Propagation Methods Affect Fruit Morphology and Antioxidant Properties but Maintain Clonal Fidelity in Lowbush Blueberry

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Abstract. The berry morphology (size and weight), phytochemical content (polyphenols, flavonoids, anthocyanins, and proanthocyanidins), and antioxidant activity of lowbush blueberry (*Vaccinium angustifolium* Ait.) wild clone QB 9C and cultivar Fundy, propagated by tissue culture (TC) and softwood cutting (SC), were studied over two growing seasons to evaluate the effect of propagation methods on fruit yield and the content of antioxidant metabolites. Number of flower clusters, number of berries and berry weight per plant, diameter and weight of individual berry were higher in SC plants than those of TC plants. Significant interaction between genotypes and propagation methods were observed for total phenolic and flavonoid content of fruits. Berries from TC plants contained more polyphenols and flavonoids than those of SC plants. Twenty microsatellite markers were used to assess the clonal fidelity of TC regenerants and SC plants. The identical monomorphic amplification profiles within the TC plants of each genotype confirmed the clonal fidelity of micropropagated blueberry plants. These results indicate that propagation methods affected the morphology and antioxidant metabolites but maintained trueness-to-type genetic makeup in blueberry.

Blueberries belong to the genus *Vaccinium* L., which has over 400 species, including several closely related small fruit species. Although many species of blueberries are native to North America, several are commercially cultivated in a number of countries in Europe and in South America, Asia, Australia, and New Zealand (Strik, 2005; Strik and Yarborough, 2005). They contain higher dietary phytochemicals like polyphenols, anthocyanins, and proanthocyanidins compared with other fruits and vegetables (Koca and Karadeniz, 2009; Prior et al., 1998; Wang et al., 1996). These phytochemicals are natural antioxidants, free radical, and metal scavengers (Wang et al., 1996) that help in mitigating oxygen-free radical damage in the human body. The consumption of dried wild blueberries increases ex vivo serum antioxidant

status (Kay and Holub, 2002) and their extract is purported to protect against carcinogenicity, and cardiovascular and neurodegenerative changes associated with aging (Ames et al., 1993; Neto, 2007).

Lowbush blueberries (*V. angustifolium* Ait.) are native to Newfoundland and Labrador (Vander Kloet, 1988) and their commercial production is localized in eastern Canada and the northeastern United States (Kalt et al., 2001). Although this species shares the common “blueberry” name with other types, its growth habit and production systems are different. Lowbush blueberry plants are slow-growing woody shrubs that grow generally in forest understory. They can form large colonies of genetically identical plants termed as clones, connected via rhizomes (underground shoots) (Vander Kloet, 1988). In commercial production, the bushes of these blueberries are managed with naturally growing native populations of plants rather than genetically improved ones. Those are propagated generally by stem cutting using softwood or rhizome or by seeds. Plants propagated from stem cuttings are difficult to establish in the field because of their slow growth and restricted spread habit compared with seedlings, and they exhibit extreme precocity of flowering (Jamieson and Nickerson, 2003). The advantages of seed propagation over SC include a lower cost of plants and better establishment

in the field with more rhizome formation than stem cuttings; but their yields are 50% lower than their respective mother clone (Aalders et al., 1979) due to lack of uniformity in fruit size and quality (Jamieson and Nickerson, 2003). This limitation may be overcome by micropropagation or in vitro culture, which combines benefits of faster spreading growth traits of seedling and the uniform productivity characteristics of stem cutting. Micropropagation ensures a rapid and continuous supply of mass production of healthy and pathogen-free planting materials of a desired plant genotype all of which are true-to-type. Taller stems with increased number of branches and leaves are reported in blueberry plants produced through TC (Debnath, 2007; Goyali et al., 2013). Although the basic objective of micropropagation is the production of trueness-to-type clonal propagules, plants derived from in vitro techniques may exhibit TC stress-induced genetic and/or epigenetic changes responsible for somaclonal variations that are often heritable and consequently unwanted for commercial plantation (Larkin and Scowcroft, 1981).

It is important to maintain and confirm clonal fidelity or genetic integrity of TC plants. Therefore, in vitro derived plants need to be carefully screened to avoid undesired and unintended clonal variability. Several strategies have been used to assess the clonal fidelity of TC derived plants of several fruit species but with limited success (Debnath, 2008). Phenotypic identification based on morphological traits is influenced by environmental factors and requires extensive observations until maturity. Although karyotype analysis cannot reveal alteration in specific genes or in small DNA segments; isozyme electrophoresis can detect only the genetic changes of DNA segments that are coded for proteins and those are prone to environmental and developmental variations. Deoxyribonucleic acid-based molecular techniques are more attractive to detect clonal fidelity and sequence variation between source plants and regenerants, since they are more informative and are not developmentally or environmentally influenced. Based on the specific requirements, different types of molecular marker systems detecting variability in different regions of DNA are available to assess genetic integrity in micropropagated plants in *Vaccinium* spp. These include restricted fragment length polymorphism, amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), microsatellite or simple sequence repeat (SSR) and expressed sequence tag-polymerase chain reaction (EST-PCR). RAPD and EST-PCR markers have been used to control the genetic fidelity of TC-raised *Vaccinium* spp. plants (Debnath, 2011; Gajdošová et al., 2006). SSR markers of genomic loci and expressed sequence tags containing SSR (EST-SSRs) (Boches et al., 2005) are considered the markers of choice in ascertaining the clonal fidelity because they are PCR based, codominant, multiallelic, highly prone to mutation, hyper-variable and randomly dispersed throughout the plant genome (Qureshi et al., 2004). The main limitation of microsatellite

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markers is that they have to be isolated de novo for new species. Although microsatellite markers have been used to assess genetic stability of clonal materials of different plant species (Agrawal et al., 2014; Lopes et al., 2006), to the best of our knowledge, this is the first report on the assessment of genetic fidelity of micropropagated *Vaccinium* species using EST-SSR markers.

