Shoot Regeneration and Plantlet Formation by Cascade Huckleberry, Mountain Huckleberry, and Oval-leaf Bilberry on a Zeatin-containing Nutrient Medium

Samir C. Debnath[1,3] and Danny L. Barney[2]

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Summary. **A plant regeneration protocol was developed for cascade huckleberry (*Vaccinium deliciosum*), mountain huckleberry (*V. membranaceum*), and oval-leaf bilberry (*V. ovalifolium*) clones. The effects of zeatin concentrations (0, 4.6, 9.1, and 13.7 μM) and explant type (leaf or stem segment) on adventitious shoot regeneration were studied on a nutrient medium of low ionic concentration. Adventitious bud and shoot regeneration was greatly influenced by clone, explant type, and zeatin concentration. Zeatin at 9.1 to 13.7 μM supported the best bud and shoot regeneration. At low concentrations (2.3 to 4.6 μM), zeatin enhanced shoot elongation and produced usable shoots after one additional subculture. The three clones differed significantly with respect to multiplication rate of adventitious shoots. Oval-leaf bilberry and mountain huckleberry clones produced six to seven 5-cm-long shoots per explant and cascade huckleberry clone produced five 3-cm-long shoots per explant, when 2.3 μM zeatin was used in the medium. Increasing the concentration of zeatin in the culture medium increased shoot number per explant, but decreased shoot height, leaf number per shoot, and shoot vigor. Proliferated shoots were rooted on the same medium but without any plant growth regulators (PGRs). Rooted plantlets were transferred to a 2 peat:1 perlite (v/v) medium for acclimatization and eventually established in the greenhouse with 75% to 90% survival rate. This in vitro protocol will be useful for micropropagation, in vitro selection, and genetic manipulation of *Vaccinium* species.**

Cascade huckleberry, mountain huckleberry, and oval-leaf bilberry belong to the genus *Vaccinium* that contains ≈400 species distributed worldwide except Antarctica and Australia (Vander Kloet, 1988) and is typically characterized as woody perennial vines or shrubs producing moderate-sized fleshy, and more-or-less edible fruit. Plants may be terrestrial or epiphytic and are generally found on acidic, sandy, peaty, or organic soils. Huckleberries and bilberries belong to the section *Myrtillus* of this genus and are native to the northwestern United States and western Canada. Mountain huckleberry (black huckleberry, thin-leaf huckleberry, or mountain bilberry) and cascade huckleberry (cascade bilberry or blue huckleberry) are tetraploid species rich in aroma and flavor chemicals (Fellman et al., 1998). The oval-leaf bilberry (oval-leaf blueberry or alaska blueberry) also is a tetraploid species [occasionally diploid (Vander Kloet, 1988)] and is rich in antioxidant compounds (Lee et al., 2004; Taruscio et al., 2004) creating opportunities for value-added nutritional products. These berries are harvested from wild stands and used in fresh, frozen, and processing markets.

In vitro methods complement traditional breeding programs to introduce new traits into selected plants, to multiply clonal plants, and to develop suitable cultivars in a minimum time. The ability to regenerate plants is crucial to the successful application of in vitro methods (Cao and Hammerschlag, 2000; Qu et al., 2000). Additionally, a shoot regeneration system can be used to identify and/or induce somaclonal variants and to develop transgenic plants following genetic transformation of plant cells.

To produce superior *Vaccinium* clones more rapidly, we began in vitro techniques at our research center in St. John's, NL, Canada, in 1999 (Debnath, 2000). A successful system for cloning cascade and mountain huckleberries and oval-leaf bilberries from axillary shoot meristems was developed so that superior germplasm could be more rapidly introduced to the *Vaccinium* industry (Barney et al., 2007). Generating shoots reliably from somatic plant tissues, such as leaves or stem segments, would permit the genetic manipulation of these species. Although adventitious shoot regeneration by cascade and mountain huckleberries and by oval-leaf bilberry was absent, shoot proliferation from nodal explants of these species has been reported (Barney et al., 2007) and adventitious shoot regeneration also has been reported in other *Vaccinium* species, including lowbush blueberry [*V. angustifolium* (Debnath, 2009, 2011)], highbush blueberry [*V. corymbosum* (Cao and Hammerschlag, 2000)], southern highbush blueberry [predominantly *V. corymbosum* germplasm with germplasm from *V. darrowii*, *V. ashei*, and/or *V. tenellum* (Liu et al., 2010; Meiners et al., 2007)], half-high blueberry [*V. corymbosum* × *V. angustifolium* (Graham et al., 1996)], cranberry [*V. macrocarpon* (Qu et al., 2000)], bilberry [*V. myrtillus* (Shibli and Smith, 1996)], ohelo [*V. pahalae* (Shibli and Smith, 1996)], and lingonberry [*V. vitis-idaea* (Debnath, 2003, 2005; Debnath and McRae, 2002)].

Cytokinins play an important role in the regulation of plant growth

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[1]Atlantic Cool Climate Crop Research Centre, Agriculture and Agri-Food Canada, Building 25, 308 Brookfield Road, St. John's, NL A1E 0B2, Canada

[2]Danny L. Barney, USDA ARS Arctic and Subarctic Plant Gene Bank, 1509 South Georgeson Drive, Palmer, AK 99645

[3]Corresponding author. E-mail: samir.debnath@agr.gc.ca.

