CHARACTERISTICS OF *IN VITRO*- AND *EX VITRO*- PROPAGATED BLUEBERRY PLANTS AT MORPHOLOGICAL, CHEMICAL AND MOLECULAR LEVELS

By

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This work is dedicated to my beloved wife

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ABSTRACT

The lowbush blueberry (*Vaccinium angustifolium* Ait.), a commercially important fruit crop in Canada and USA, is one of the richest sources of antioxidant metabolites which have highly potential to reduce the incidence of several degenerative diseases. The aim of this study is to investigate the effect of propagation on the morphological, chemical and molecular characteristics of blueberries. The study evaluated the genetic and epigenetic variation in micropropagated plants. A lowbush wild clone 'QB9C' and the cultivar 'Fundy' were studied after being propagated by conventional softwood cutting (SC), and by tissue culture (TC) using nodal explants. The antioxidant metabolites in leaves and fruits of both genotypes were investigated in different maturity stages. The TC-regenerated plants were grown more vigorously and produced higher number of stems, branches, and larger leaves compared to SC plants. However, TC plants of both genotypes produced less flowers and fruits compared with SC counterparts. Micropropagation influenced the synthesis of phenolic and flavonoid compounds, and their antioxidant activities in blueberry which were genotype specific. 'QB9C' plants were highly influenced by micropropagation for their phytochemical content and antioxidant capacity. Leaves contained substantially higher levels of polyphenolics, flavonoids and proanthocyanidins than berries. The total soluble phenolic and flavonoid content and reducing power of ferric ion were boosted in fruits of the micropropagated plants, whereas the levels of these metabolites and total antioxidant activity were decreased in the leaves of TC plants. Red leaves had higher phenolic and flavonoid content and antioxidant potential than the green leaves, and green fruits had higher levels of bioactive phytochemicals than semi-ripe and full ripe berries. In

contrary, anthocyanin content increased with the advancement of fruit maturity. Molecular marker analysis with expressed sequence tag (EST)-simple sequence repeat (SSR) and EST-polymerase chain reaction (PCR) makers detected the identical monomorphic amplification profiles within the TC plants of each genotypes which confirmed their genetic integrity. Methylation sensitivity amplification polymorphism (MSAP) analysis demonstrated that TC plants of both genotypes had higher DNA methylation compared to SC plants. Discrete methylation polymorphism was observed among the tissue culture regenerated plants. These results indicate that although *in vitro* derived plants maintained trueness-to-type genetic makeup, tissue culture induces DNA methylation alterations and the possibility of involvement of these DNA fragments in the dynamic processes regulating plant growth and development under prevailing growth conditions.

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2,4-D	2,4-dichlorophenoxyacetic acid
4CL	4-coumarate:coenzyme A ligase
AFLP	amplified fragment length polymorphism
ANM	Anderson's rhododendron medium
ANOVA	analysis of variance
ANR	anthocyanidin reductase
ANS	anthocyanidin synthase
CA	cold acclimated
CHI	chalcone isomerase
CHS	chalcone synthase
CoA	coenzyme A
COMT	caffeic acid *O*-methyltransferase
DHFR	dihydroflavonol reductase
DPPH	1,1-diphenyl-2- picrylhydrazyl
EST-PCR	expressed sequence tag - polymerase chain reaction
EST-SSR	expressed sequence tag - simple sequence repeat
F3H	flavanone-3β-hydroxylase
F.F.	fresh fruit
F.L.	fresh leaf
F.W.	fresh weight
HPLC	high performance liquid chromatography

ISSR	inter simple sequence repeat
MBM-C	modified basal medium for cranberry
MSAP	methylation sensitive amplification polymorphism
MSM	Murashige and Skoog medium
PAL	phenylalanine ammonia lyase
PCR	polymerase chain reaction
RAPD	random amplified polymorphic DNA
RDMs	random DNA markers
RFLP	restriction fragment length polymorphism
SBSM	sodium bisulfite modification
SC	softwood cutting
SSAP	sequence specific amplification polymorphism
SSR	simple sequence repeat
STMS	sequence tagged microsatellite site
TBE	tris borate EDTA
TC	tissue culture
TDZ	thidiazuron
UFGT	UDP-glucose flavonol-3-*O*-glucosyl transferase
WPM	woody plant medium
ZBM	Zimmerman and Broome medium

