A Scale-up System for Lowbush Blueberry Micropropagation Using a Bioreactor

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Abstract. In an attempt to improve the micropropagation protocol for lowbush blueberry (*Vaccinium angustifolium* Ait.), a protocol using a bioreactor system combined with a semisolid gelled medium has been developed. Cultures of cultivar Fundy and two wild clones ('NB1' and 'QB1') were established in vitro on a gelled modified cranberry basal medium (BM) containing 5 μ M zeatin or 10 μ M N6-[2-isopentenyl]adenine. Multiple shoots were obtained within 8 weeks by transferring zeatin-induced shoots from the gelled BM to a bioreactor containing liquid BM with 1 to 4 μ M zeatin. Genotypes differed significantly with respect to multiplication rate in liquid and gelled BM containing 1 μ M zeatin with 'NB1' producing 8.5 ± 1.1 and 2.9 ± 0.3 shoots per explant in liquid and gelled media, respectively, after one subculture followed by 'QB1' (7.1 ± 0.6 and 2.6 ± 0.4 shoots per explant, respectively) and 'Fundy' (5.8 ± 0.4 and 2.0 ± 0.2 shoots per explant, respectively). With subculture, there was an increase of shoot multiplication rate for all genotypes. Bioreactor- and gelled medium-proliferated shoots were treated with 39.4 mM indole-3-butyric acid powder, rooted in a 2 peat:1 perlite (v/v) medium, plantlets acclimatized, and eventually established in the greenhouse with 64% to 74% rooting of microshoots and 90% to 99% survival of rooted shoots. Results obtained suggested the possibility of large-scale multiplication of lowbush blueberry shoots in bioreactors.

Lowbush blueberry (*Vaccinium angustifolium* Ait.), a perennial, rhizomatous, cross-pollinated shrub (Vander Kloet, 1988), is a commercially important crop in Maine, Quebec, and the Canadian Atlantic Provinces in North America. The berries are known to possess great potential health benefits, mostly attributable to the high content of antioxidant phenolic compounds: anthocyanins, flavonols, and phenolic acids. The high anthocyanin content of lowbush blueberry (Kalt et al., 1999) is believed to have important therapeutic values (Cristoni and Magistretti, 1987; Kamei et al., 1995). The wild stands, made up of numerous heterogenous genetically diverse clones, are commercially managed and harvested. The genetic variation for traits affecting yield (berry size, number of berries per cluster, and stem density) results in great yield variability within a field. The genetic diversity between wild clones has been

verified by molecular techniques (Debnath, 2009). The variability in fruit yield is especially important because unlike a uniformly high-yielding highbush blueberry field made up of two or three genotypes, a lowbush blueberry field includes many low-yielding genotypes (Jamieson, 2008). Partial coverage results from inadvertent kills of plants from applied herbicides, erosion that had been prevented by weeds, and scalping by machinery is very common in commercial lowbush blueberry fields (Morrison et al., 2000) and in young fields, which may have less than 50% coverage (Smagula and Yarborough, 1990). Increasing the blueberry cover by the introduction of improved selections could greatly improve the productivity of native lowbush blueberry fields (Hepler and Yarborough, 1991). The limited availability of plant material, the high cost of establishment, and the slow rate of plant spread have prevented growers from establishing cultivated lowbush blueberry fields.

Plants for establishing new blueberry fields can be produced from softwood or rhizome cuttings of selected clones. Although stem or rhizome cuttings are relatively easy to root, the extreme precocity of flowering results in very slow establishment of plantings (Galletta and Ballington, 1996). This problem can be largely avoided by using plants produced through micropropagation (Smagula and Lyrene, 1984). Previous information indicated that seedlings and micropropagated lowbush blueberries became

established and spread faster than conventional rooted cuttings (Debnath, 2007). Although plants grown from seeds produced rhizomes more freely, variation in fruit characteristics was problematic, and fruit yields were often 50% lower than those of the parental clones (Jamieson and Nickerson, 2003). In vitro cloning is a potentially more effective method for improving lowbush blueberry fields, because tissue culture-propagated plants exhibited uniform productivity characteristics of rooted cuttings (Frett and Smagula, 1983). Removing the less productive clones and interplanting high-yielding clones into the interclonal spaces in existing fields would greatly increase the average yields. Using improved micropropagated selections or seedlings from polycrosses of selected clones in natural stands will preserve the variable nature of the lowbush blueberry fruit and markedly increase yields.