The growth habit especially the rhizome and branch number, fruit size and berry yield propagated by TC have been reported (Goyali et al., 2013; Jamieson and Nickerson, 2003; Morrison et al., 2000); however, little is known about antioxidant metabolite content or antioxidant activity of fruits of micropropagated blueberries. Although enhanced levels of health promoting phytochemicals like polyphenols, flavonoids, and anthocyanins as well as antioxidant activities in micropropagated lingonberry and strawberry fruits have been identified (Debnath, 2009; Foley and Debnath, 2007; Vyas et al., 2013), lower concentration of phenolic compounds has been found in leaves of established TC-derived *V. angustifolium* (Goyali et al., 2013) and *Fragaria*

vesca L. species (Yildirim and Turker, 2014). Therefore, it is necessary to evaluate the phytochemical content of micropropagated blueberry to explore and possibly promote in vitro culture in blueberry production. This research was carried out to investigate the effect of propagation methods on fruit morphology, to estimate the antioxidant metabolites of fruits obtained from SC and TC propagation methods and to evaluate the clonal fidelity of in vitro regenerated plants using EST-SSR markers. The main goal was to assess the possibility of using in vitro technique as a sustainable propagation method to increase production and fruit quality.

Materials and Methods

Plant materials. Two lowbush genotypes were used for this study: one was wild clone QB 9C collected from Longue-Rive in Quebec, which was well established through TC at the Atlantic Cool Climate Crop Research Center (ACCCRC), Agriculture and Agri-Food Canada (AAFC), St. John's, Newfoundland and Labrador, Canada; another

was cultivar Fundy developed at the Atlantic Food and Horticulture Research Center, AAFC, Kentville, Nova Scotia, Canada, which had consistently good yield in eastern Canada (Hall et al., 1988). Plants were propagated by conventional SC and TC from the source plants maintained in a greenhouse at the ACCCRC, St. John's. For rooting of SC plants, individual shoot tips (4–5 cm long) of both genotypes were planted in a cell (5.9 cm diameter × 15.1 cm depth) in a 45-cell plastic tray with peat: perlite [2:1 (v/v)] and placed in a humidity chamber equipped with a vaporizer (Controlled Environments Ltd., Winnipeg, MB, Canada) at 22 ± 2 °C, 95% relative humidity and 16-h photoperiod provided by fluorescent lights (55 µmol·m⁻²·s⁻¹). For micropropagation, explants obtained from nodal segments of young, actively growing shoots were cultured on the modified cranberry medium (Debnath and McRae, 2001) supplemented with sucrose (25 g·L⁻¹), agar (3.5 g·L⁻¹), and Gelrite (1.25 g·L⁻¹) (Sigma Chemical Co., St. Louis, MO) and the growth hormone zeatin (5 µmol) following a technique developed by Debnath (2007). Elongated shoots obtained

Table 1. List of microsatellite markers employed to analyze the clonal fidelity of micropropagated blueberry genotypes, their sequences, annealing temperature (T_A), and number and size of amplified allele(s). Locus name prefixes reflect origin of GenBank source sequence.

Primer name	Forward (F) and reverse (R) primer sequences (5'–3')	T_A (°C)	QB 9C		Fundy	
			No. of amplified alleles	Size(s) of amplified alleles (bp)	No. of amplified alleles	Size(s) of amplified alleles (bp)
CA23F	F: GAGAGGGTTTCGAGGAGGAG R: GTTTAGAAACGGGACTGTGAGACG	62	1	152	1	152
CA 112F	F: TCCACCCACTTCACAGTTCA	56	1	110	1	110
	R: GTTTATTGGGAGGGAATTGGAAAC	62	1	127	1	127
CA169F	F: TAGTGGAGGGTTTGTCTTGG R: GTTTATCGAAGCGAAGGTCAAAGA	61	2	209, 1751	2	209, 1751
CA236F	F: GTTAAAGCTTTTAGATGAGTTGATGG R: GTTTAACCAGTCCCAGACCCAAAT	60	2	180, 1084	2	180, 1084
CA421F	F: TCAAAATTCAAAGCTCAAAATCAA R: GTTTAAGGATGATCCCGAAGCTCT	61	1	302	1	318
CA483F	F: GTCTTCCTCAGGTTCGGTTG R: GAACGGCTCCGAAGACAG	60	1	331	1	331
CA787F	F: TCCTCGTTCTCTCCCTCTCA R: GTTTCGCTGAAGTTGGAGTCCTT	62	1	266	1	266
CA855F	F: CGCGTGAAAAACGACCTAAT R: GTTTACTCGATCCCTCCACCTG	61	2	216, 336	1	216
NA398	F: TCCTTGCTCCAGTCCTATGC R: GTTTCCTTCCACTCCAAGATGC	58	3	212, 295, 415	2	182, 295
NA741	F: GCCGTCGCCTAGTTGTTG R: GTTTGATTTTGGGGGTTAAGTTTGC	62	3	130, 213, 252	2	213, 252
NA800	F: CAATCCATTCCAAGCATGTG R: GTTTCCTTAGACCAGTGCCACTTA	61	2	121, 176	2	121, 176
NA961	F: TCAGACATGATTGGGGAGGT R: GTTTGGAATAATAGAGGCGGTGGA	56	3	200, 376, 664	1	182
NA1040	F: GCAACTCCAGACTTTCTCC R: GTTTAGTCAGCAGGGTGCACAA	62	1	216	1	216
VCC_I2	F: AGGCGTTTTTGAGGCTAACA R: TAAAAGTTCGGCTCGTTTGC	59	2	153, 270	3	121, 182, 225
VCC_I8	F: TTCAGCATTCATCCATCCA R: GTTTCTCTTCTCCAATCTTTTCCA	62	2	227, 507	2	227, 507
VCC_J1	F: CTCATGGGTTCCTATAGACAA R: TGCAGTGAGGCAAAAGATTG	58	1	194	1	194
VCC_J3	F: TGATTACATTGCCAGGGTCA R: TGGAAACAACCGGGTTACAT	61	2	216, 392	2	216, 238
VCC_J9	F: GCGAAGAACTTCCGTCAAAA R: GTGAGGGCACAAAGCTCTC	53	2	171, 242	2	191, 242
VCC_K4	F: CCTCCACCCCACTTTCATTA R: GCACACAGGTCCAGTTTTTG	61	1	154	2	139, 174
VCC_S10	F: ATTTGGTGTGAAACCCCTGA R: GTTTGCGGCTATATCCGTGTTTGT					