CHAPTER 1

Introduction and Overview

1.1 Introduction

Blueberry belongs to the genus *Vaccinium* L., family Ericaceae. Many species of blueberries are native to North America. Several of them especially highbush (*Vaccinium corymbosum* L.), lowbush (*V. angustifolium* Ait.) and rabbiteye (*V. ashe* Reade) are commercially cultivated in many countries in Europe and in South America, Asia, Australia and New Zealand (Strik, 2005; Strik & Yarborough, 2005). Blueberry has significant contribution in the Canadian dynamic and diverse berry industries. According to Statistics Canada (2017) report, the blueberry production was highest among the small fruit crops in Canada in last three years, followed by cranberry, grape and strawberry (**Table 1.1**). In 2016, the farmgate value of blueberry production was 262 million dollars which was accounted for one-quarter of total national fruit value.

Lowbush or wild blueberry is indigenous to northeastern regions of North America (Vander Kloet, 1988). Although domestication of the lowbush blueberry has been started through selection and breeding technique many years ago (Hall, 1983), extensive planting has not taken place in this continent because of the slow establishment and lack of rhizome production from stem cuttings which are generally used as propagation materials (Yarborough, 2012). Wild blueberries are naturally grown in acidic, low-fertile glacial soils in cold, harsh winter areas in boreal forests, bogs and barrens in the Atlantic Provinces and

Quebec in Canada which is the largest lowbush blueberry producer in the world, followed by Maine in USA (Wetzel et al., 2006). Although, the lowbush blueberry in these countries is mostly harvested from fields composed of managed native existing plants, planting area of lowbush blueberry cultivars has rapidly increased in several provinces in China (Li & Hong, 2009). High yielding cultivars are planted in backyard gardens and to fill up bare areas in commercial gardens.

Table 1.1 Canadian small fruit production and farmgate value in 2016[z]

Common name	Production (million kg)			Farmgate value (million $)
	2014	2015	2016	2016
Blueberries	182	192	241	262
Cranberries	176	161	175	135
Strawberries	20	25	27	99
Cherries	20	18	23	61
Raspberries	13	11	12	32
Grapes	89	88	107	151

[z] Statistics Canada (2017)

Blueberries especially wild blueberries have become more popular around the world as a 'Superfruit' due to their elevated levels of bioactive phytochemicals (phenolic acids, anthocyanins, proanthocyanidins and flavonols), antioxidant potential and nutritional value (Prior et al., 1998; Piljac-Zegarac et al., 2009; Skrovankova et al., 2015). It has been proved

in numerous *in vitro* and *in vivo* research that those bioactive compounds in fruits and leaves can reduce risk for development of different degenerative diseases such as cancer, cardiovascular disorders, obesity, diabetes and urinary tract infection (Faria et al., 2010; Yuan et al., 2011; Kang et al., 2015). Due to awareness of these health-promoting properties, the market demand and growing area of blueberry have been dramatically increased during the last two decades. In China, the average annual increase rate of cultivated area of lowbush blueberry is 161% from year 2005 to 2010 (Li & Hong, 2009). The production area of lowbush blueberries in Canada is increased from 155 thousand acres in 2012 to 171 thousand acres in 2016 (Statistics Canada, 2017). To cope with the high demand for planting materials essential to cover the production area, conventional propagation with stem or rhizome cuttings is not sufficient because of its poor spreading habit and lower planting materials from a source plant. Therefore, tissue culture has been attractive to researchers in wild blueberry improvement programs for its incredible potential to produce a large number of starting materials from a selected genotype in a short time all year round. *In vitro* propagation influences spreading capacity with high number of rhizome and branches (Jamieson & Nickerson, 2003). Tissue culture (TC) plants of some *Vaccinium* species demonstrates higher yield compared to conventionally propagated plants (Read et al., 1988; El-Shiekh et al., 1996; Gustavsson & Stanys, 2000). However, micropropagation does not result in higher yields in some lowbush blueberry clones.