Units

To convert U.S. to SI, multiply by	U.S. unit	SI unit	To convert SI to U.S., multiply by
29.5735	fl oz	mL	0.0338
2.54	inch(es)	cm	0.3937
25.4	inch(es)	mm	0.0394
28.3495	oz	g	0.0353
0.001	ppm	$g \cdot L^{-1}$	1000
(°F – 32) ÷ 1.8	°F	°C	(1.8 × °C) + 32

and morphogenesis (Miller, 1961). They induce cell division and shoot differentiation (Skoog and Miller, 1957). Zeatin and its derivatives are the most abundant cytokinins in plants (Letham and Palni, 1983). Zeatin was found effective for shoot regeneration in lingonberry (Debnath and McRae, 2002). The objective of the study is to obtain plantlets using leaves and stem segments derived from micropropagated shoots. Hormonal, physical, and genetic experimental factors were investigated to optimize plant regeneration from the leaves and stem segments of a cascade huckleberry clone, a mountain huckleberry clone, and of an oval-leaf bilberry clone.

Materials and methods

Establishment and maintenance of in vitro shoot cultures. Shoot tips of mountain huckleberry clone 'VAME 031C', cascade huckleberry clone 'VADE 004A', and oval-leaf bilberry clone 'VAOF 032D', selected from in vitro-grown open-pollinated individual vigorous seedlings (Barney et al., 2007), were established and maintained following the protocol of Barney et al. (2007) with some modifications (Debnath, 2009). In vitro-grown shoot tips were inoculated into 175-mL glass baby food jars (Sigma Chemical Co., St. Louis, MO) containing 35 mL of modified cranberry medium (Debnath and McRae, 2001a), which contained 0.75% macrosalts and microsalts of Debnath and McRae's (2001b) shoot proliferation medium D [referred to as basal medium (BM) hereafter] supplemented with (per liter) 25 g sucrose, 3.5 g Sigma A 1296 agar, 1.25 g Gelrite (Sigma Chemical Co.), and 4.6 μM zeatin. The medium pH was adjusted to 5.0 before autoclaving at 121 °C for 20 min. Cultures were maintained at 20 ± 2 °C under a 16-h photoperiod [photosynthetic photon flux (*PPF*) density of 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the culture level] provided by cool white fluorescent lamps and were subcultured every 10 weeks.

Expt. 1. The goal of this experiment was to study the effects of clones, zeatin concentration, and explant type on adventitious shoot regeneration. Young, actively growing leaves and stem segments (10- to 12-mm stem sections without leaves) were obtained from 10-week-old proliferating shoot cultures of three huckleberry and bilberry clones. All preculture operations of the explants were completed on sterile filter paper that was saturated with BM to prevent desiccation. Four almost-fully or fully expanded leaves nearest the apical meristem were excised with a scalpel at the petiole base and five transverse cuts were made through the midrib that did not sever the leaf completely. Stem segments were obtained from the tip of the shoots. Prepared leaves and stem segments were transferred to 100- × 25-mm sterilized, disposable petri plates containing 25 mL of BM containing 0, 4.6, 9.1, or 13.7 μM zeatin. Plates were sealed along the rim with two layers of parafilm M (Fisher Scientific, Chicago, IL) and arranged in the incubator randomly across treatments. Six explants were placed in each plate, and four plates were used for each treatment; the experiment was conducted three times with a total of 72 explants per treatment. Cultures were incubated in darkness at 20 ± 2 °C for 2 weeks, before transfer to the same photoperiod, light intensity, and temperature used for multiplication.

Expt. 2. The purpose of this experiment was to study the effects of two zeatin concentrations for elongation of adventitious shoots. Buds and shoot clumps from leaf and stem explants of clones 'VAME 031C', 'VADE 004A', and 'VAOF 032D' cultured on BM with 9.1 μM zeatin were collected 10 weeks following culture initiation and randomly transferred to 175-mL glass baby food jars (Sigma Chemical Co.) containing 35 mL of BM with 2.3 or 4.6 μM zeatin. Vessels were capped with polypropylene clear lids. Four jars were used for each treatment, and each jar contained five explants. The experiment was conducted three times.

Expt. 3. In this experiment, effects of clone and zeatin concentration on proliferation of leaf culture-derived shoots from nodal explants were studied. A 3 × 4 factorial experiment (completely randomized) compared all combinations of three clones 'VAME 031C', 'VADE 004A', and 'VAOF 032D' and four zeatin concentrations (0, 2.3, 4.6, or 9.1 μM). Three-node stem sections with leaves intact from in vitro shoots obtained from leaf culture with 9.1 μM zeatin and maintained for 10 weeks in BM without PGRs, were cultured in 175-mL glass baby food jars (Sigma Chemical Co.) containing 35 mL media. Four jars were used per treatment for each clone, and each jar contained five explants. The experiment was conducted three times.

Rooting and acclimatization. Elongated shoots (4- to 5-cm long) from BM with zeatin (2.3 or 4.6 μM) were transferred to BM void of PGR for rooting in vitro. Rooted shoots were planted in 45-cell plug trays (5.9 cm cell diameter, 15.1 cm cell depth; Beaver Plastics, Edmonton, AB, Canada) containing 2 peat : 1 perlite (v/v). Trays were placed in a humidity chamber with a vaporizer at 22 ± 2 °C, 95% relative humidity (RH) at 16-h photoperiod at 55 $\mu mol \cdot m^{-2} \cdot s^{-1}$ *PPF* for rooting. Plantlets were transferred to 1-L plastic pots (12.5-cm diameter) 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 ± 2 °C, 85% RH, and 16-h photoperiod at a maximum *PPF* of 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The number of surviving plants was recorded when they were removed from the humidity chamber (6 weeks).