CA = cold acclimated EST library; NA = non-acclimated EST library; VCC = enriched genomic library.

Table 2. Mean values of the main factors across all the treatments for combined effect of genotypes, propagation methods, and growing seasons on flowering and fruiting characteristics of two lowbush blueberry genotypes assessed in two growing seasons.

Parameters	No. of flower clusters per plant	No. of flowers per cluster	No. of fruits per plant	Berry diam (mm)	Wt of individual berry (g)	Berry wt per plant (g)
Genotypes (G)						
QB 9C	30 a ^z	3.4 a	16 a	7.5 b	0.17 b	3.0 a
Fundy	16 b	3.4 a	7.5 b	11 a	0.48 a	3.3 a
PM						
SC	30 a	4.1 a	19 a	10 a	0.33 a	4.9 a
TC	16 b	2.7 b	4.5 b	9.0 b	0.31 a	1.5 b
GS						
2012	26 a	3.5 a	17 a	9.1 b	0.31 a	4.7 a
2013	20 b	3.3 a	6.2 b	9.8 a	0.33 a	1.6 b
	G, PM, GS, G × PM, G × GS, G × PM × GS	PM, G × PM, PM × GS, G × PM × GS	G, PM, GS, G × PM, PM × GS, G × GS, G × PM × GS	G, PM, GS, G × PM	G	PM, GS, G × PM, PM × GS, G × PM × GS
Significant effects						

^zMeans within columns and parameters followed by different letters indicate significant differences at $P \leq 0.05$.

PM = propagation methods; SC = stem cutting; TC = tissue culture; GS = growing seasons.

from TC were rooted following the same technique used for SC propagation. Rooted SCs and TC-regenerated plantlets were transplanted into plastic pots ($10.5 \times 10.5 \times 12.5$ cm³) with the same medium used for rooting in 2007. Since then, plants were grown in a greenhouse at the ACCCRC under natural light conditions (maximum $90 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), 20 ± 2 °C and 85% relative humidity, and they were pollinated naturally. Fertilization ($100 \text{ mg}\cdot\text{L}^{-1}$ N from Peters Azalea neutral fertilizer 20N–8P–20K; Plant Products Co., Brampton, ON, Canada) and irrigation were applied when it was necessary. Dormancy requirements were met by maintaining the plants at, or below, 6 °C for at least 12 weeks from January to March in each year.

Flowering and fruiting characteristics. Data on the number of flower clusters per plant and number of flowers per cluster were collected when $\approx 50\%$ flowers bloomed. Data on the number of fruits per plant, diameter and weight of individual berry, and berry weight per plant were collected from four plants per treatment. Fully ripened (well-developed blue color) fruits were picked from those four plants per treatment, weighed and stored at -80 °C until the antioxidant phytochemicals were extracted. All experiments were replicated four times.

Extraction of polyphenolics. The berries from each plant were homogenized with 80% aqueous acetone containing 0.2% formic acid at a ratio of 1:4 (w/v) of fruit and solvent using FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Irvine, CA). The homogenate was shaken at 4 °C for 30 min and then centrifuged at $15,000 g$, 4 °C for 15 min. The extract was separated and the residual tissue re-extracted following the same steps and conditions. The two supernatants were combined and further diluted to determine total phenolic, flavonoid, anthocyanin and proanthocyanidin (condensed tannin) content, and total antioxidant activity.

Determination of total phenolics. Total phenolic content was determined by the photometric method with Folin-Ciocalteu reagent following Singleton and Rossi (1965) with slight modifications (Goyali et al., 2013). The samples and standard (gallic acid) were analyzed with a spectrophotometer at the wave-

length of 725 nm. Eighty percent aqueous acetone was used as a control. The phenolic content was measured as gallic acid equivalents (GAE) in $\text{mg}\cdot\text{g}^{-1}$ of fresh fruit. The test was performed three times on each sample and the mean was calculated.

Determination of total flavonoids. Total flavonoid content was assessed using colorimetric method developed by Zhishen et al. (1999) with few modifications (Goyali et al., 2013). Absorbance was measured at 510 nm using an ultraviolet spectrophotometer (Libra S32 PC; Biochrom Ltd., Cambridge, UK) and the flavonoid content was expressed as catechin equivalents (CE) in $\text{mg}\cdot\text{g}^{-1}$ of fresh fruit.