There have been some reports of in vitro propagation of *V. angustifolium* (Brissette et al., 1990; Debnath, 2004; Frett and Smagula, 1983; Nickerson, 1978). Each of these techniques used semisolid gelled medium and were difficult to automate as well as high in production costs rendering the systems less suitable for large-scale production. Automated bioreactors for large-scale production of micropropagated plants are important for the micropropagation industry. Bioreactors are self-contained, sterile environments that capitalize on liquid nutrient or liquid-air inflow and outflow systems designed for intensive culture and control over microenvironmental conditions (aeration, agitation, and dissolved oxygen) (Paek et al., 2005). The use of large-scale liquid cultures and automation has the potential to resolve the manual handling of the various stages of micropropagation. Bioreactor systems have been introduced for mass propagation of horticultural plants (Levin and Vasil, 1989) and have proven potential for large-scale micropropagation of oriental lily (Lian et al., 2003) and potato (Piao et al., 2003). The present study sought to develop an effective protocol for lowbush blueberry micropropagation using a combination of gelled medium and bioreactor system. One cultivar and two native clones were tested. Bioreactor micropropagation has not been documented in *Vaccinium* species.

Materials and Methods

Plant material and establishment of shoot cultures. Eight-week-old shoot tip cultures of cultivar Fundy developed at the Atlantic Food and Horticulture Research Center, Agriculture and Agri-Food Canada, Kentville, Nova Scotia, Canada, and two wild clones, 'NB1' and 'QB1', collected from Newfoundland and Labrador and from Quebec in Canada, respectively, were established following the method of Debnath (2004). Briefly, 3- to 5-cm shoot tips were surface-disinfected in a 15% commercial bleach solution [0.79% NaOCl plus 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate)]

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for 20 min followed by a quick rinse in 70% ethanol and then three rinses in sterile distilled water. Shoot tips were cultured for 8 weeks in glass baby food jars (98.5 mm height, 59 mm diameter; Sigma Chemical Co., St. Louis, MO) containing 35 mL modified cranberry basal medium [BM, three-fourth macrosalts and microsalts of Debnath and McRae's (2001a) shoot proliferation medium D] supplemented with 25 g·L⁻¹ sucrose, 3.5 g·L⁻¹ Sigma A 1296 agar (Sigma A 1296), and 1.25 g·L⁻¹ Gelrite (Sigma Chemical Co.). The pH of the medium was adjusted to 5.0 before autoclaving at 121 °C for 20 min. Five micromolar zeatin or 10 μ M N6-[2-isopentenyl]adenine (2iP) was filter-sterilized and added to autoclaved and cooled (40 to 50 °C) BM medium. Cultures were maintained at 20 $\pm$ 2 °C under a 16-h photoperiod (30 μ mol·m⁻²·s⁻¹ at the culture level) provided by cool-white fluorescent lamps.

Effect of zeatin on shoot proliferation in liquid bioreactor cultures over three subculture periods. Three-node stem sections with leaves intact from in vitro 'NB1' shoots maintained for 8 weeks in gelled BM without plant growth regulators (PGRs), originally derived from gelled BM with 5 μ M zeatin, were transferred at random to a temporary immersion bioreactor vessel [RITA® bioreactors (VITROPIC, Saint-Mathieu-de-Treiviers, France; Teisson and Alvard, 1995)] with 0, 1, 2, or 4 μ M zeatin. RITA® bioreactors contain 15-cm high cylindrical vessels that consist of two compartments; the upper part holds the plant material on a polyurethane filter (1 cm) and the lower part contains the culture medium. The container was connected to an automated air pump, which set overpressure to the lower part of the container, pushing the medium to the upper part through the filter. The overpressure escaped through an air vent in the lid of the container. The air pump was controlled with a timer that set the duration and frequency of the liquid immersion. Temporary immersion cultures were established with immersion of explants for 15 min every 4 h. Subculturing was conducted at 8-week intervals with the three-node stem sections (leaves intact) randomly selected from 8-week-old in vitro-grown shoots. There were three vessels for each treatment and each vessel contained eight explants. The experiment was conducted three times.