Data collection and statistical analysis. In Expt. 1, the frequency of explants with callus and adventitious buds (longer than 1 mm) or shoots (longer than 2 mm); callus size; the number of buds and shoots per regenerating explant; and shoot vigor were recorded after 10 weeks of incubation for each treatment. Vigor was assessed visually on a scale of 1 (strongly hyperhydric, necrotic, and/or malformed shoots) to 8 (fully normal and healthy shoots). Callus was rated on a scale of no callus formation (0) and least (1) to highest (8) growth.

For Expts. 2 and 3, the following growth characteristics of surviving explants were measured for each treatment at 10 weeks: number of shoots (>1-cm long) per responding explant, shoot height, number of leaves per shoot, and shoot vigor. Vigor was determined by visual assessment on a scale of 1 (strongly hyperhydric, necrotic, and/or malformed shoots) to 8 (fully normal and healthy shoots with excellent vigor); with 2 = less hyperhydric, necrotic, and/or malformed shoots; 3 = no vitrification, but with very poor vigor; 4 = possessing poor shoot vigor;

5 = average shoot vigor; 6 = good shoot vigor; and 7 = very good shoot vigor.

Data for all characters except shoot vigor and callus size were subjected to analysis of variance with SAS (release 8.2; SAS Institute, Cary, NC). In Expt. 1, data for the control treatment (0 PGR) were excluded from analysis because all explants failed to respond. Statistical F tests were evaluated at $P \leq 0.05$. Differences among treatments were further analyzed using Duncan's multiple range test. Shoot vigor and callus size were analyzed separately by categorical analysis (CATMOD procedure in SAS), and differences between treatment combinations were contrasted using the contrast statement in the CATMOD procedure. This method is appropriate for the analysis of categorical data (Compton, 1994).

Results and discussion

EXPT. 1. Explants grown on medium with different concentrations of zeatin initially responded by callus formation not only on the cut edges but also on the surface and edges of the explants (Fig. 1A). These changes were absent on controls. Calli were initially green with reddish areas that were related to the sites of bud differentiation. Using a dissecting microscope, bud initials were observed on explants 4 weeks after culture initiation. Multiple shoot buds often formed as tight bud clusters with some leaf expansion which varied by zeatin concentration. Adventitious buds appeared to regenerate on the callus from both the abaxial and adaxial sides of the leaves and on the whole surface of stem explants. Some developed into shoots after 6 to 7 weeks of culture. Explants receiving treatments with a higher regeneration rate tended to have more buds and shoots per regenerating explant after 9 to 10 weeks of culture (Fig. 1B).

Clones interacted ($P \leq 0.05$) with explant type and zeatin concentration for the number of bud formed (Table 1). These three factors affected callus regeneration percentage and number of buds and shoots developed per regenerating explant (Table 1). Although bud regeneration percentage was affected by clone and zeatin concentration (Table 1), shoot vigor was affected by only zeatin concentration ($\chi^2 = 7.07$, $P = 0.0288$) (Table 2).

The induction of callus and buds was dependent on the addition of zeatin to the medium. Responding leaf and stem explants produced higher percentages of callus and buds with increasing levels of zeatin up to 9.1 μM. Across explant types and clones, numbers of buds and shoots per explant were highest at 13.7 μM zeatin (Table 1). However, across explant types, number of buds formed by clone 'VAOF 032D' was similar at 9.1 and 13.7 μM zeatin [8.2 ± 0.6 and 9.1 ± 1.3, respectively (mean ± SD)], which might be due to clone by zeatin concentration interaction. With increasing concentration of zeatin in culture medium, shoot vigor increased on medium with zeatin up to 9.1 μM and then decreased for both explant types in all three clones. Callus size was unaffected by all three factors (Table 2).

Choice of explant is important when propagation is based on shoot initiation (George, 1993). Explants excised from different organs vary in morphogenic capacity (George, 1993). In the present study, shoots formed from cascade huckleberry, mountain huckleberry, and oval-leaf bilberry on both sides of the leaves as was reported previously for other *Vaccinium* species (Debnath, 2009; Debnath and McRae, 2002; Marcotrigiano et al., 1996). However, Qu et al. (2000) reported that shoot regeneration by cranberry was almost exclusively from the adaxial side of the leaf. For blueberry, both adaxial down (Billings et al., 1988; Rowland and Ogden, 1992) and up (Callow et al., 1989) have been used for shoot regeneration from leaf