Determination of anthocyanin content. Quantification of monomeric anthocyanin content of the blueberry extract was done using the pH-differential method (Chen et al., 2012) with few modifications. Two aliquots of each sample extract and the standard (cyanidin-3-glucoside) were diluted, one with the 0.025 M potassium chloride buffer (pH 1.0) and another with 0.4 M sodium acetate buffer (pH 4.5). The absorbance of each mixture was measured at 510 nm and 700 nm using an ultraviolet spectrophotometer after incubating in the dark at room temperature for 20 min. Total anthocyanin content was calculated using the following formula:

$$\text{Anthocyanin content (mg}\cdot\text{L}^{-1}) = (A \times \text{MW} \times \text{DF} \times 1000) / (\epsilon \times l),$$

where A (absorbance) = $(A_{\lambda 510} - A_{\lambda 700})$ pH 1.0– $(A_{\lambda 510} - A_{\lambda 700})$ pH 4.5; MW (molecular weight) = $449.2 \text{ g}\cdot\text{mol}^{-1}$ for cyanidin-3-glucoside; DF = dilution factor; ϵ = $26,900 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ for cyanidin-3-glucoside; and l = path length in cm. The total anthocyanin pigment concentration was expressed as cyanidin-3-glucoside equivalents (C3GE) in $\text{mg}\cdot\text{g}^{-1}$ of fresh fruit.

Determination of proanthocyanidin content. Proanthocyanidin content of fruit extract was measured by colorimetric assay developed by Price et al. (1978) with few modifications. A 0.5% (w/v) solution of vanillin-HCl reagent (0.5% vanillin in 4% concentrated HCl in methanol; 2.5 mL) was added with 0.5 mL of diluted extracts and standard (catechin)

solutions, mixed thoroughly and incubated at 30 °C in the dark for 20 min. The absorbance was recorded at 500 nm against the corresponding blanks. Proanthocyanidin content of fruits was expressed as CE in $\text{mg}\cdot\text{g}^{-1}$ of fresh fruit.

Determination of antioxidant activity. The radical scavenging activity of lowbush blueberry extract was carried out using a stabilized artificial free radical, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) following a published method (Hatano et al., 1988) with slight modifications. A freshly prepared DPPH solution ($60 \mu\text{M}$) in absolute methanol was mixed with an aliquot of fruit extract or standard solution (gallic acid) and left to stand for 45 min in the dark, and the absorbance of the resulting solution was recorded at 517 nm. The DPPH scavenging activity of fruit extract was measured as a percentage of inhibition of DPPH radicals, which is the concentration of the test compound required to give a decrease of the absorbance from that of the blank solution (mixture of 80% aqueous acetone and DPPH solution). The gallic acid standard curve was used to express the results as GAE in $\text{mg}\cdot\text{g}^{-1}$ of fresh fruit.

Genetic fidelity assessment using SSR markers. Sample size for this experiment was 13 (2 SCs and 11 TCs) randomly selected plants of both genotypes. Genomic DNA was isolated from 80–90 mg of actively growing young leaves. The leaves were shock-frozen in liquid nitrogen immediately after collection and stored at -80 °C until DNA isolation. DNA was isolated using DNeasy Plant Mini Kits (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions with few modifications. The leaf tissue was homogenized with $450 \mu\text{L}$ buffer AP1 using FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals). RNase A ($4 \mu\text{L}$) was added to the mixture and incubated for 15 min at 65 °C. The rest of the steps were the same as those were described in the manufacturer instructions. The concentration and purity of DNA were estimated spectrophotometrically. The DNA with an A_{260}/A_{280} absorbance ratio of 1.7–1.9 was diluted (concentration: $12.5 \text{ ng}\cdot\mu\text{L}^{-1}$) to use as template DNA for PCR reactions.

A total of 13 EST-SSR (prefix CA or NA) and seven genomic SSR (prefix VCC) primer pairs (Table 1) synthesized by Integrated

Fig. 1. Effect of propagation method on number of flower clusters per plant (A, B), number of flowers per cluster (C, D), number of berries per plant (E, F), diameter of berry (G, H), weight of individual berry (I, J), and berry weight per plant (K, L) of blueberry genotypes ('QB 9C' and 'Fundy') obtained by softwood cutting (light gray bars) and tissue culture (dark gray bars) measured in 2012 and 2013. Different letters (a, b) within columns indicate significant differences at $P \leq 0.05$ by least significant difference test. Bars indicate mean $\pm$ SE ($n = 4$).

DNA Technologies (Coralville, IA) were used to assess the genetic fidelity of TC blueberry plants. PCR was carried out in an

optimized amplification reaction mixture (25 μ L) containing 1 $\times$ PCR buffer (1.5 mM $MgCl_2$, pH 8.7; Qiagen), 200 μ M of each

