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In vitro multiplication of lingonberry – Short Communication

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Abstract

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Although plants of *Vaccinium* genus have not been cultivated on a large scale in the Czech Republic, there is a potential for commercial lingonberry production in some mountain regions. The purpose of this study was to develop an efficient in vitro system for a quick multiplication of lingonberry cvs Koralle, Linnea, and Runo Bielawskie. McCown woody plant medium (WPM), Anderson's rhododendron medium (AN) and half-strength Murashige and Skoog medium (half-MS) containing cytokinin zeatin in concentrations 0.5, 1 or 2 mg/l were tested for repeated subcultures. The number of newly formed shoots varied with the cultivar, medium tested and concentration of zeatin. Across all experiments, the highest multiplication rate (8.9 ± 0.6) was obtained for cv. Runo Bielawskie on WPM medium with the highest concentration 2 mg/l of zeatin. The lowest multiplication rates 1.1 ± 0.0 were noted on half-MS medium with the lowest concentration of zeatin (0.5 mg/l). In conclusion, micropropagation techniques described in this paper increased multiplication mainly in lingonberry cv. Runo Bielawskie on WPM medium. However, some cultivars of lingonberry would still require further research to optimize proliferation media.

Keywords: explant; *Vaccinium vitis-idaea*; zeatin; sterilization; medium

Vaccinium vitis-idaea L. (lingonberry) is a small evergreen creeping plant of the *Ericaceae* family. The plants grow densely in the forest understory of high north temperate regions of Europe, Asia and America. The fruit is a true globose berry 7–10 mm across, carmine in colour, ripening in late summer to autumn. Lingonberries are primarily grown in Scandinavian and European countries. They were also studied as a possible new crop for the Pacific Northwestern, United States. The fruit is used for jelly and juice by northern Europeans and by Scandinavians in the United States (Debnath, McRae 2001; Penhallegon 2009). Lingonberry is also used as medicinal plant due to its high content of anthocyanins, organic acids and phenolic compounds with antioxidant activity (Hokkanen 2009).

Lingonberries have never been grown commercially in the Czech Republic. However, the berries are traditionally collected in the wild and used as compote or sauce, which often accompany Czech meat dishes (marinated beef sirloin with lingonberry sauce). Due to high nutritional quality of lingonberries, we initiated a programme to evaluate selected cultivars with superior fruit characteristics suitable for growing in selected Czech sub-moun-

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tainous regions with low soil pH (Paprštein et al. 2006). If suitable cultivars are to receive wide distribution, rapid vegetative propagation techniques will be necessary. In addition to traditional nursery methods (cuttings, division of the rhizome), micropropagation can be used as potentially more effective method to propagate desirable cultivars in sterile laboratory conditions all year round.

Unfortunately, micropropagation of lingonberry is still less developed and less efficient in comparison with other *Vaccinium* species. Moreover it was confirmed that in vitro regeneration ability is highly genotype-dependent (Debnath, McRae 2001; Gajdošová et al. 2006, 2007; Debnath 2007).

Our project was initiated to develop a shoot proliferation in vitro system for three commercial cultivars of ligonberry (Koralle, Linnea, and Runo Bielawskie), exhibiting valuable horticultural attributes including satisfactory quality of fruit and frost hardiness. Cytokinin zeatin, which was found to be more effective for shoot initiation in lingonberry in vitro culture (Debnath, McRae 2001), was used in different concentrations to induce proliferation in our experiments.