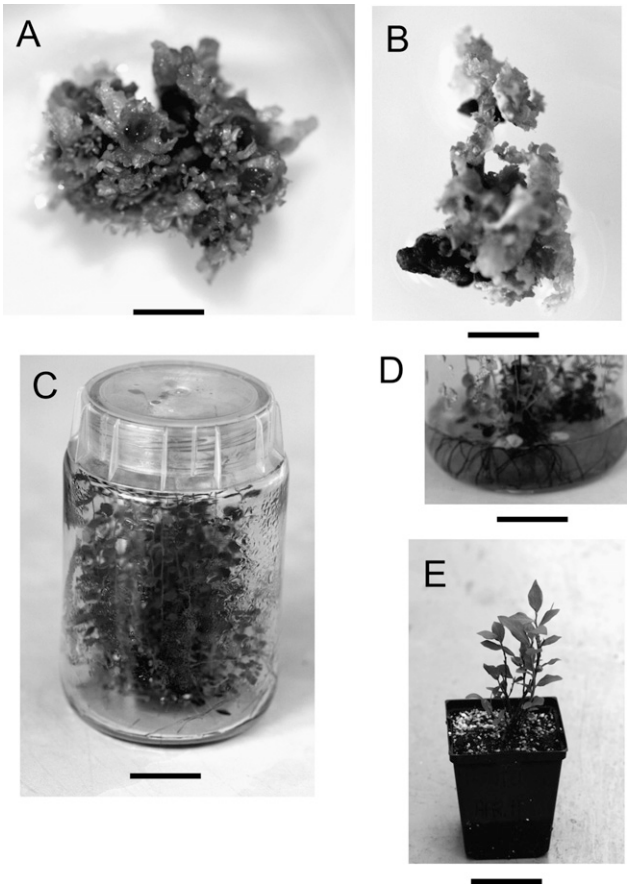

Fig. 1. Morphogenesis of cascade huckleberry clone 'VADE 004A'. (A) Bud and shoot cluster on a leaf explant 10 weeks after plating on a basal medium (BM) with 9.1 μM zeatin [bar = 0.5 cm (0.197 inch)]. (B) Bud and shoot cluster on a stem segment 10 weeks after plating on a BM with 9.1 μM zeatin (bar = 0.5 cm). (C) Shoot elongation of 10-week-old adventitious shoots (developed from a leaf explant on 9.1 μM zeatin) after 10 weeks of transfer to a BM with 2.3 μM zeatin [bar = 2 cm (0.787 inch)]. (D) Rooting of 10-week-old elongated shoots (developed on 2.3 μM zeatin) 10 weeks after transferring to a growth regulator-free BM (bar = 2 cm). (E) One-year-old greenhouse grown plant [bar = 10 cm (3.937 inch)].

explants. In this study, although shoots formed with both explant orientation treatments, the highest explant response occurred when the adaxial side was in contact with the medium. Ten percent to 15% more leaves responded for bud regeneration when adaxial sides were in contact with the medium. Shoots also formed on stem explants. Adventitious shoot regeneration from stem explants were previously not reported for *Vaccinium* species. Adventitious shoot production, with respect to callus regeneration percentage and number of buds and shoots per explant, was higher on leaves than on stem segments (Table 1). Possibly, the leaves are more responsive to nutrients than those of the stem segments. However, across zeatin concentrations, the number of buds formed by clone 'VADE 004A' was similar with leaf and stem explants [7.4 ± 2.7 and 7.2 ± 2.5, respectively (mean ± SD)], which might be due to clone by zeatin concentration interaction.

Table 1. Effects of zeatin (Z) concentration and explant type (E) on the frequency of callus formation and bud regeneration and on the numbers of buds and shoots formed per regenerating explant from leaves and stem segments of mountain huckleberry clone (C) 'VAME 031C', cascade huckleberry clone 'VADE 004A', and oval-leaf bilberry clone 'VAOF 032D', collected after 10 weeks in culture on modified cranberry medium (Debnath and McRae, 2001a, 2001b); *n* = 72.

Treatment	Callus formation (%)	Bud regeneration (%)	Buds (no./explant)	Shoots (no./explant)
Zeatin concn (μM)				
4.6	68 b[z]	54 b	5.2 c	4.1 c
9.1	83 a	76 a	8.7 b	9.0 b
13.7	84 a	80 a	11 a	9.7 a
Explant type				
Leaf	81 a	72 a	8.6 a	7.9 a
Node	75 b	69 a	7.8 b	7.3 b
Clone				
'VAME 031C'	85 a	78 a	9.7 a	8.7 a
'VADE 004A'	73 b	63 c	7.3 b	6.2 c
'VAOF 032D'	77 b	69 b	7.5 b	7.9 b
Significant effects[y]	C, E, Z	C, Z	C × E, C × Z, C, E, Z	C, E, Z

[z]Mean separation within columns and factors by Duncan's multiple range test, $P \leq 0.05$, whereby means associated with different letters signify significant differences ($n = 72$).
[y]Significant effects ($P \leq 0.05$).

The clones in this study, which belong to three different species, differed in their shoot regeneration and development potential (Table 1). This result is reported in other adventitious shoot regeneration studies on *Vaccinium* species including lingonberry (Debnath and McRae, 2002) and lowbush blueberry (Debnath, 2009). Because cells within the same plant can have different endogenous levels of PGRs and additional variation in receptor affinity or cellular sensitivity to PGRs (Minocha, 1987), in vitro response will vary with clone. More studies on diverse clones, particularly within each species, are required to further characterize genotypic variation of huckleberry and bilberry responses to in vitro conditions. In this study, clone 'VAME 031C' was the best followed by clones 'VAOF 032D' and 'VADE 004A' for adventitious shoot regeneration (Tables 1 and 2).

Expt. 2. Leaf explants produced more but shorter shoots per explant than those produced on stem segments by all three clones (Fig. 2). Across all treatments, ≈17 shoots were produced on each leaf compared with ≈15 on a stem segment. Shoot elongation was better on 2.3 than 4.6 μM zeatin although more shoots were produced on 4.6 μM zeatin (Fig. 2). Among the three clones, 'VAME 031C' was the best for shoot elongation with maximum number of shoots and leaves per explant followed by clones 'VAOF 032D' and 'VADE 004A' (Fig. 2).

Table 2. Effects of zeatin (Z) concentration and explant type (E) on shoot vigor and callus size of regenerating explants from leaves and stem segments of mountain huckleberry clone 'VAME 031C', cascade huckleberry clone 'VADE 004A', and oval-leaf bilberry clone 'VAOF 032D', collected after 10 weeks in culture on modified cranberry medium (Debnath and McRae, 2001a, 2001b); *n* = 72.