deoxynucleotide triphosphate, 0.2 μ M of each of the 20 forward and reverse primers, 0.63 unit of *Taq* DNA polymerase (Qiagen) and 25 ng of template DNA. DNA was amplified in a Mastercycler ep Gradient S (Eppendorf AG, 22331 Hamburg, Germany) programmed for an initial 10 min denaturation step "hot start" at 94 $^{\circ}$ C, followed by 40 cycles of 40 s of denaturation step at 92 $^{\circ}$ C, 70 s annealing step at the appropriate annealing temperature (Table 1) and 2 min extension step at 72 $^{\circ}$ C, followed by a final extension step at 72 $^{\circ}$ C for 10 min before holding the sample at 4 $^{\circ}$ C. Annealing temperature of 20 SSR primers was standardized using temperature gradient PCR. Amplified products, along with a low range 100 base pair (bp) DNA ladder (Norgen Bioteck Corp., Thorold, ON, Canada) were separated by electrophoresis using 1.6% agarose 3:1 high-resolution blend (HRB) (Amersco, Solon, OH) gel precasted with 2 $\times$ tris-borate-ethylenediaminetetraacetic acid buffer and 1 $\times$ GelRed nucleic acid stain (Biotium Inc., Hayward, CA) solution and digitally photographed under ultraviolet light using the InGenius 3 gel documentation system (Syngene, Cambridge, UK). Scoring and recording of DNA banding patterns were carried out using image analysis software (GeneTools; Syngene).

Statistical analysis. Data for the flower and fruit characteristics were subjected to statistical analysis using the SAS statistical software package (Release 8.2; SAS Institute Inc., Cary, NC). All data are presented as the means $\pm$ SE of four replications. Significant differences between the factors (genotypes, propagation methods, and growing seasons) were calculated by analysis of variance (ANOVA). Statistical *F* tests were evaluated at $P \leq 0.05$ for the number and size of flower clusters, diameter and weight of individual berries, berry number and weight per plant, and total polyphenol, flavonoid, anthocyanin, proanthocyanidin content of fruits and for their antioxidant activities. The treatment means were compared by the least significant difference using the *F* test. The relationships among antioxidant activity and flower and fruit characteristics and phytochemical content of fruits were determined using Pearson's correlation coefficients calculated with Microsoft Excel 2010.

Results and Discussion

Flowering and fruiting characteristics. Analysis of variance for combined effects of two (genotypes $\times$ propagation methods) and three factors (genotypes $\times$ propagation methods $\times$ growing seasons) were significant ($P \leq 0.05$) for all the characteristics except berry diameter and individual berry weight (Table 2). Jamieson and Nickerson (2003) reported significant genotypes $\times$ propagation methods interaction for berry weight and yield in lowbush blueberry. In this study, the number and size of flower clusters, berry diameter, number and weight of berries per plant were affected by propagation methods. The SC plants had more and bigger flower clusters, greater numbers of fruits, and higher

Table 3. Mean values of the main effects across all the treatments for combined effect of genotype, propagation method, and growing season on total phenolic, flavonoid, anthocyanin and proanthocyanidin content, and antioxidant activity of two lowbush blueberry genotypes measured in two growing seasons.

Parameters	Total phenolic content (mg GAE/g F.F.)	Total flavonoid content (mg CE/g F.F.)	Monomeric anthocyanin content (mg C3GE/g F.F.)	Proanthocyanidin content (mg CE/g F.F.)	DPPH scavenging activity (mg GAE/g F.F.)
Genotypes (G)					
QB 9C	8.4 a ^z	3.2 a	2.5 a	2.1 a	2.9 a
Fundy	6.5 b	2.6 b	2.2 b	1.9 b	2.5 b
PM					
Stem cutting	6.9 b	2.7 b	2.3 a	2.0 a	2.6 a
TC	8.1 a	3.1 a	2.3 a	2.0 a	2.7 a
GS					
2012	6.5 b	2.8 b	2.6 a	1.8 b	2.5 b
2013	8.4 a	3.0 a	2.0 b	2.2 a	2.9 a
Significant effects	G, PM, GS, G × PM	G, PM, GS, G × PM, G × PM × GS	G, GS, G × GS, PM × GS	G, GS, PM × GS	G, GS

^zMeans within columns and parameters followed by different letters indicate significant differences at $P \leq 0.05$.

DPPH = 2,2-diphenyl-1-picrylhydrazyl; GAE = gallic acid equivalents; CE = catechin equivalents; C3GE = cyanidine-3-glucoside equivalents; F.F. = fresh fruit; PM = propagation methods; TC = tissue culture; GS = growing seasons.

berry weight per plant than those of TC plants. Across propagation methods and growing seasons, the number of flower clusters and fruits per plant were higher in 'QB 9C' than in 'Fundy', whereas 'Fundy' had bigger fruits than 'QB 9C'. Flowering and fruiting performances were better in the growing season of 2012 than in 2013 (Table 2).

The performance of individual genotypes of the wild clone QB 9C and the cultivar Fundy propagated by SC and TC in two different growing seasons is presented in Figure 1. All the characters of 'QB 9C' studied except individual berry weight were affected more by the propagation methods than those of 'Fundy'. Number of flower clusters per plant, number of flowers per cluster, number and weight of berries per plant, and berry diameter were higher in 'QB 9C' SC plants compared with TC plants. Whereas in 'Fundy', none of the above characters except berry weight per plant was changed significantly in both growing seasons. Berry weight per plant was less in 'Fundy' TC plants than in SC counterparts. Lower numbers of flower buds were reported in in vitro regenerated lowbush blueberry (Goyali et al., 2013; Jamieson and Nickerson, 2003; Morrison et al., 2000) and lingonberry plants (Foley and Debnath, 2007) compared with plants derived from stem cutting. El-Shiekh et al. (1996) and Read et al. (1989) reported higher numbers of flower buds and berry yields in TC plants of half-high "North-blue" blueberry. Whereas, no significant variation in the number of flower buds per branch was found by Grout et al. (1986) who evaluated micropropagated and stem cutting plants of the same blueberry cultivar. In general, micropropagation enhances growth and metabolism in vegetative parts of plants. The residual action of growth hormones, especially cytokinin used to multiply and elongate the shoots during micropropagation, might have stimulated the vegetative growth of in vitro regenerated blueberry plants (Debnath et al., 2012; Grout et al., 1986; Morrison et al., 2000) and of other *Vaccinium* species (Debnath, 2005; Debnath and McRae, 2005). In the 'QB 9C' wild clone, in vitro derived plants had mostly single flowers rather than a standard size

cluster (4–5 flowers per cluster), which might affect pollination and ultimately fruit set. Since lowbush blueberries are genetically heterozygous and self-incompatible in nature, natural pollinators play an important role in successful and adequate pollination and on fruit setting (Hicks, 2011). The cluster may be more attractive to natural pollinators.

