Micropropagation of cranberry (*Vaccinium macrocarpon*) through shoot tip cultures – Short communication

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Abstract

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The goal of this study was to determine an efficient micropropagation system for cranberry (*Vaccinium macrocarpon* Ait.). Cranberry cultivar Howes was successfully established *in vitro* using mercuric chloride in a concentration of 0.15% as a sterilization solution. Anderson's rhododendron medium (AN), half-strength Murashige and Skoog medium (half-MS) and McCown woody plant medium (WPM) containing the cytokinin zeatin in concentrations 0.5, 1 or 2 mg/l were tested. Generally, the highest multiplication rate (2.7) was obtained for cv. Howes on AN medium with the concentration 1 mg/l of zeatin. The effects of indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and naphtalen acetic acid (NAA) on root induction were tested in WPM medium. The percentage of rooting was 100% for NAA, 86% for IBA and 81% for IAA. From the tested variants, auxin NAA also promoted the highest development of good quality roots (more than 5) per shoot without callus formation.

Keywords: *in vitro*; multiplication; rooting; explant; Howes

Vaccinium macrocarpon Ait. (American cranberry) is a native North American perennial species, which belongs to family Ericaceae. Cranberries are low-growing, creeping shrubs with small, alternate, glossy leaves. The leaves are dark green in summer and turn a variety of colours in fall. The fruit is a dark red, edible epigynous berry (up to 2 cm in diameter) that is larger than the leaves of the shrub (Vodičková 1999). Cultivated cranberry varieties were selected from native species. Cranberry was identified as having beneficial medicinal properties namely the role of cranberry in the maintenance of urinary tract health (Howell 2009).

Although American cranberry has not been grown on a large scale in the Czech Republic, there is potential for commercial ornamental or fruit production in selected sub-mountainous regions with favourable soil conditions and low soil pH. If suitable genotypes are to receive wide distribution, rapid propagation methods will be necessary. Generative propagation of *Vaccinium macrocarpon* in a greater scale is hardly usable, because it does not give homogeneous progeny. Most *Vaccinium* plants can be propagated vegetatively by multiple-node softwood or hardwood cuttings. This method, although generally successful, is slow and labor-intensive. Furthermore, rates of rooting and shoot growth are often insufficient. These traditional nursery techniques are also restricted with respect to vegetation period.

Micropropagation can potentially multiply selected cranberry genotypes more rapidly than standard nursery propagation techniques. Over the last three decades, micropropagation methods on various cultivation media using axillary bud proliferation and adventitious shoot regeneration was utilized for different species from the *Vaccinium* genus with varying success (Reed, Abdelnour

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1991; Qu et al. 2000; Debnath, McRae 2001a; Sedlák, Paprštein 2009).

As a part of the program to introduce *Vaccinium* culture to the Czech Republic, studies were carried out to investigate new genetic resources and the possibility of their multiplication by micropropagation (Paprštein et al. 2006). The aim of this study was to compare different agar solidified media for *in vitro* cultivation of cranberry. Cultivar Howes was tested. This frost-resistant late cranberry cultivar was selected from the wild by Elias Howes in Massachusetts some time prior to 1880 (Hedrick 1922; Drain 1925). Because of its frost resistance, cv. Howes is being tested in the Research and Breeding Institute of Pomology (RBIP) Holovousy, Ltd. as a prospective cultivar for the climatic conditions in sub-mountainous regions of the Czech Republic.

MATERIAL AND METHODS

In February 2008, 13 stem segments ca. 3 cm long were collected from containerized plants growing in greenhouse in RBIP Holovousy. Following removal of withered leaves and a wash in running water, the stem segments were immersed in 0.15% solution of mercuric chloride with Tween-20 (0.05%) for 1 min. This was carried out under sterile conditions in a laminar air flow cabinet. After sterilization, the explants were rinsed in sterile demineralised water and cultured in 200 ml glass culture flasks (seven shoots per flask), each with 35 ml of woody plant medium (WPM) according to Lloyd and McCown (1981). Zeatin was added to the initial WPM medium in concentration 1 mg/l. Glass bottles capped with clear permeable polypropylene caps were used for cultivation. Following sterilization, contamination rate, survival and development of shoots were observed. Established shoots were placed on a fresh proliferation medium after 4 weeks. All shoot cultures were serially subcultured for at least 4 months on a WPM medium supplemented with 1.0 mg/l zeatin. This provided a stock collection of shoots for proliferation studies.

All initiation and multiplication media contained 7.0 g/l Difco agar. The pH of the media was adjusted to 5.2 before autoclaving at 120°C at 100 kPa for 15 min. Cultures were grown in rooms under cool-white fluorescent tubular lamps at 22 ± 1°C and 16-hour photoperiod.

To determine favourable conditions for shoot initiation and multiplication, three basal nutrient media WPM, AN (Anderson's rhododendron medium) 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. Zeatin was filter-sterilized (25 mm, Acrodisc Syringe Filter 0.2 μm, Pall Gelman, Ann Arbor, 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.

The morphology of the shoots (hyperhydricity, primary callus formation etc.) was also noted. Multiplication rate was defined as the number of newly formed shoots (> 10 mm) per initial shoot tip after one month of culture. The shoot formation was recorded between the fifth 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. Data were analysed in the Statistica 6 programme (StatSoft CR, Ltd., Prague, Czech Republic).

The effects of indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) and naphtalen acetic acid (NAA) on root induction were tested in WPM medium. Although several media were evaluated for induction of roots, only results from medium that showed maximal root induction are presented in this report. Each auxin was tested separately at a 1 mg/l concentration.