Clone	Zeatin concn (μM)	Shoot vigor [mean ± SD (1–8 scale)][z]		Callus size [mean ± SD (0–8 scale)][y]	
		Leaf	Stem	Leaf	Stem
'VAME 031C'	4.6	4.5 ± 0.5	4.5 ± 0.5	5.7 ± 1.0	4.5 ± 0.9
'VADE 004A'	4.6	3.3 ± 0.6	4.5 ± 1.3	3.3 ± 0.8	3.4 ± 1.0
'VAOF 032D'	4.6	4.3 ± 0.6	4.2 ± 0.8	4.3 ± 0.8	4.0 ± 1.3
'VAME 031C'	9.1	6.8 ± 0.8	6.8 ± 0.8	6.3 ± 0.6	5.5 ± 0.9
'VADE 004A'	9.1	4.8 ± 0.8	5.7 ± 0.6	5.5 ± 0.5	5.2 ± 1.0
'VAOF 032D'	9.1	5.8 ± 0.8	6.0 ± 0.9	5.5 ± 0.5	5.3 ± 0.3
'VAME 031C'	13.7	5.5 ± 0.5	5.8 ± 0.3	6.3 ± 0.6	6.0 ± 0.5
'VADE 004A'	13.7	2.7 ± 0.3	4.2 ± 0.8	5.8 ± 0.3	5.7 ± 0.3
'VAOF 032D'	13.7	4.2 ± 0.3	4.2 ± 0.3	6.0 ± 1.0	5.5 ± 0.9
Significant effects[x]		*Z*			
Significant contrasts (*Z*)		4.6 vs. 13.7 ($P = 0.0013$), 9.1 vs. 13.7 ($P = 0.0040$)			

[z]Assessed visually: strongly hyperhydric, necrotic, and/or malformed shoots (1); fully normal and healthy shoots (8).
[y]No callus formation (0) and least (1) to greatest (8) growth per explant.
[x]Significant effects ($P \leq 0.05$).

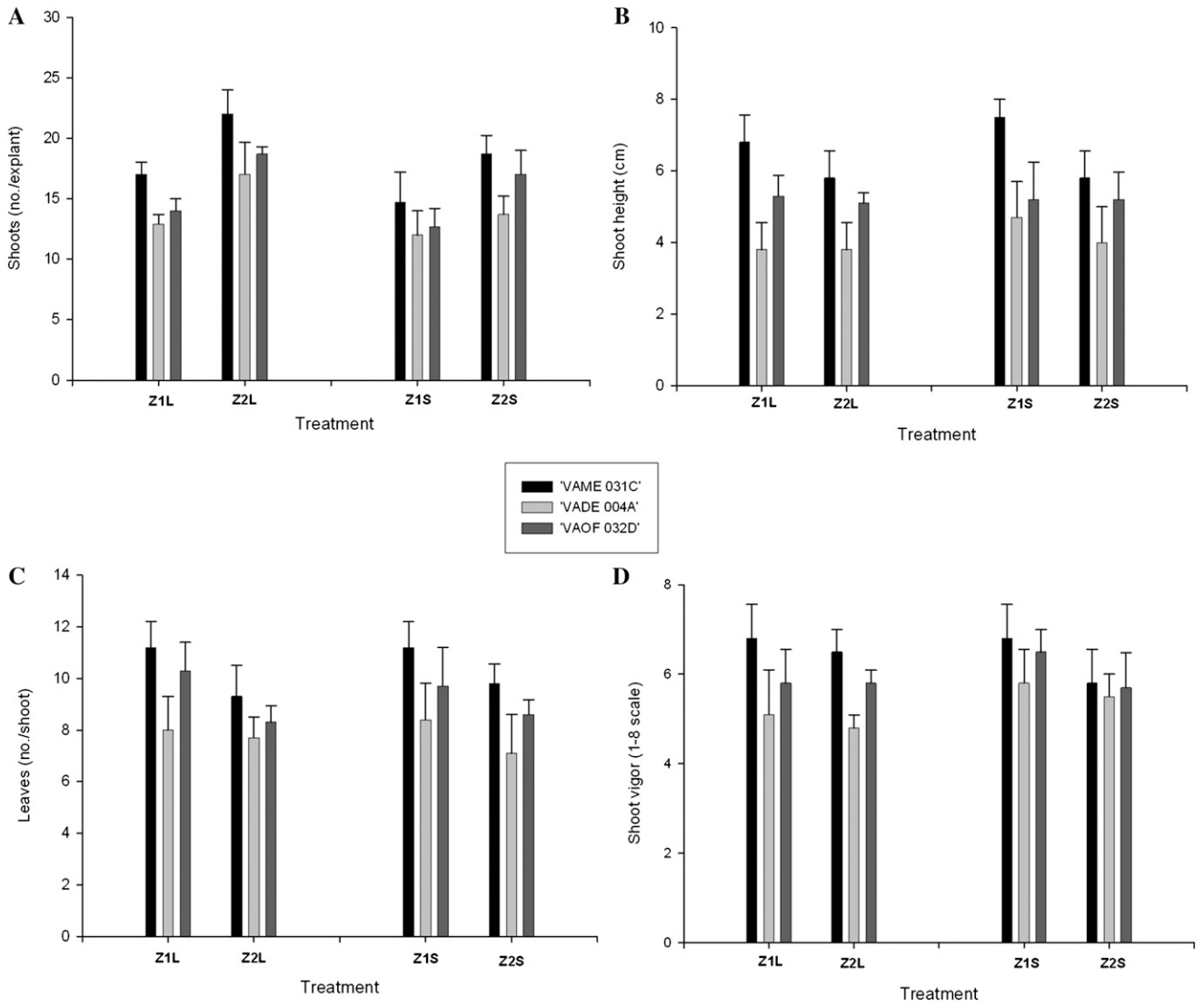

Fig. 2. Effects of zeatin concentrations on (A) mean number of shoots per explant, (B) shoot height, (C) number of leaves per shoot, and (D) shoot vigor scored a scale from 1 to 8, with the poorest shoot being 1 and 8 the best of (L) leaf and (S) stem explant-derived mountain huckleberry clone 'VAME 031C', cascade huckleberry clone 'VADE 004A', and oval-leaf bilberry clone 'VAOF 032D' shoots 10 weeks after transferring 10-week-old shoots [developed on a basal medium (BM) with 9.1 μM zeatin] on a BM with 2.3 μM zeatin (Z1) and 4.6 μM zeatin (Z2). Error bars indicate SD; 1 cm = 0.3937 inch.