Effect of genotypes on shoot proliferation in liquid bioreactor cultures over two subculture periods. The response of 'Fundy', 'NB1', and 'QB1' was studied over two subculture periods following the same procedure as was followed in the previous experiment. Explants were grown in BM with 1 μ M zeatin. The experimental unit consisted of eight explants in each vessel, and there were two vessels for each treatment. The experiment was replicated three times.

Effect of genotypes on shoot proliferation on gelled medium over two subculture periods. Three-node stem sections of 'Fundy', 'NB1', and 'QB1' were cultured in Sigma baby food jars containing 35 mL gelled BM with 1 μ M zeatin. There were four jars per treatment for

each clone and each jar contained five explants. The experiment was conducted three times.

Rooting and acclimatization. Elongated shoots (3 to 4 cm long) of all genotypes from gelled and liquid BM with 1 μ M zeatin were excised, dipped in 39.4 mm indole-3-butyric acid (IBA) powder (Stim-Root#3; Plant Products Co. Ltd., Brampton, Ontario, Canada), and planted in 45-cell plug trays (cell diameter: 5.9 cm, cell depth: 15.1 cm; Beaver Plastics, Edmonton, Alberta, Canada) containing 2 peat:1 perlite (v/v). Trays were placed in a humidity chamber with a vaporizer at 22 $\pm$ 2 °C, 95% relative humidity (RH), with a 16-h photoperiod (55 μ mol·m⁻²·s⁻¹) for rooting. Thirty to 40 shoots from each genotype were used for rooting and the experiment was repeated three times. Rooting percentage was recorded after 4 weeks. Plantlets were transferred to 10.5 (L) $\times$ 10.5 (W) $\times$ 12.5 (D) -cm³ plastic pots containing the same medium as used for rooting and acclimatized by gradually lowering the humidity over 2 to 3 weeks. Hardened-off plants were maintained in the greenhouse at

20 $\pm$ 2 °C, 85% RH, under a 16-h photoperiod (90 μ mol·m⁻²·s⁻¹). The number of surviving plants was recorded when plants were removed from the humidity chamber (6 weeks).

Data collection and statistical analysis. The following growth characteristics of surviving explants were measured for each treatment at 8 weeks: number of shoots (greater than 1 cm long) per responding explant, shoot length (cm), number of leaves per shoot, and shoot vigor. Vigor was determined by visual assessment on a scale of 1, strongly vitrified, necrotic, and malformed shoots; 2, less vitrified, necrotic, and malformed shoots; 3, no vitrification but with very poor vigor; 4, poor shoot vigor; 5, average shoot vigor; 6, good shoot vigor; 7, very good shoot vigor; and 8, fully normal and healthy shoots with excellent vigor.

Data for number of shoots per explant, shoot length, number of leaves per shoot, and rooting and survival percentages were subjected to analysis of variance using the SAS statistical software package (SAS Institute, Inc., 2002). The number of shoots per explant was transformed to the square root scale

Fig. 1. In vitro propagation of lowbush blueberry clone 'NB1'. (A) Shoot proliferation after 8 weeks of culture on semisolid gelled basal medium (BM) supplemented with 5 μ M zeatin (bar = 1 cm). (B) Shoot proliferation after 8 weeks in liquid BM with 1 μ M zeatin (bar = 2.3 cm). (C) Adventitious shoot regeneration after 14 weeks in liquid BM with 4 μ M zeatin (bar = 1 cm). (D) One-year-old hardened-off plants (bar = 9 cm).

before the analysis of variance to stabilize the variance and then back-transformed for presentation. Statistical F-tests were evaluated at $P \leq 0.05$. Differences among treatments were further analyzed using Duncan's multiple range test. Shoot vigor was analyzed separately by categorical analysis (CATMOD procedure in SAS) and differences between treatment combinations were contrasted using the contrast statement in the CATMOD procedure (Compton, 1994).

