assessment. The assessment of viability was carried out under standard culture conditions described above. The percentage of viable shoots (shoots that proliferated) was determined four weeks after being subcultured. After retrieval from storage and subculture onto multiplication medium, microbial contamination rate was also evaluated. Ten replicated flasks (i.e. 100 shoots) were used for each genotype experiment.

RESULTS AND DISCUSSION

In vitro culture initiation

Results of sterilization procedures and development of shoots from initial explants are shown in Table 1. The use of mercuric chloride in a concentration of 0.15% as a sterilization solution proved to be an effective treatment to disinfect the starting plant material of selected four *Vaccinium* genotypes. The rate of contamination was generally low. Of the 80 shoot tips taken only 9 explants were visibly contaminated with micro-organisms. These explants were later discarded. The use of mercuric chloride had a direct beneficial effect and overcame the contaminations from the microflora of the field germplasm collections of blueberry and lingonberry. On the other hand, the toxicity to tissues caused by mercuric chloride was high. In the case of cultivars Bluecrop and Linnea 50% of initial uncontaminated explants did not produce shoots and turned brown. However, the remaining uncontaminated initial explants of these two cultivars had greenish color and developed shoots. Debnath and McRae (2001) reported that although regeneration from primary explants is a first necessary step in any micropropagation of *Vaccinium* genus, the regeneration frequency has no effect on the further success of the micropropagation program. Many shoots could be obtained from a few clean shoots regenerated from the primary explant.

Shoot multiplication

The results of multiplication of *Vaccinium* genotypes are shown in Tables 2 and 3. The number of newly formed shoots varied with the cultivar, medium tested and concentration of zeatin. Across all experiments, the highest multiplication rate (4.8) was obtained for Berkeley on WPM medium with the highest concentration 2 $mg.l^{-1}$ of zeatin. On both tested media, Berkeley was the cultivar with the highest ability

Table 1

Surface sterilization of highbush blueberry and lingonberry cultivars by 0.15% mercuric chloride

Cultivars[1]	Explants contaminated		Explants died without contamination		Established explants which developed shoots	
	Number	[%]	Number	[%]	Number	[%]
Bluecrop	2	10	10	50	8	40
Blueray	5	25	9	45	6	30
Berkeley	1	5	5	25	14	70
Linnea	1	5	10	50	9	45

[1]Twenty actively growing shoot tips were used for each cultivar experiment.

to produce new shoots. On the contrary, for cultivar Blueray, neither of two tested media containing different concentrations of zeatin promoted markedly *in vitro* shoot formation and the number of newly formed shoots was thus very low (from 1.0 to 1.8). Cultivars Bluecrop and Linnea had intermediate results on both tested media. Similar significant differences in intensity of shoot proliferation among highbush blueberry genotypes were also observed by Ostrolucká et al. (2004). In these experiments, the highest multiplication rate was achieved for Duke (5.3), while the lowest for Blueray (1.7) on Anderson's medium with 2 mg.l^{-1} of zeatin. Cultivar Blueray also gave the lowest multiplication rates in the present study.

Within the same range of zeatin concentration, the four genotypes gave higher multiplication rates on WPM medium. Woody plant medium (WPM) was found to be more effective than the AN medium for initiation of new shoots in our study. Multiplication rate 4.8 for cultivar Berkeley on WPM medium with 2 mg.l^{-1} of zeatin was higher than multiplication rate 3.8 reported by Ostrolucká and Šimala (2002) for Berkeley with the same concentration of zeatin in AN medium. However in comparison with our experiments, the same authors achieved higher multiplication rate (3.9) for cultivar Bluecrop on AN medium with zeatin 2 mg.l^{-1}.

In the present work, the increasing zeatin concentration in both tested media also increased the shoot multiplication without excessive callus formation in the all selected *Vaccinium* genotypes. The highest multiplication rates were always noted on media with the highest concentration of zeatin (2 mg.l^{-1}). Zeatin level 2 mg.l^{-1} can be recommended for multiplication of cultivars used in our study. It was previously observed that zeatin could be used successfully to induce *in vitro* shoot production in *Vaccinium* micropropagation (Reed and Abdelnour 1991; Debnath and Mcrae 2001; Ostrolucká et al. 2004). According to Reed and Abdelnour (1991), cultivation medium with relatively high levels of zeatin (4 mg.l^{-1}) promoted significantly higher initiation of axillary shoots in eight of twelve *Vaccinium corymbosum* genotypes than control medium. On the contrary, Gajdošová et al. (2006) pointed out the effectiveness of zeatin in low concentration (0.5 mg.l^{-1}) for inducing multiple shoot development in meristem cultures of *Vaccinium* sp. Zeatin concentrations of 2 mg.l^{-1} and higher promoted callus formation and suppressed shoot regeneration, which is contradictory to our findings.