EXPT. 3. All cuttings placed on the culture media responded by internal swelling and developed callus around the cut surface at the basal end of the explants at 0 to 9.1 μM zeatin between 10 and 12 d of culture. Usually, callus was creamy-white to pale brown in color, nodular and compact, and sometimes contained patches of red pigments. Basal callusing was particularly severe at the higher concentrations (data not shown). Basal callus frequently formed on shoot cultures with strong apical dominance (Preece et al., 1991). Basal callusing has been attributed to the action of accumulated auxin at the basal ends (Marks and Simpson, 1994), which initiates cell proliferation, especially in the presence of cytokinins (Tao and Verbelen, 1996).

The number of shoots formed increased with increasing dose of zeatin, whereas shoot height, leaf number per shoot, and shoot vigor diminished for all three clones when zeatin concentrations increased (Fig. 3). A concentration of 2.3 μM zeatin, with its best effects on shoot height, leaf number per shoot, and shoot vigor, was sufficient to induce shoot proliferation although the number of shoots formed per explant was less than that of explant on 4.6 μM. Across all treatments, an average of six shoots were found per explant when 2.3 μM zeatin was used.

In this study, clones affected shoot proliferation of adventitious shoots. This result is reported for shoot proliferation of other *Vaccinium* species including lingonberry (Debnath and McRae, 2001b; Serres et al., 1994) and cranberry (Debnath and McRae, 2001a; Marcotrigiano and McGlew, 1991; Smagula and Harker, 1997). Although shoot number per explant was highest for clone 'VAOF 032D', the tallest shoots with maximum leaf formation per shoot were produced by clone 'VAME 031C' followed by clone 'VAOF 032D' (Fig. 3).