1.2 Overview

1.2.1 Taxonomy of blueberry

The genus *Vaccinium* consists of 30 sections and about 150 to 450 species (the number varies by authority) which are widely spread in the Himalayas in India, New Guinea, North America and the Andean region (Vander Kloet, 1988; Luby et al., 1991; Galletta & Ballington, 1996; Hancock et al., 2008). The commercially important species are found in the section *Cyanococcus, Oxycoccus, Vitis-Idea* and *Myrtillus* (Hancock et al., 2008). Blueberry has been placed under section *Cyanococcus* Gray. The recent classification of the *Cyanococcus* species includes a total of 7 diploid species (*V. boreale* Hall & Aald., *V. corymbosum* L., *V. darrowi* Camp, *V. elliottii* Chapm., *V. myrtilloides* Michx., *V. pallidum* Ait. and *V. tenellum* Ait.), 6 tetraploid species (*V. angustifolium* Ait., *V. corymbosum*, *V. hirsutum* Buckley, *V. myrsinites* Lam., *V. pallidum*, and *V. simulatum* Small), and 2 hexaploid species (*V. ashei* Reade and *V. constablaei* Gray), with *V. corymbosum* and *V. pallidum* occurring at diploid and tetraploid levels (Vander Kloet, 1988; Galletta & Ballington, 1996; Rowland & Hammerschlag, 2005). The origin of tetraploid lowbush blueberries (*V. angustifolium*) is as an autotetraploid of *V. boreale* (Camp, 1945) or an allotetraploid of either *V. boreale* × *V. palladium* or *V. boreale* × *V. myrtilloides* (Vander Kloet, 1977).

1.2.3 Blueberry types

Blueberry species are commonly grouped into five major types according to stature and

referred to as the lowbush, highbush, half-high, southern highbush and rabbiteye blueberries (**Figure 1.1**) (Debnath, 2007a). (i) Highbush blueberries (*V. corymbosum*; 2n = 4x = 48) found mainly in the east coast of North America from Florida and Quebec,

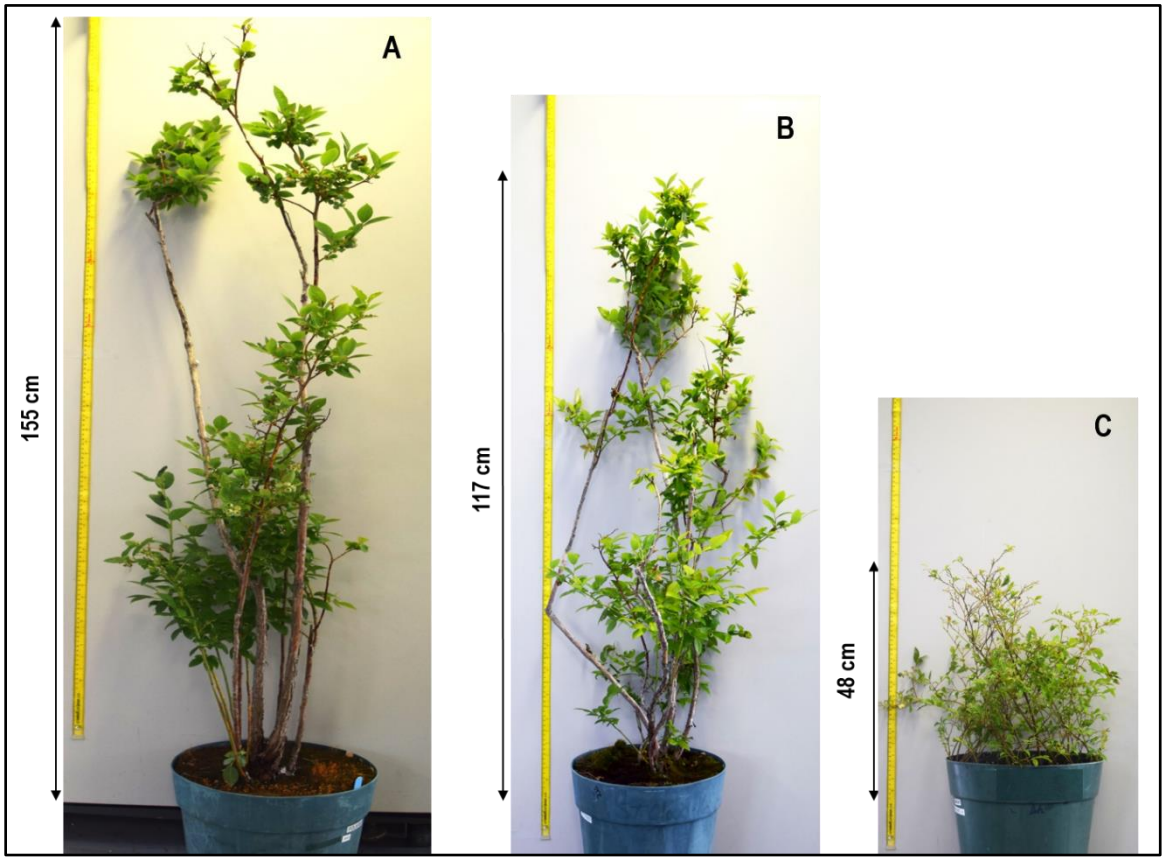

Figure 1.1 Matured plants of highbush blueberry cultivar 'Polaris' (A); half-high blueberry cultivar 'St. Cloud' (B) and a lowbush blueberry wild clone (C) grown in pot mixture (2:1 peat and perlite)