Table 2

Multiplication rates for highbush blueberry and lingonberry cultivars on WPM medium with zeatin

Zeatin [mg.l^{-1}]	Cultivar[1]			
	Bluecrop	Blueray	Berkeley	Linnea
0.5	1.3 ± 0.1	1.1 ± 0.1	3.0 ± 0.1	1.4 ± 0.1
1	1.5 ± 0.1	1.2 ± 0.1	4.0 ± 0.2	2.8 ± 0.1
2	2.0 ± 0.1	1.8 ± 0.1	4.8 ± 0.2	3.3 ± 0.2

[1]Means ± SE (n=100)

Table 3

Multiplication rates for highbush blueberry and lingonberry cultivars on AN medium with zeatin

Zeatin [mg.l^{-1}]	Cultivar[1]			
	Bluecrop	Blueray	Berkeley	Linnea
0.5	1.2 ± 0.0	1.0 ± 0.0	1.4 ± 0.0	1.0 ± 0.0
1	1.2 ± 0.1	1.0 ± 0.0	2.2 ± 0.1	1.6 ± 0.1
2	1.9 ± 0.1	1.3 ± 0.0	2.3 ± 0.1	1.6 ± 0.1

[1]Means ± SE (n=100)

In vitro storage

The results of survival of *in vitro* plants of *Vaccinium* genotypes in particular months are shown in Table 4. After three months of storage under normal growing conditions, the survival rate was very high (99–100%). The appearance of cultures was affected by prolonged stay on the multiplication medium without subculture. The leaves from stored cultures became progressively yellow and red during the storage period of three months. However, there were no visible substantial morphological changes on the shoots. No roots were developed by any of the explants in either of the preservation periods.

After three months of storage and subsequent transfer to fresh WPM medium, shoots of three *Vaccinium* genotypes remained viable

Table 4

Survival and viability of *in vitro* plants of *Vaccinium* cultivars during and after storage at normal cultivation temperature 22°C.

Cultivar	Survival in particular months[1]			Viable shoots after transfer to fresh WPM medium[1]
	1	2	3	
Bluecrop	100	100	100	100
Berkeley	100	100	100	100
Linnea	100	100	99	98

[1]In sum, 100 shoots were used for each cultivar experiment. Hence the number of surviving shoots in particular months and viable shoots also represents the percentage of survival and regrowth.

(Table 4). Only one explant of Linnea died after subculturing. The remaining *in vitro* shoots resumed growth. The results obtained in this study demonstrate that *in vitro* shoots of selected cultivars stored under normal culture conditions could survive and regenerate shoots for at least three months without subculture. Previous work on plant species has indicated that shoots of some species can remain viable without subculturing or under minimal growth conditions up to ten months (Nirmal Babu et al. 1999; Redenbaugh et al. 1991; Watt et al. 2000). In the case of three tested cultivars, contamination levels during storage ranged from 0 to 5%. This contamination did not appear to be related to the storage time. The inevitable presence of endogenous bacterial and fungal contaminants in some species (Watt et al. 1996; Watt et al. 2000) must be taken into consideration when managing germplasm storage systems.

CONCLUSION

Micropropagation techniques described in this paper increased multiplication mainly in highbush blueberry cultivar Berkeley and lingonberry cultivar Linnea on WPM medium. However, some cultivars of highbush blueberry would still require further research to optimize proliferation media. The presented results showed that established shoot cultures of highbush blueberry and lingonberry can be stored effectively for three months without subculture. This will help to save time and labor costs in the research and production laboratories. Extended subculture intervals also lower the risk of contamination during manipulation with plant material in flow box. In this preliminary study, a storage period longer than three months was not investigated. Future efforts will focus on testing of other fruit species and long term storage strategies. Stored shoots provide an alternative to the field germplasm collections.

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SOUHRN

Bezpečné uchování kolekcí genetických zdrojů vyžaduje použití různých metod. Ve Výzkumném a šlechtitelském ústavu ovocnářském v Holovousích je v současné době zkoumána možnost založení duplicitní kolekce vybraných odrůd kanadské borůvky (*Vaccinium corymbosum* L.) a brusnice brusinky (*Vaccinium vitis-idaea* L.) ve formě aktivně rostoucích vrcholových *in vitro* kultur. S použitím sterilizace pomocí chloridu rtuťnatého v koncentraci 0,15 % byly do *in vitro* kultur prozatím úspěšně zavedeny tři odrůdy kanadské borůvky (Blueray, Bluecrop a Berkeley) a jedna odrůda brusnice brusinky (Linnea). Po ukončení sterilizační procedury se viditelné kontaminace vyskytovaly u 25 % explantátů odrůdy Blueray, 10 % počátečních explantátů odrůdy Bluecrop a 5 % explantátů odrůd Berkeley a Linnea. Nejlepšího výsledku bylo dosaženo u odrůdy Berkeley, kde byl zaznamenán nejnižší výskyt kontaminací a kde 70 % počátečních explantátů vyvinulo výhony. Pro opakované subkultivace byla testována dvě média WPM (Woody plant medium) a AN (Anderson's rhododendron medium) s přídavkem tří různých koncentrací cytokininu zeatin (0,5; 1 nebo 2 $mg.l^{-1}$). Na základě dosažených výsledků se WPM médium jeví jako více vhodné pro kultivaci vybraných odrůd v *in vitro* kultuře. Při stejné koncentraci zeatinu vykazovaly čtyři vybrané genotypy vyšší multiplikační koeficient na WPM médiu. Celkově nejvyšší multiplikační koeficient (4,8) byl získán u odrůdy Berkeley na WPM médiu s nejvyšší koncentrací zeatinu 2 $mg.l^{-1}$. Odrůda Berkeley vykazovala zároveň na obou testovaných multiplikačních médiích nejvyšší schopnost produkovat nové výhony. Postupy *in vitro* rozmnožování popsané v tomto příspěvku zvýšily koeficient multiplikace zejména u odrůd kanadské borůvky Berkeley a brusnice brusinky Linnea na kultivačním médiu typu WPM. Po tříměsíční době skladování v normálních růstových podmínkách přežilo velmi vysoké procento výhonů (99–100 %), které po následném přenosu na čerstvě připravené WPM medium obnovily růst.

Klíčová slova: kanadská borůvka, brusnice brusinka, odrůdy, sterilizace, multiplikace, genofond