Explants on cytokinin-free medium produced one unbranched shoot

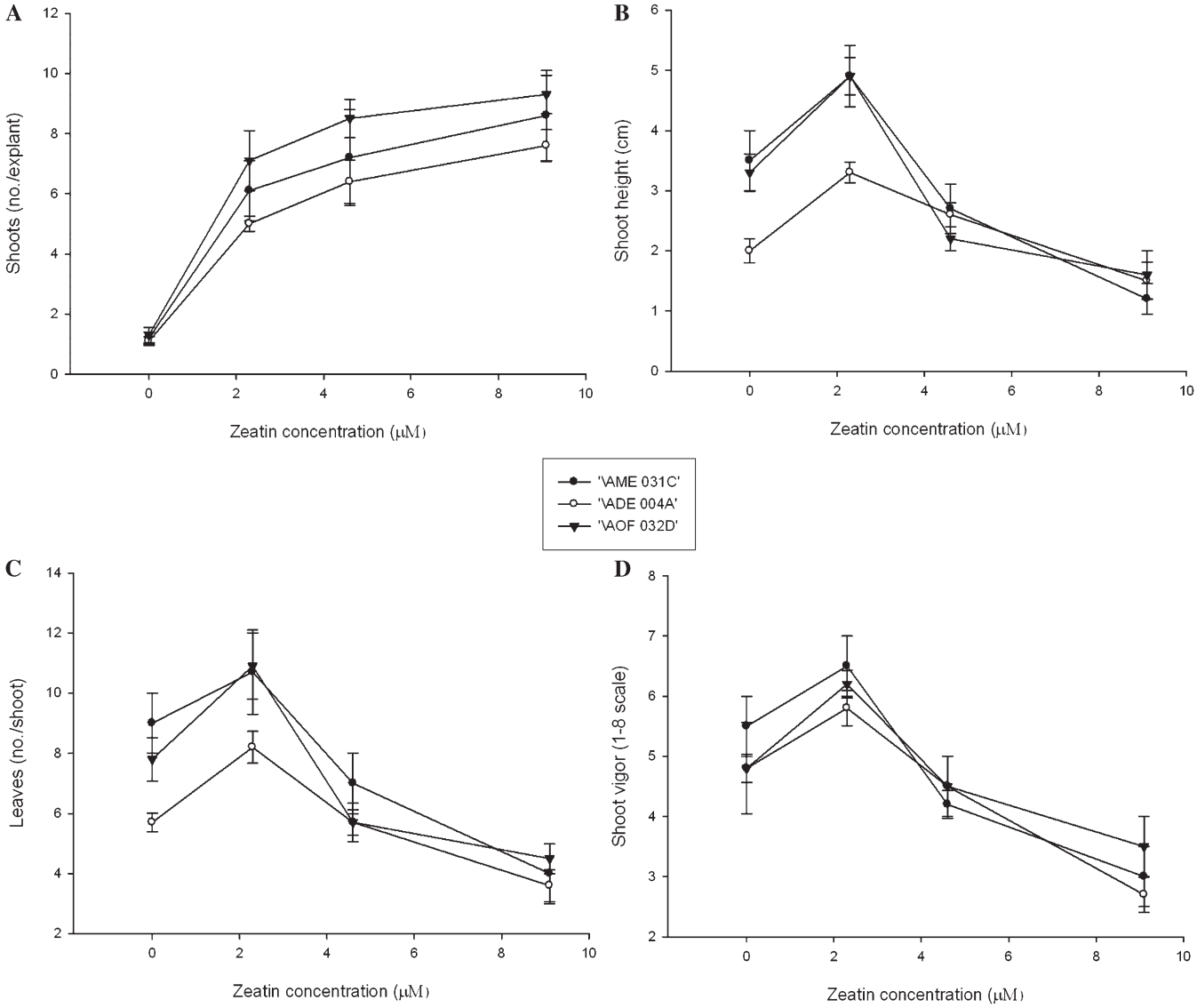

Fig. 3. Effects of zeatin concentrations on (A) mean number of shoots per explant, (B) shoot height, (C) number of leaves per shoot, and (D) shoot vigor scored a scale from 1 to 8, with the poorest shoot being 1 and 8 the best of nodal explant-derived mountain huckleberry clone 'VAME 031C', cascade huckleberry clone 'VADE 004A', and oval-leaf bilberry clone 'VAOF 032D' shoots proliferated 10 weeks after transferring on a basal medium supplemented with varying concentrations of zeatin. Error bars indicate SD; 1 cm = 0.3937 inch.

each, indicating strong apical dominance. Persistence of strong apical dominance is a major constraint in the development of efficient in vitro procedures for clonal propagation of some plant species (George and Sherrington, 1984). Axillary branching by nodal explants occurred only when zeatin was applied exogenously in the present study. Zeatin might release apical dominance, that, in turn, accelerates shoot proliferations which was observed in this study. The apical dominance associated with zeatin treatment has also been reported for lowbush blueberry (Debnath, 2009).

In this experiment, shoots arising from node-associated callus in the medium at the explant base were termed "short shoots" (basal shoots <1-cm long) and were excluded as axillary shoots because distinguishing whether "short shoots" were of axillary or adventitious origin was difficult. Nevertheless, "short-shoot" numbers appeared to increase with increasing zeatin concentration for three clones (data not shown). One objective of this study was to establish clonal material of specific clones. Axillary shoot material, which is easier to handle, was more useful than short shoots for subsequent rooting and plantlet establishment. Because adventitious shoots may have an increased frequency of somaclonal variation (Huetteman and Preece, 1993) and the clonal fidelity of "short shoots" is more questionable than that of axillary shoots, zeatin concentrations of 9.1 μM or more should be avoided if nodal explants of these berries are to be cultured for clonal propagation. However, if the objective is to generate variability in a crop, a short callus step before adventitious shoot induction may prove effective.

Rooting and acclimatization. In vitro proliferated shoots for three clones rooted easily within 4 to 6 weeks (Fig. 1D). Roots at or below

the medium surface were typically plump or white, whereas aerial roots were thin, brown, and wiry for all three clones corroborating previous report (Barney et al., 2007). Rooted microcuttings grew well in the greenhouse, and plants acclimatized readily to the greenhouse (Fig. 1E) with survival rates of 75% to 90% for all three clones. The percentages of rooting and survival were similar among the clones.

Conclusion

This study presents, for the first time, protocols for adventitious shoot regeneration and for shoot proliferation and plantlet formation from adventitious shoots of cascade huckleberry, mountain huckleberry, and oval-leaf bilberry using a low concentration nutrient medium containing zeatin. Multiple shoot regeneration can be obtained using leaf and stem explants from in vitro-grown shoots by incorporating 9.1 to 13.7 μM zeatin in the semisolid culture medium for 10 weeks, and including a dark treatment for 14 d before incubating the explants under a 16-h photoperiod, followed by culturing in the same medium but with 2.3 to 4.6 μM zeatin for another 10 weeks and rooted in the same medium but without PGR. The present protocol excluded auxin from the culture medium, which lowers the cost and reduces the probability of somaclonal variation among the proliferated plants. The new procedure is expected to be simpler and requires less time to produce plants. The results indicated in vitro protocols for regeneration of plants from these *Vaccinium* species that will be used in a *Vaccinium* berry improvement program including micropropagation, in vitro selection, and genetic manipulation.

Literature cited

Barney, D.L., O.M. Lopez, and E. King. 2007. Micropropagation of cascade huckleberry, mountain huckleberry, and oval-leaf bilberry using woody plant medium and Murashige and Skoog medium formulations. HortTechnology 17:279–284.

Billings, S.G., C.K. Chin, and G. Jelenkovic. 1988. Regeneration of blueberry plantlets from leaf segments. HortScience 23:763–766.

Callow, P., K. Haghighi, M. Giroux, and J. Hancock. 1989. In vitro shoot regeneration on leaf tissue from micropropagated highbush blueberry. HortScience 24:373–375.

Cao, X. and F.A. Hammerschlag. 2000. Improved shoot organogenesis from leaf explants of highbush blueberry. HortScience 35:945–947.

Compton, E.C. 1994. Statistical methods suitable for the analysis of plant tissue culture data. Plant Cell Tissue Org. Cult. 37:217–242.

Debnath, S.C. 2000. Combined application of classical and biotechnological techniques in the development of small fruits. Can. J. Plant Sci. 80:233 (abstr.).

Debnath, S.C. 2003. Improved shoot organogenesis from hypocotyls segments of lingonberry (*Vaccinium vitis-idaea* L.). In Vitro Cell. Dev. Biol. Plant 39:490–495.

Debnath, S.C. 2005. A two-step procedure for adventitious shoot regeneration from in-vitro-derived lingonberry leaves: Shoot induction with TDZ and shoot elongation using zeatin. HortScience 40:189–192.