Texas and Illinois and far West British Colombia. Mature plant height of this type varies by cultivar and typically ranges from 120 cm and 330 cm with varying degrees of bushiness (Vander Kloet, 1980). Among all the types of blueberries, they produce largest fruits upto

a 25 mm in diameter. (ii) Lowbush blueberries (*V. angustifolium*; 2n = 4x = 48, *V. myrtilloides*; 2n = 2x = 24 and *V. boreale*; 2n = 2x = 24) are low-growing, variable shrubs that range in height from 5 cm to 60 cm. After establishment, they can form large colonies of genetically identical plants which are connected via subterranean rhizomes (Vander Kloet, 1988). (iii) Half-high blueberries are essentially *V. corymbosum* genetic background (2n = 4x = 48), and are developed by highbush × lowbush hybridization. They are intermediate in height between highbush and lowbush blueberries (60–125 cm (Ratnaparkhe, 2007). (iv) Southern highbush blueberries (2n = 4x = 48) are predominantly *V. corymbosum* germplasm but they have been developed from hybridization of *V. corymbosum* with one or more low-chilling species mainly *V. darrowi*, *V. angustifolium* or *V. ashei* in some cases. (v) Rabbiteye blueberries (*V. ashei*; 2n = 6x = 72) are localized in the southern states of USA especially in southern Georgia and northern Florida which can reach height upto 600 cm (Vander Kloet, 1980; Galletta & Ballington, 1996). Among the blueberry species, rabbiteye blueberries are tallest and lowbush blueberries are shortest plants.

1.2.3 Biology of blueberry

Lowbush blueberries are a diverse group of woody small perennial shrubs, mostly deciduous, which bear flower and fruit in clusters. Shoots of lowbush blueberry are erect forming dense, extensive colonies; twigs are green to glaucous, glabrous or hairy. Woody rhizomes are in average 4.5 mm in diameter and grow around 6 cm underground. Stems are smooth and vary in color from tan to red (Flinn & Pringle, 1983). Leaf blade is pale to

dark green, elliptic to narrowly elliptic, 5–20 mm × 16–40 mm in size; margins are sharply, uniformly serrated; surfaces are glabrous/smooth predominantly or hairy. Flowers are bell-shaped and usually white or pinkish-white borne in short, few-flowered terminals or axillary racemes (Mohr & Kevan, 1987). Flowers are generally self-incompatible. Fruits are globular, ovate and blue to dark blue in color with or without waxy coating (Camp, 1945). The fruit is intermediate in size between the larger highbush or rabbiteye blueberry and the European bilberry (*V. myrtillus*) or bog blueberry (*V. uliginosum*) averaging 4 - 10 mm in diameter (Mohr & Kevan, 1987; Yarborough, 2012). Cluster of berries are generally held on upright stem. The pedicel scar is medium, and the calyx end is closed.

Plants of highbush blueberries are crown-forming woody shrubs with several stems or suckers to form compact colony (Camp, 1945). Twigs are angular to terete; glabrous to densely pubescent. Leaves are ovate to narrowly elliptic; 20 - 30 mm wide and 40 - 80 mm long. Leaf blades are pubescent or glabrous with entire or sharply serrate margins. Flower is cylindrical; white, white tinged with pink or pink in color with green or glaucous calyx (Camp, 1945; Vander Kloet, 1980). Berries are blue, dull black, or even black.

Half-high blueberry plants are crown-forming shrubs with medium height. Leaves are narrow to broadly elliptic with entire or serrate margins; 30 - 60 mm long. Pubescence of leaves depends on the leaf characters of the parent type of *V. corymbosum* (Camp, 1945). Fruits are dark blue, dull or even black.