Polyphenol, flavonoid, anthocyanin, and proanthocyanidin content. The 'QB 9C' fruits had higher total polyphenols, flavonoids, anthocyanins, and proanthocyanidins compared with those of 'Fundy' (Table 3). A significant interaction between genotypes and propagation methods (G × PM) was observed for total phenolic and flavonoid content of fruit extracts. This demonstrated that propagation methods could impact the capacity of blueberry plants to synthesize polyphenols and flavonoids in berries and certain genotypes varied in their capacity under different conditions of propagation methods. The wild clone QB 9C was influenced more by micropropagation for all the phytochemical characters than the cultivar Fundy (Fig. 2). Total phenolic, flavonoid, anthocyanin, and proanthocyanidin content in 'QB 9C' fruits were higher in TC plants than in SC counterparts at least in one growing season. None of the phytochemical contents of 'Fundy' fruits studied was changed significantly when propagated by either SC or micropropagation. The higher quantity of polyphenols and flavonoids in TC plants of *Vaccinium* spp. agree with previous studies. Foley and Debnath (2007) and Vyas et al. (2013) and reported higher phenolic and anthocyanin content, respectively in the fruits of micropropagated lingonberry (*Vaccinium vitis-idaea* L. ssp. *vitis-idaea* Britton) cultivars than in berries of conventionally propagated plants. They reported higher antioxidant metabolites in leaves compared with fruits, and in leaves of SC plants compared with their TC counterparts. Goyali et al. (2013) reported that the leaves of SC blueberry plants had higher content of polyphenols and proanthocyanidins than the leaves of TC plants.

The increased levels of phenolics, flavonoids, and anthocyanins of micropropagated 'QB 9C' fruits can be attributed to the fruit

size. The fruits of 'QB 9C' TC plants were smaller in size (Fig. 1G–H) with a higher proportion of berry peel, which was enriched by anthocyanin pigments (Gao and Mazza, 1994; Kalt and Dufour, 1997). The type, amount and localization of these phytochemicals, especially flavonoids, anthocyanins, and proanthocyanidins, were influenced by genetic differences. For example, different types of epidermal and sub-epidermal layers of peel containing variable amounts of pigments (Allan-Wojtas et al., 2001) and by other factors like fruit size, developmental stages of the fruit, and the specific weather conditions of growing seasons (Connor et al., 2002; Howard et al., 2003; Kalt and Dufour, 1997; Prior et al., 1998; Wang et al., 1996). However, Kalt et al. (2001) reported that there was no relationship between fruit size and anthocyanin content in blueberry species, but the method of extraction had an influence on the composition of fruit extracts. They observed that lowbush blueberries contained higher anthocyanins, total phenolics, and antioxidant capacity than those of highbush blueberries.

The content of total polyphenolics, flavonoids, and proanthocyanidins in fruits were significantly higher in the growing season of 2013 than in 2012. However, anthocyanin content was higher in 2012 compared with in 2013. Significant main effects for growing seasons and genotypes × growing seasons for anthocyanin content showed that environmental conditions can affect anthocyanin synthesis, which is also genotype specific.

Total antioxidant activity. Total antioxidant activity of fruit extract from blueberry genotypes 'QB 9C' and 'Fundy' measured using DPPH radical scavenging method were influenced by two main factors: genotypes and growing seasons (Table 3). This agrees with previous studies on blueberry leaves (Goyali et al., 2013); they also reported a significant combined effect of propagation methods and growing seasons for total antioxidant activity. This was absent for DPPH radical scavenging activity of fruits of the same blueberry species under the same propagation conditions. In other words, some other external factors or metabolites, which have not been taken in consideration, might have affected the antioxidant capacity of

Fig. 2. Effect of propagation method on the content of phenolics (A, B), flavonoids (C, D), anthocyanins (E, F) and proanthocyanidins (G, H), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (I, J) in fruits of blueberry wild clone QB 9C and cultivar Fundy, obtained by softwood cutting (SC) (light gray bars) and tissue culture (TC) (dark gray bars) measured in 2012 and 2013. GAE = gallic acid equivalents; CE = catechin equivalents; C3GE = cyanidine-3-glucoside equivalents. Different letters (a, b) within columns indicate significant differences at $P \leq 0.05$ by least significant difference test. Bars indicate mean $\pm$ SE ($n = 4$).

fruits. On the other hand, the flavonoid biosynthesis pathway is involved in synthesizing a number of antioxidant metabolites like flavonols, anthocyanins, and proanthocyanidins.

Most of the genes and enzymes involved in this pathway in plants are typically controlled by the tissue-specific expression of transcription factors (Lepiniec et al., 2006),

which might be the reason for the differences in antioxidant activity between fruits and leaves.