Results and Discussion

Establishment of shoot cultures. The frequency of contamination varied from 4.5% to 7.5% whether the stems were grown on the basal medium with zeatin or 2iP. Nodal explants that were slightly tender and having greenish axillary buds responded efficiently for bud sprouting compared with hard nodal explants with brownish buds, which showed no sign of growth. The explants produced elongated shoots on both zeatin and 2iP. However, better initiation rates (percentage of explants showing new shoot growth) were on zeatin-containing medium. Zeatin allowed 35% to 50% more shoots per explant to sprout than those on 2iP for all three genotypes. Zeatin was also more effective than 2iP in the shoot initiation and proliferation of *Vaccinium* species. Shoot initiation percentage for 12 *V. corymbosum* genotypes ranged from 14% to 100% on gelled medium with 18.2 μM zeatin and from 0% to 88% with 49.2 to 73.8 μM 2iP (Reed and Abdelnour-Esquivel, 1991). Shoot proliferation of low-bush and highbush blueberries was very effective on gelled medium with 4.6 μM (Kaldmäe et al., 2006) and 20 μM zeatin, respectively (Tetsumura et al., 2008). However, Litwiniczuk and Wadas (2008) reported that 5.7 μM IBA in combination with 9.8 to 73.8 μM 2iP significantly enhanced axillary shoot elongation in highbush blueberry.

Like in another studies (Reed and Abdelnour-Esquivel, 1991), differences existed between genotypes. Shoot initiation was best in 'NB1' (85% $\pm$ 7.5%) followed by 'QB1' (78% $\pm$ 5.3%) and 'Fundy' (69% $\pm$ 8.2%). For shoots both on 2iP and zeatin supplemented media, shoots proliferated directly from the node through the axillary branching of buds from the original explants (Fig. 1A).

Effect of zeatin on shoot proliferation in liquid bioreactor cultures over three subculture periods. In the first subculture, all 'NB1' explants placed in the culture medium responded by swelling and developed callus around the basal end of the explants at 0 to 2 μM zeatin from Day 6 to 8 of culture. Callus size increased with increasing concentration of zeatin. Axillary buds and multiple shoots developed at all zeatin concentrations (Fig. 1B).

An interaction ($P \leq 0.05$) between zeatin concentration and subculture period was observed for shoot number, shoot height, and number of leaves per shoot (Table 1). Zeatin affected shoot number, shoot height, leaf number per shoot, and shoot vigor ($\chi^2 =$

8.38, $P = 0.0388$). Across subculture periods, responding explants produced more shoots with increasing levels of zeatin up to 4 μM . Shoot height, leaf number per shoot, and shoot vigor were best at 1 to 2 μM zeatin and declined at higher zeatin concentrations (Table 1). These results suggest that a threshold level of endogenous growth regulators accumulated during culture initiation, which enabled the explants to produce shoots optimally at reduced levels of zeatin.

Nodal explants cultured on zeatin-free medium produced one or two unbranched axillary shoots each, and the shoot growth was not vigorous. This might be because of persistence of a strong apical dominance, a major constraint in the development of efficient in vitro clonal propagation of some plant species (George and Sherrington, 1984). Axillary branching in nodal explants oc-

curred only when cytokinin was applied exogenously in the present study. Lingonberry responds similarly (Debnath and McRae, 2001a).

Shoot proliferation and development improved significantly ($P \leq 0.05$) with subculturing (Table 1); shoots per explant of rootable size (greater than 1.0 cm long) in the first subculture were fewer than in the second and third subcultures (Table 1). Similar results were also reported by Debnath and McRae (2001b) for cranberry. Shoot height and leaf number per shoot were also better in second and third subcultures, although shoot vigor was similar in three culture periods (Table 1).