MATERIAL AND METHODS

In March, 22 actively growing shoot tips (5 to 15 mm in length) were taken from shoots of three lingonberry cultivars (Koralle, Linnea, and Runo Bielawskie) sprouting in laboratory conditions. These shoots were forced for 14 days in laboratory conditions at 22°C. Donor shoots were collected from containerized plants growing in Research and Breeding Institute of Pomology Holovousy Ltd., Czech Republic. After removal of the most of the leaves, shoot tips were dipped in a solution of 0.15% $HgCl_2$ with several drops of the wetting agent Tween-20 (polyoxyethylene sorbitan monolaurate) for 1 minute. After rinsing in sterile distilled water and removing any injured tissue, the explants were placed in 200 ml glass culture flasks (seven shoots per flask), each with 35 ml of modified woody plant medium (WPM) according to Lloyd and McCown (1981). Zeatin was added to the initial WPM medium in concentration 1 mg/l. All initiation and multiplication media contained 7.0 g/l Difco agar (Becton, Dickinson and Company, Franklin Lakes, USA). Glass bottles capped with clear permeable polypropylene caps were used for cultivation. The medium was adjusted to pH 5.2 prior to dispensing and autoclaving at 120°C at 100 kPa for 15 minutes. Cultures were grown on shelves in rooms with 16 h cool white fluorescent lighting provided by tubular lamps (F18W/840-TB; Osram Sylvania, Danvers, USA) at 22 ± 1°C.

Contamination rate, survival and development of shoots from excised shoot tips were analysed after sterilization. Uncontaminated cultures were transferred to fresh medium once a month. After 6 months of culture, a stock collection of shoots, which showed active and uniform growth, was created for proliferation studies. The shoot tip cultures were multiplied by removing elongating shoots from the basal mass and subculturing the shoots on fresh medium.

To determine favourable conditions for shoot initiation and multiplication, three basal nutrient media WPM, Anderson's rhododendron medium (AN) according to Anderson (1980) and modified MS (Murashige, Skoog 1962) medium containing half macro and micronutrients (half-MS) were supplemented with three different concentrations 0.5, 1 or 2 mg/l of the cytokinin zeatin. Thermolabile cytokinin zeatin was filter-sterilized (0.2 mm Supor® Membrane; Pall Corporation, Port Washington, USA) and added to multiplication media after autoclaving.

Uniform developing shoots (5 to 10 mm in length including the apex) were detached from previously cultured explants and transferred to fresh medium for shoot proliferation. After 30 days, the number of newly formed shoots and the morphological appearance (primarily callus formation, hyperhydricity etc) were determined for each cultivar, medium and concentration of zeatin.

The shoot formation was recorded between the sixth and fifteenth subculture. In all experiments 25 shoot tips were used. Each experiment was repeated four times. Data from four independent experiments were pooled and expressed as the mean. To evaluate the accuracy of estimate of the mean of population, treatment means were compared with the standard error (SE) of the mean as a measure of variance.

RESULTS AND DISCUSSION

Bacterial and fungal contaminations were infrequent. Of the shoot tips taken only one explant of cv. Runo Bielawskie became contaminated with

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Table 1. Multiplication rates for lingonberry cultivars on WPM, AN and half-MS medium with zeatin

Zeatin (mg/l)	Cultivar		
	Koralle	Linnea	Runo Bielawskie
WPM medium with zeatin			
0.5	1.4 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
1	1.4 ± 0.1	2.8 ± 0.1	4.9 ± 0.3
2	2.6 ± 0.1	3.3 ± 0.2	8.9 ± 0.6
AN medium with zeatin			
0.5	1.2 ± 0.0	1.1 ± 0.0	1.4 ± 0.1
1	1.3 ± 0.0	1.7 ± 0.1	1.5 ± 0.1
2	2.0 ± 0.1	1.7 ± 0.1	1.8 ± 0.1
Half-MS medium with zeatin			
0.5	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
1	1.5 ± 0.1	1.6 ± 0.1	1.1 ± 0.0
2	1.9 ± 0.1	1.7 ± 0.1	1.3 ± 0.1

WPM – woody plant medium; AN – Anderson's rhododendron medium; Half-MS – half-strength Murashige and Skoog medium

micro-organisms during the establishment phase. This explant was later discarded. Selected three genotypes were successfully established in vitro using mercuric chloride in a concentration of 0.15% as a sterilization solution. The regeneration frequency of uncontaminated explants was very high 90%. The explants produced axillary shoots after one month. In agreement with our results, Debnath and McRae (2001) obtained for five lingonberry genotypes 40–90% viability of explants and fairly low contamination (4.5–6.5%) after the disinfestation procedure with a solution of 1.05% sodium hypochlorite. During and after our sterilization procedure we did not observe browning of lingonberry explants or cultivation medium caused by oxidation of phenolic substances from the cut surface of the explant, which is otherwise associated with woody species.