Higher DPPH radical scavenging activity of fruits in 2013 compared with 2012 (Table 3) was attributed to the content of total phenolics, flavonoids, and proanthocyanidins, which were also higher in 2013, and was confirmed by the correlation studies. Significant positive correlations were observed between DPPH radical scavenging activity and other bioactive metabolites: phenolic, flavonoid, anthocyanin, and proanthocyanidin content (Table 4). Significant positive correlation between antioxidant activity and total phenolic content was reported in blueberry leaves (Goyali et al., 2013) and fruits (Connor et al., 2002; Giovanelli and Buratti, 2009; Koca and Karadeniz, 2009; Krupa and Tomala, 2007). Diameter of berry and individual berry weight were negatively correlated with DPPH radical scavenging activity of fruits. Total phenolic, flavonoid, anthocyanin, and proanthocyanidin content also had negative correlations with berry diameter and individual berry weight. Data in this study revealed that the antioxidant activity of fruits increased with the increase in quantity of the secondary metabolites and with the decrease in berry size. Similar results were reported in rabbiteye blueberry (Yuan et al., 2011). Fruit size that was affected by propagation method plays an important role in antioxidant metabolic pathways of blueberry plants. The general phenomenon in micropropagation of plant is the reversion from mature stage of cell to juvenile characteristics. In the previous reports on the same genotypes under similar propagation conditions (Goyali et al., 2013), it was found that TC plant showed higher vegetative growth (i.e., higher number of rhizomes and branches) than SC plants. However, an inverse trend in berry number, size, and yield was observed in greenhouse grown TC blueberry plants. Fruit production requires substantial metabolic inputs in the form of nutrients and energy. In micropropagation, plants direct significant amounts of nutrients and energy into the production of new axillary shoots and rhizomes, and hence are limited by an obligation to vegetative production that might restrict the diameter, number, and yield of fruit per plant (Foley and Debnath, 2007). SC plants conserve energy by producing fewer or no rhizome and only one or two primary shoots and thereby allowed higher production of flowers and fruits (Debnath, 2006). Moreover, the stressful environment of TC system may be responsible for the induction of abiotic stress during plant regeneration (Miguel and Marum, 2011) that influences the defense system of the metabolic pathways. The DPPH radical scavenging capacity of blueberry fruits was attributed to the high concentration of anthocyanins, since they are relatively low in antioxidant vitamins and minerals (Bushway et al., 1983). Antioxidant activity is the result of a combination of different compounds and environmental factors having synergistic and antagonistic effects (Hassimotto et al., 2005) and its effectiveness is influenced by the

Table 4. Pearson's correlation coefficients for number of flower clusters per plant, number of flowers per cluster, number of fruits per plant, fruit diameter (mm), individual berry weight (g), berry weight per plant (g), total phenolic (mg GAE/g F.F.), flavonoid (mg CE/g F.F.), anthocyanin (mg C3GE/g F.F.) and proanthocyanidin (mg CE/g F.F.) content, and DRSA (mg GAE/g F.F.) in blueberries.

Characters	NFC	NFP	BD	WIB	BWP	TPC	TFC	MAC	PAC	DRSA
Number of flower clusters per plant	0.87	0.98*	-0.35	-0.41	0.76	-0.12	-0.08	0.01	0.11	0.06
NFC	—	0.90	0.18	0.12	0.95*	-0.62	-0.57	-0.47	-0.39	-0.46
NFP	—	—	-0.25	-0.30	0.87	-0.24	-0.23	-0.03	0.05	-0.03
BD	—	—	—	0.99*	0.24	-0.88	-0.86	-0.83	-0.93	-0.96*
WIB	—	—	—	—	0.19	-0.85	-0.84	-0.86	-0.92	-0.95*
BWP	—	—	—	—	—	-0.66	-0.68	-0.38	-0.34	-0.47
TPC	—	—	—	—	—	—	0.98*	0.90	0.91	0.97*
TFC	—	—	—	—	—	—	—	0.80	0.82	0.93
MAC	—	—	—	—	—	—	—	—	0.99*	0.96*
PAC	—	—	—	—	—	—	—	—	—	0.97*

*Significant at $P \leq 0.05$.

GAE = gallic acid equivalents; CE = catechin equivalents; DRSA = DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity; F.F. = fresh fruit; NFC = number of flowers per cluster; NFP = number of fruits per plant; BD = berry diameter; WIB = weight of individual berry; BWP = berry weight per plant; TPC = total phenolic content; TFC = total flavonoid content; MAC = monomeric anthocyanin content; PAC = proanthocyanidin content.

Fig. 3. Simple sequence repeat profiles of blueberry plants obtained from 'QB 9C' softwood cutting (SC: lanes 1–2) and tissue culture (TC: lanes 3–13), and 'Fundy' SC (lanes 14–15) and TC (lanes 16–26) generated using primer VCC_K4. The 100-bp DNA marker ladder is shown in lane L. Size of marker fragments (bp) is indicated at the left.

chemical composition of antioxidants and their structure, especially the number and position of hydroxyl and methoxyl groups on the phenolic ring of the molecule (Seeram and Nair, 2002).

Lowbush blueberries (*V. angustifolium*) are tetraploid and the proposed origin of this species is allotetraploid of two diploid species either *Vaccinium boreale* × *Vaccinium palladium* or *V. boreale* × *Vaccinium myrtilloides* (Vander Kloet, 1977). 'Fundy' and 'QB 9C' are tetraploid but genetically different as shown in the SSR marker system analysis (Table 1; Fig. 3). Since both clones originated from open pollinated genotypes and are different at the genetic level, they responded differently to the propagation methods for fruit morphology and metabolite content.