Shoots when allowed to grow for more than 12 weeks on media that contained more than 4 μM zeatin occasionally produced adventitious shoot masses, which appeared

Table 1. Effects of zeatin and subculture periods on shoot proliferation of blueberry clone 'NB1' in liquid bioreactor cultures.^z

Zeatin concn (μM)	Subculture period	Shoots (no./explant)	Shoot ht (cm)	Leaves (no./shoot)	Shoot vigor (scale: 1–8) ^y
0	1	2.0 $\pm$ 0.2 d ^x	4.3 $\pm$ 0.3 abc	12.0 $\pm$ 1.7 ab	4.8 $\pm$ 0.3 a
	2	1.4 $\pm$ 0.2 d	3.3 $\pm$ 0.3 bc	9.0 $\pm$ 1.0 ab	3.5 $\pm$ 0.5 a
	3	1.1 $\pm$ 0.1 d	2.8 $\pm$ 0.3 c	6.3 $\pm$ 1.5 b	2.7 $\pm$ 0.3 a
1	1	6.0 $\pm$ 0.5 c	4.9 $\pm$ 0.3 abc	13.7 $\pm$ 1.5 ab	6.3 $\pm$ 0.3 a
	2	7.1 $\pm$ 0.6 bc	5.8 $\pm$ 0.3 a	17.0 $\pm$ 2.0 a	7.3 $\pm$ 0.3 a
	3	7.0 $\pm$ 0.6 bc	6.1 $\pm$ 0.4 a	17.3 $\pm$ 2.3 a	7.3 $\pm$ 0.3 a
2	1	6.5 $\pm$ 0.8 c	4.7 $\pm$ 0.3 abc	12.0 $\pm$ 2.0 ab	6.3 $\pm$ 0.3 a
	2	7.2 $\pm$ 0.5 abc	5.4 $\pm$ 0.2 ab	16.0 $\pm$ 1.0 a	7.2 $\pm$ 0.3 a
	3	7.6 $\pm$ 0.5 abc	6.0 $\pm$ 0.2 a	18.0 $\pm$ 2.0 a	7.2 $\pm$ 0.6 a
4	1	7.9 $\pm$ 0.8 abc	2.9 $\pm$ 0.3 c	10.7 $\pm$ 1.5 ab	4.5 $\pm$ 0.2 a
	2	9.1 $\pm$ 0.3 a	3.8 $\pm$ 0.3 abc	13.7 $\pm$ 1.5 ab	5.5 $\pm$ 0.5 a
	3	8.6 $\pm$ 0.4 ab	3.7 $\pm$ 0.4 abc	14.7 $\pm$ 1.2 ab	5.2 $\pm$ 0.3 a
Significant effects ^w		Z, S, Z $\times$ S	Z, S, Z $\times$ S	Z, S, Z $\times$ S	Z

^zData were collected after 8 weeks in culture.

^yShoot vigor was scored on a scale of 1, strongly vitrified, necrotic, and malformed shoots; 2, less vitrified, necrotic, and malformed shoots; 3, no vitrification but with very poor vigor; 4, poor shoot vigor; 5, average shoot vigor; 6, good shoot vigor; 7, very good shoot vigor; and 8, fully normal and healthy shoots with excellent vigor.

^xValues represent means $\pm$ sd. Different letters within a column represent significant difference at $P \leq 0.05$.

^wSignificant effects ($P \leq 0.05$): Z = zeatin concentration, S = subculture period; data for shoot number, shoot height, and leaf number per shoot were subjected to analysis of variance with the SAS statistical software package (Release 8.2, SAS Institute, Inc., Cary, NC) and for shoot vigor by categorical analysis (CATMOD procedure in SAS).