After the 15th subculture on multiplication media, tissue culture shoots (10 to 20 mm in length) were excised and transferred to rooting media (ten shoots per flask). Cultivation conditions during initiation and growth of roots were the same as during the multiplication phase. Hundred shoots were used for this treatment. The percentage of rooted *in vitro* shoots and the number of roots per plant were recorded five weeks after the transfer to rooting medium. Treatment means were compared with the standard error (SE) of the mean. Shoots with roots were rinsed in water to remove remnants of the medium and then transferred to Jiffy 7 peat pellets (AS Jiffy Products, Morten, Norway) soaked with water. The shoots were misted with water to prevent wilting during transplanting. The Jiffy 7 pellets with rooted plants were placed in a plastic box equipped with transparent plastic cov-

Table 1. Multiplication rates of *V. macrocarpon* cv. Howes

Zeatin (mg/l)	Medium		
	AN	Half-MS	WPM
0.5	2.4 ± 0.1	1.4 ± 0.1	1.6 ± 0.1
1	2.7 ± 0.1	1.3 ± 0.1	1.6 ± 0.1
2	2.6 ± 0.1	1.3 ± 0.0	1.9 ± 0.1

WPM – woody plant medium; AN – Anderson's rhododendron medium; half-MS – modified MS medium containing half macro and micronutrients

ers (100% air humidity) under standard greenhouse condition. The plants were gradually acclimated by opening the covers over fourteen days.

RESULTS AND DISCUSSION

Of the 13 explants taken in February 2008, only two uncontaminated explants survived and developed shoots. Of the other explants, seven were contaminated with microorganisms and were discarded whilst the reminder turned brown. These brownish explants did not show any sign of growth even after three months in culture. *In vitro* culture was established from the two surviving explants by monthly subculture to fresh WPM medium. Debnath and McRae (2001b) 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.

The use of mercuric chloride in a concentration of 0.15% as a sterilization solution proved to be an applicable treatment method to disinfect the starting plant material of selected *Vaccinium macrocarpon* genotype Howes.

Table 2. Rooting of *V. macrocarpon* cv. Howes on WPM medium

Auxin (mg/l)	Rooting shoots (%)	Root number per shoot ± SE
IAA	81%	1.7 ± 0.1
IBA	86%	2.9 ± 0.2
NAA	100%	5.3 ± 0.4

IBA – indole-3-butyric acid; IAA – indole-3-acetic acid; NAA – naphtalen acetic acid; WPM – woody plant medium

After 5 months in culture, surviving explants showed active and uniform shoot growth and multiplication. Dividing and subculturing the basal shoot mass did not cause tissue breakdown or exudation.

The results of multiplication are stated in Table 1. The number of newly formed shoots varied with the medium tested and concentration of zeatin. Generally, the highest multiplication rate (2.7) was obtained for cv. Howes on AN medium with the concentration 1 mg/l of zeatin. Further increase in zeatin concentration to 2 mg/l had no appreciable effect on shoot number. Without zeatin, the explants did not multiply. Earlier reports have demonstrated that zeatin was an important plant growth regulator for efficient multiplication and growth in *Vaccinium* micropropagation (Reed, Abdelnour 1991; Debnath, McRae 2001b; Ostrolúcka et al. 2004; Jiang et al. 2009; Sedlák, Paprštein 2009). Gajdošová et al. (2006) stated the effectiveness of zeatin in low concentration (0.5 mg/l) for inducing multiple shoot development in meristem cultures of *Vaccinium* sp. Zeatin concentrations of 2 mg/l and higher promoted callus formation and suppressed shoot regeneration in Gajdošová's experiments, which is contradictory to our findings.

Within the same range of zeatin concentration, cv. Howes gave higher multiplication rates on AN medium. On the contrary, the lowest multiplication rates were noted on half-MS medium. Out of three media tested in our study, AN medium was found to be more effective than the WPM medium and half-MS medium for shoot proliferation. Many contradictions among reports on the best medium for *Vaccinium* micropropagation have been noted. Ostrolúcka and Šimala (2002) and Gajdošová et al. (2006) used modified AN medium with zeatin for successful micropropagation of different *Vaccinium* species and cultivars. In our previous experiments with *V. corymbosum* and *V. vitis-idaea*, WPM was the best medium for maximum multiplication (Sedlák, Paprštein 2009). On the contrary, Debnath and McRae (2001b) reported that modified MS medium was found to be more effective than the WPM for shoot multiplication of *V. vitis-idaea*.

In our experiments on all media, any physiological disorders or morphological abnormalities such as excessive callus formation or production of abnormally narrow leaves were not observed during *in vitro* shoot proliferation stage. Shoots in our experiments evolved from preformed meristems on the original explants and not from callus. In accordance with several authors (Lloyd, McCown 1981; Debnath, McRae 2001b), who have described micro-

propagation of species from Ericaceae family, the genetic stability of the culture should remain high.

Omission of cytokinin and exposure to a high auxin concentration (1 mg/l) in WPM medium was effective for root induction (Table 2). Root initiation started within 2 weeks. The percent of rooting was 100% for NAA, 86% for IBA and 81% for IAA. The best results were achieved with NAA, which promoted an average development of more than 5 good quality roots per shoot without callus formation. Cranberry micropropagated plants performed very well after acclimatization in *ex vitro* conditions with 90–100% survival. The acclimatized plants exhibited normal growth and developmental characteristics with no apparent differences between micropropagated shoots and conventionally grown mother plants.

In conclusion, *V. macrocarpon* can be efficiently propagated by using a zeatin-supplemented AN medium. Thousands of plants could be produced per year from several initial shoot cultures. In comparison, conventional nursery techniques using cuttings produce only a few plants annually. *In vitro* propagation techniques described in this study may also be applied to other *Vaccinium* species. However because of the great variability within this genus, some species and cultivars would still require further research to optimize micropropagation system.

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