Genetic fidelity assessment of TC plants. All of the 20 primers produced clear, reproducible, good quality bands in two SCs and eleven TC regenerated plants of 'QB 9C' clone and 'Fundy' cultivar. Each primer generated a set of amplification products ranging from 110 to 1751 bp in size (Table 1). Since the micropropagation technique is the industry standard method of propagation,

large number of sample size should be tested for clonal fidelity. However, previous studies have been done with as few as 10 micropropagated berry plants to confirm trueness-to-type using different molecular markers (Debnath, 2011). Out of 20 primer pairs, seven detected one band, six detected two bands, four detected three bands, two detected four bands, and one detected five bands (Table 1). From the 20 primer pairs considered for genetic analysis, a total of 44 SSR bands were scored, resulting in an average about two bands per primer pair. Representative amplified band patterns produced by primer VCC_K4 in SC and TC plants of 'QB 9C' and 'Fundy' are illustrated in Figure 3 and three fragments were considered for analysis. The entire fragment patterns of TC plants appeared as bands in 'QB 9C' and 'Fundy' and were found to be monomorphic (i.e., no variation based on fragments size was observed in SC and TC plants of either genotype). Amplicons of different size and/or number were observed in 'QB 9C' from 'Fundy' genotypes for nine primer pairs (CA483F, NA398, NA741, NA800, NA1040, VCC_I8, VCC_J9, VCC_K4, and VCC_S10).

Trueness-to-type regenerated plants and their genetic uniformity are essential for the application of micropropagation in *Vaccinium* spp. Since somaclonal variation may escalate under certain stress conditions in vitro, especially high levels of growth hormones (Larkin and Scowcroft, 1981), the genetic fidelity of regenerated clones for commercial propagation need to be ascertained. Microsatellite markers selected for this study were found to be a reliable technique to differentiate the types, cultivars, clones of *Vaccinium* spp. (Boches et al., 2006; Česonienė et al., 2013; Gajdošová et al., 2006), and used to develop phylogenetic relationships among lowbush blueberry clones collected from different provinces of Canada (Debnath, 2014). In this study, polymorphic banding pattern at nine out of 20 SSR loci detected for 'QB 9C' compared with 'Fundy' confirmed the diversification between the wild clone and named cultivar studied, and confirmed the utility of using EST-SSR markers to control the clonal fidelity of micropropagated blueberry plants. Twenty SSR loci were used in this study to increase the polymorphism and thus reduce the probability of false assessment regarding

clonal fidelity of the TC regenerated plants. The absence of any variation in the banding pattern at 20 microsatellite loci clearly indicated the genetic integrity among the blueberry TC plants of both 'QB 9C' and 'Fundy' genotypes. Since no artificial medium or growth hormone was used, and none of the TC-induced stresses applied during SC propagation, it was assumed that SC plants had identical genetic structures to source plants. The genetic pattern of TC plants was the same as their SC counterparts, confirming that the micropropagated progenies were of the same genotype and maintained the same genetic features as the SC plants. Molecular analyses of micropropagated plants using other DNA markers have revealed genetic fidelity in *Vaccinium* spp. Using SSR markers, Agrawal et al. (2014) found identical genetic profiles among banana plants regenerated from sucker meristem through in vitro propagation. Micropropagation using node, axillary buds, or meristems as explants is generally considered to be a low-risk method for genetic variation (Pierik, 1991) because those organized tissues perform dedifferentiation or redifferentiation of cells or tissues with few genomic aberrations and consequently maintain clonal fidelity of in vitro raised plants. The plants used in this study were regenerated from nodal explants. Therefore, TC plants might have shown clonal fidelity. Another reason for showing genetic integrity may be due to a very small fraction of the genome in most cases being involved in molecular marker analysis. The RAPD, EST-PCR, and ISSR markers were used to testify the genetic fidelity of berry crops and reported complete similarity among TC derived blueberry, lingonberry, and strawberry progenies (Debnath, 2009; Debnath, 2011; Gajdošová et al., 2006). However, a few reports suggested that SSRs were not powerful tools neither for detection of clones of a specific cultivar nor for the identification of somaclonal variation among somaclones (Imazio et al., 2002; Schellenbaum et al., 2008). Imazio et al. (2002) could not distinguish 24 accessions of a grape cultivar Traminer when they used nine microsatellite markers. In contrast, they could separate 16 out of 24 examined 'Traminer' clones using AFLP and methyl-sensitive amplified length polymorphism techniques. Moreover, this technique could not detect the point mutation in the length of a microsatellite product. To ensure whether the clonal variation resulting from genetic modification or epigenetic changes during the micropropagation processes has occurred or not, more sensitive techniques or a combination of two or more techniques should be used to detect it.

In conclusion, this study showed that lowbush blueberries had a substantial amounts of antioxidants especially polyphenols and flavonoids with high antioxidant capacity, which were influenced by micropropagation, whereas conventional stem cutting stimulated various horticultural characters: number and size of flower clusters, fruit number, size, and yield per plant. The genotype response of blueberry

plants was not consistent for those antioxidant metabolites and fruit characters. Although propagation methods and growing seasons appeared to have a clear effects on flowering and fruiting characteristics and phenolic biosynthesis, in vitro regenerated plants maintained genetic integrity. Morphological differences between TC and SC plants were probably because of the synergetic effect of genetic and epigenetic modifications, as well as the artificial stress of TC. This study established the feasibility of using propagation directly to change some important horticultural characters without undergoing any genetic change.

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