Debnath, S.C. 2009. A two-step procedure for adventitious shoot regeneration on excised leaves of lowbush blueberry. In Vitro Cell. Dev. Biol. Plant 45:122–128.

Debnath, S.C. 2011. Adventitious shoot regeneration in a bioreactor system and EST PCR based clonal fidelity in lowbush blueberry (*Vaccinium angustifolium* Ait.). Sci. Hort. 128:124–130.

Debnath, S.C. and K.B. McRae. 2001a. An efficient in vitro shoot propagation of cranberry (*Vaccinium macrocarpon* Ait.) by axillary bud proliferation. In Vitro Cell. Dev. Biol. Plant 37:243–249.

Debnath, S.C. and K.B. McRae. 2001b. In vitro culture of lingonberry (*Vaccinium vitis-idaea* L.): The influence of cytokinins and media types on propagation. Small Fruits Rev. 1:3–19.

Debnath, S.C. and K.B. McRae. 2002. An efficient adventitious shoot regeneration system on excised leaves of micropropagated lingonberry (*Vaccinium vitis-idaea* L.). J. Hort. Sci. Biotechnol. 77:744–752.

Fellman, J., J. Thorngate, and D. Barney. 1998. Identification of western huckleberry flavor components. Proc. Northwest Ctr. Small Fruit Res. 7:62–63.

George, E.F. 1993. Plant propagation by tissue culture. Part 1. The technology. Exegetics, Edington, United Kingdom.

George, E.F. and P.D. Sherrington. 1984. Plant propagation by tissue culture. Exegetics, Reading, United Kingdom.

Graham, J., K. Greig, and R.J. McNicol. 1996. Transformation of blueberry without antibiotic selection. Ann. Appl. Biol. 128: 557–564.

Huetteman, C.A. and J.E. Preece. 1993. Thidiazuron: A potent cytokinin for woody plant tissue culture. Plant Cell Tissue Org. Cult. 33:105–119.

Lee, J., C.E. Finn, and R.E. Wrolstad. 2004. Anthocyanin pigment and total phenolic content of three *Vaccinium* species native to the Pacific Northwest of North America. HortScience 39:959–964.

Letham, D.S. and L.M.S. Palni. 1983. The biosynthesis and metabolism of cytokinins. Annu. Rev. Plant Physiol. 34:163–197.

Liu, C., P. Callow, L.J. Rowland, J.F. Hancock, and G. Song. 2010. Adventitious shoot regeneration from leaf explants of southern highbush blueberry cultivars. Plant Cell Tissue Org. Cult. 103:137–144.

Marcotrigiano, M. and S.P. McGlew. 1991. A two-stage micropropagation system for cranberries. J. Amer. Soc. Hort. Sci. 116:911–916.

Marcotrigiano, M., S.P. McGlew, G. Hackett, and B. Chawla. 1996. Shoot regeneration from tissue-cultured leaves of the American cranberry (*Vaccinium macrocarpon*). Plant Cell Tissue Org. Cult. 44:195–199.

Marks, T.R. and S.E. Simpson. 1994. Factors affecting shoot development in apically dominant *Acer* cultivars in vitro. J. Hort. Sci. 69:543–551.

Meiners, J., M. Schwab, and I. Szankowski. 2007. Efficient in vitro regeneration systems for *Vaccinium* species. Plant Cell Tissue Org. Cult. 89:169–176.

Miller, C.O. 1961. Kinetin and related compounds in plant growth. Annu. Rev. Plant Physiol. 12:395–408.

Minocha, S.C. 1987. Plant growth regulators and morphogenesis in cell and tissue culture of forest trees, p. 50–66. In: J.M. Bonga and D.J. Durzan (eds.). Cell and tissue culture in forestry, Vol. I. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.

Preece, J.E., C.A. Huetteman, W.C. Ashby, and P.L. Roth. 1991. Micro- and cutting propagation of silver maple. I. Results with adult and juvenile propagules. J. Amer. Soc. Hort. Sci. 116:142–148.

Qu, L., J. Polashock, and N. Vorsa. 2000. A highly efficient in vitro cranberry regeneration system using leaf explants. HortScience 35:948–952.

Rowland, L.J. and E.I. Ogden. 1992. Use of a cytokinin conjugate for efficient shoot

regeneration from leaf sections of highbush blueberry. HortScience 27:1127–1129.

Serres, R.A., S. Pan, B.H. McCown, and E.I. Stang. 1994. Micropropagation of several lingonberry cultivars. Fruit Var. J. 48:7–14.

Shibli, R.A. and M.A.L. Smith. 1996. Direct shoot regeneration from *Vaccinium pahalae* (ohelo) and *V. myrtillus* (bilberry) leaf explants. HortScience 31: 1225–1228.

Skoog, F. and C.O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissue cultured in vitro. Symp. Soc. Exp. Biol. 11:118–30.

Smagula, J.M. and J. Harker. 1997. Cranberry micropropagation using a lowbush blueberry medium. Acta Hort. 446:343–347.

Tao, W. and J. Verbelen. 1996. Switching on and off cell division in cultured mesophyll protoplasts of tobacco. Plant Sci. 116:107–115.

Taruscio, T.G., D.L. Barney, and J. Exon. 2004. Content and profile of flavonoid and phenolic acid compounds in conjunction with the antioxidant capacity for a variety of Northwest *Vaccinium* berries. J. Agr. Food Chem. 52:3169–3176.

Vander Kloet, S.P. 1988. The genus *Vaccinium* in North America. Agr. Can. Publ. 1828.