Table 2. Performance of 'Fundy', 'QB1', and 'NB1' blueberries on shoot proliferation in liquid bioreactor cultures with 1 μM zeatin over two subculture periods.^z

Genotype	Subculture period	Shoots (no./explant)	Shoot ht (cm)	Leaves (no./shoot)	Shoot vigor (scale: 1–8) ^y
Fundy	1	5.2 $\pm$ 0.5 d ^x	4.5 $\pm$ 0.5 ab	10.7 $\pm$ 1.6 a	6.0 $\pm$ 0.3 a
	2	5.8 $\pm$ 0.4 d	4.8 $\pm$ 0.4 ab	11.3 $\pm$ 2.5 a	6.4 $\pm$ 0.4 a
QB1	1	6.1 $\pm$ 0.3 cd	5.1 $\pm$ 0.5 ab	13.7 $\pm$ 1.5 a	6.3 $\pm$ 0.3 a
	2	7.1 $\pm$ 0.6 bc	6.1 $\pm$ 0.7 a	18.0 $\pm$ 1.7 a	6.7 $\pm$ 0.3 a
NB1	1	7.5 $\pm$ 0.5 ab	4.0 $\pm$ 0.5 b	11.3 $\pm$ 2.5 a	5.5 $\pm$ 0.2 a
	2	8.5 $\pm$ 1.1 a	4.4 $\pm$ 0.5 ab	12.0 $\pm$ 2.0 a	6.4 $\pm$ 0.4 a
Significant effects ^w		G, S	G, S	G	—

^zData were collected after 8 weeks in culture.

^yShoot vigor was scored on a scale of 1, strongly vitrified, necrotic, and malformed shoots; 2, less vitrified, necrotic, and malformed shoots; 3, no vitrification but with very poor vigor; 4, poor shoot vigor; 5, average shoot vigor; 6, good shoot vigor; 7, very good shoot vigor; and 8, fully normal and healthy shoots with excellent vigor.

^xValues represent means $\pm$ sd. Different letters within a column represent significant difference at $P \leq 0.05$.

^wSignificant effects ($P \leq 0.05$): G = genotype, S = subculture period; data for shoot number, shoot height, and leaf number per shoot were subjected to analysis of variance with the SAS statistical software package (Release 8.2, SAS Institute, Inc., Cary, NC) and for shoot vigor by categorical analysis (CATMOD procedure in SAS).

to arise from dense calli growing at the base of the shoots in the medium (Fig. 1C). Adventitious bud formation in lingonberries was reported by Debnath and McRae (2001a) for shoots grown on medium with high cytokinin concentrations. Because the formation of adventitious shoots was more likely to produce somaclonal variation (Larkin and Scowcroft, 1981), zeatin concentrations of more than 4 μM should be avoided if lowbush blueberries were to be cultured for more than 12 weeks.

Effect of genotypes on shoot proliferation in liquid bioreactor cultures over two subculture periods. The genotypes differed significantly ($P \leq 0.05$) for shoot number, shoot height, and leaf number per shoot, but not for shoot vigor ($\chi^2 = 0.49$, $P = 0.7833$). 'NB1' produced the maximum shoots per explant followed by 'QB1' and 'Fundy'. 'QB1' shoots were the longest with maximum leaves per shoot (Table 2). Because cells within the same plant can have different endogenous levels of PGRs and additional variation in receptor affinity or cellular sensitivity to plant growth regulators (Minocha, 1987), it was reasonable to expect that in vitro responses will vary with genotype. Genotypic differences for number of in vitro-proliferated shoots were also noticed on a gelled medium (Debnath, 2004).

Effect of genotypes on shoot proliferation on gelled medium over two subculture periods. Like in the liquid medium, the genotypes differed significantly ($P \leq 0.05$) for shoot number, shoot height, and leaf number per shoot, but not for shoot vigor ($\chi^2 = 0.27$, $P = 0.8748$). 'NB1' produced the maximum shoots per explant followed by 'QB1' and 'Fundy'. 'QB1' shoots were the longest with maximum leaves per shoot (Table 3).

Liquid culture was ideal in micropropagation for reducing plantlet production costs and automation, and many plants have been mass-propagated in the liquid medium using bioreactors (Ziv, 2005). Liquid culture systems improved the efficiency of in vitro propagation of three lowbush blueberry genotypes; shoot number per explant was approximately three times more than those on gelled medium for all three selections (Tables 2 and 3). Less zeatin (1 μM) was required in liquid culture compared with gelled medium (2 to 4 μM) for maximum shoot proliferation (Debnath, 2004). Similar observations were reported for *Chrysanthemum* by Hahn and Paek (2005) who found higher propagation rates in bioreactors than on gelled medium. Working with a gelled medium containing 4.6 μM zeatin, Kaldmäe et al. (2006) observed poor shoot proliferation (1.8 shoots per explant) in lowbush blueberry. The presumed reasons for faster growth in the liquid medium were thought to be better availability of nutrients and faster uptake.