In our experiments on all media, no physiological disorders or morphological abnormalities such as excessive callus formation or production of abnormally narrow leaves were observed during in vitro shoot proliferation stage. Further dividing and subculturing the basal shoot mass did not cause tissue breakdown or exudation.

The rates of multiplication for particular cultivars are shown in Table 1. Increasing the zeatin concentration in all three media tested also increased the shoot proliferation without excessive callus formation in the case of the three studied lingonberry cultivars. Our results are in accordance with previous observations, confirming that zeatin enhances in vitro shoot production of *Vaccinium* species (Reed, Abdelnour 1991; Debnath, McRae 2001; Ostrolucká et al. 2004; Sedlák, Paprštein 2009). However, the cultivars in this study differed in their multiplication and development potential. Generally, the highest rate was obtained for cv. Runo Bielawskie that produced 8.9 ± 0.6 new shoots on WPM medium containing the highest concentration 2 mg/l of zeatin. On the contrary, for cv. Koralle, neither of three tested media containing different concentrations of zeatin promoted markedly in vitro shoot formation and the number of newly formed shoots was thus relatively low (from 1.1 to 2.6). Concerning the cv. Koralle, our results are similar to the observations of Gajdošová et al. (2006), who reported relatively low proliferation intensity 2.8 on AN medium containing 0.5 mg/l zeatin for this cultivar after six weeks.

Our results confirmed that in vitro proliferation depends not only on the concentration of particular plant growth regulator in culture medium, but also on the response of individual genotype. The

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effect of the genotype on the various aspects of the performance of tissue-cultured material and significant differences in multiplication rates among genotypes were also reported in other studies on *Vaccinium* species (Debnath, McRae 2001; Gajdošová et al. 2006, 2007). The studies of Debnath and McRae (2001) on the *in vitro* culture of a range of *Vaccinium vitis-idaea* cultivars and clones have shown clear differences in genotypic multiplication rates and cultural behaviour, which suggests a link between multiplication rate and genetic composition related to geographical origin and various auxin and cytokinin metabolism of plant tissue.

Out of three media tested for shoot proliferation, woody plant medium (WPM) was found to be more effective than the AN medium and half-MS medium for shoot multiplication. On WPM medium, the three genotypes gave the highest multiplication rates. Basic characteristic of WPM macronutrients is a low total ionic concentration, which is almost half of that of MS. WPM macronutrients are comparable to AN. Moreover WPM total nitrogen concentration is approximately one-fourth of that of MS. WPM macronutrients are also characterized by a higher sulfate concentration almost five times that of MS or AN. Similar beneficial effects of WPM medium were reported with some other woody crops including *Cornus florida* (Declerk, Korban 1994) and *Cornus nuttallii* (Edson et al. 1994). In contrast, the lowest multiplication rates 1.1 ± 0.0 were noted on half-MS medium with the lowest concentration of zeatin (0.5 mg/l). Half-MS medium appears to be less suitable for micropropagation of lingonberry. Micropropagated shoots were rooted directly under ex vitro conditions in peat 'jiffy' pots (Jiffy 7; AS Jiffy Products, Stange, Norway) soaked with water. Rooting was achieved in all genotypes tested with 90–95 % survival of acclimatised plants. Rooting microshoots ex vitro lowers micropropagation costs and makes easier commercial production. The plants established after period of in vitro culture showed no visible morphological differences from conventionally propagated plants.

CONCLUSION

In summary, in vitro multiplication of lingonberry cultivars ranged from easy cv. Runo Bielawskie (8.9 new shoots) to difficult cv. Koralle (2.6 new shoots). The best shoot proliferation was supported on WPM medium. The best multiplication rate was obtained with media containing 2 mg/l of zeatin. By using a zeatin supplemented WPM medium for shoot initiation and proliferation, thousands of in vitro plants could be produced from a single initial explant a year. A high proliferation rate in the case of cv. Runo Bielawskie is promising for *V. vitis-idaea* micropropagation. However, some cultivars of lingonberry would still require further research to optimize proliferation.

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