Rooting and acclimatization. In vitro regenerated shoots from gelled and liquid BM with 1 μM zeatin rooted easily within 4 weeks (Fig. 2). Lowbush blueberry microcuttings performed well in the greenhouse and rooted plants acclimatized readily to the

Table 3. Performance of 'Fundy', 'QB1', and 'NB1' blueberries on shoot proliferation on gelled basal medium with 1 μM zeatin over two subculture periods.^z

Genotype	Subculture period	Shoots (no./explant)	Shoot ht (cm)	Leaves (no./shoot)	Shoot vigor (scale: 1–8) ^y
Fundy	1	1.8 $\pm$ 0.2 d*	2.5 $\pm$ 0.3 ab	6.7 $\pm$ 1.5 ab	6.0 $\pm$ 0.5 a
	2	2.0 $\pm$ 0.2 cd	3.0 $\pm$ 0.2 ab	8.0 $\pm$ 1.0 ab	6.3 $\pm$ 0.8 a
QB1	1	2.3 $\pm$ 0.3 bc	2.9 $\pm$ 0.2 ab	8.7 $\pm$ 2.1 ab	6.5 $\pm$ 0.5 a
	2	2.6 $\pm$ 0.4 ab	4.0 $\pm$ 0.6 a	11.0 $\pm$ 0.6 a	6.8 $\pm$ 0.4 a
NB1	1	2.6 $\pm$ 0.4 ab	2.1 $\pm$ 0.4 b	5.8 $\pm$ 0.4 b	5.3 $\pm$ 0.3 a
	2	2.9 $\pm$ 0.3 a	2.7 $\pm$ 0.3 ab	7.3 $\pm$ 1.5 ab	6.6 $\pm$ 0.4 a
Significant effects ^w		G, S	G, S	G, S	—

^zData were collected after 8 weeks in culture.

^yShoot vigor was scored on a scale of 1, strongly vitrified, necrotic, and malformed shoots; 2, less vitrified, necrotic, and malformed shoots; 3, no vitrification but with very poor vigor; 4, poor shoot vigor; 5, average shoot vigor; 6, good shoot vigor; 7, very good shoot vigor; and 8, fully normal and healthy shoots with excellent vigor.

^xValues represent means $\pm$ SD. Different letters within a column represent significant difference at $P \leq 0.05$.

^wSignificant effects ($P \leq 0.05$): G = genotype, S = subculture period; data for shoot number, shoot height, and leaf number per shoot were subjected to analysis of variance with the SAS statistical software package (Release 8.2, SAS Institute, Inc., Cary, NC) and for shoot vigor by categorical analysis (CATMOD procedure in SAS).

Fig. 2. Rooting of 'Fundy', 'QB1', and 'NB1' blueberry microshoots after 4 weeks of transfer in a 2 peat:1 perlite (v/v) medium (A) and survival of rooted shoots when plants were removed from the humidity chamber (6 weeks) (B). Each bar represents mean $\pm$ SD.

greenhouse with survival rates of 90% to 99% (Fig. 2). Debnath (2004) reported a survival rate of 80% to 90% of micropropagated lowbush blueberry shoots. After acclimatization, plantlets have grown actively in the greenhouse with an apparent normal leaf and shoot morphology (Fig. 1D).

In conclusion, this report presents a protocol for lowbush blueberry micropropagation for the first time in a bioreactor system. Multiple shoot proliferation can be obtained by culturing in liquid medium with 1 μ M zeatin in a bioreactor system for 8 weeks to scale-up shoot multiplication. Such zeatin-induced shoots can be rooted easily in a peat-perlite medium and established in the greenhouse. The results from this investigation can be used for the conservation and large-scale propagation of lowbush blueberry and other *Vaccinium* species.

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