Commercial cultivars of southern highbush blueberry acquired the characteristics from both parents. Although most of the cultivars are taller than *V. darrowi*, high percentages of

dwarf plants are found in those populations. They don't require long winter chilling for flower development as do the northern highbush (Hancock et al., 2008). The flowers are white, bell-shaped. Berries color range from powder blue to medium blue and they have intense flavor. Some southern highbush cultivars are self-fertile, but the berries grow larger if two varieties are planted together.

Plants of rabbiteye blueberry are crown-forming shrubs with tall stature (Camp, 1945). Leaves usually deciduous or in some forms essentially evergreen. Berries are black to dull in color with 8 - 18 mm in diameter.

1.2.4 Health benefits of blueberry

Blueberries are rich in many essential nutritional components including carbohydrate (15.3%), protein (0.7%), fibre (1.5%), fat (0.5%) and water (85%) (Hancock et al., 2003). Ripe blueberries have 3.5% cellulose and 0.7% pectin (Rowland et al., 2011). The total sugars of ripe blueberries are more than 10% of fresh berry, and main reducing sugars are glucose and fructose (2.4%) (Retamales & Hancock, 2012). Compare with other fruits and vegetables, blueberries contain intermediate to low levels of vitamins A, C, E and minerals. They contain 22.1 mg of vitamin C in 100 g of fresh fruits. In addition to these essential nutrients, these berries contain a wide range of organic acids, non-nutritive antioxidants such as flavonoids, anthocyanins, proanthocyanidins and other polyphenolic compounds. The principle function of those antioxidants is to delay the oxidation of other molecules through inhibiting the initiation or propagation of oxidizing chain reactions by free radicals. The free radicals cause oxidative damage to different essential molecules in human body

such as lipids, proteins and nucleic acids, and are thus involved in the beginning phase of several degenerative diseases such as cancer and cardiovascular diseases. Blueberry consumption reduces the oxidative damage and thus prevents human body from those worsening diseases.

In vitro and *ex vivo* pharmaceutical research has conceded a great deal of information on the bioactivity of blueberry against multiple stages of carcinogenesis and the ability in treatment of different degenerative diseases (Bomser et al., 1996; Skrovankova et al., 2015). The anticancer properties of blueberries have been the subject of investigation since the late 1990's. Fruits or leaves of highbush, lowbush and rabbiteye, blueberries induce apoptosis in carcinogenic cells *in vitro* of various kinds of cancer such as blood (Skupień et al., 2006), breast (Adams et al., 2010; Faria et al., 2010), colon (Yi et al., 2006), liver and prostate (Matchett et al., 2005; Schmidt et al., 2006) cancer, and thus it is believed that blueberry can help preventing human body from cancer. Wild blueberry extracts reduce the occurrence of ageing related diseases (Papandreou et al., 2009; Uysal et al., 2013). The blueberry products demonstrate the ability to reduce high blood pressure, blood cholesterol and thus prevent cardiovascular and atherosclerosis risks (Sweeney et al., 2002; Norton et al., 2005; Basu et al., 2010; Wu et al., 2010). Blueberries exhibit anti-diabetic properties by protecting pancreatic β-cells from glucose-induced oxidative stress (Martineau et al., 2006; Kang et al., 2016). Recent surveys have identified Canadian lowbush blueberry as highly recommended by traditional practitioners and Cree Elders of Eeyou Istchee in Quebec for treatment of diabetic symptoms and complications (Haddad et al., 2003; Leduc et al., 2006). Consumption of blueberries improves blood and oxygen delivery to the eye

and scavenge free radicals, which contribute to cataract and macular degeneration (Calò & Marabini, 2014). Proanthocyanidins, anthocyanins, and flavonols in blueberries are beneficial in bone protection (Shen et al., 2012). Blueberry anthocyanins have been used for several therapeutic purposes including the treatment of fibrocystic disease, vision disorders, radiation-induced cell death (Leonardi, 1993; Liu et al., 2015). Proanthocyanidins from wild blueberry possess anti-adhesion properties which help to treat and prevent urinary tract infections (Schmidt et al., 2004). Blueberry juice has positive effect to treatment of juvenile idiopathic arthritis (Zhong et al., 2015). Consequently, blueberries prevent human health from several chronic diseases.

Numerous products from blueberry fruit and leaf extracts utilized as dietary supplements in the world market (Yuan et al., 2011). The consumption of wild blueberry powder supplements increases a diet-induced *ex vivo* serum antioxidant status in human body (Kay & Holub, 2002). The extract from leaves, the main waste products in blueberry harvesting as well as in processing industries, inhibits the Hepatitis C virus expression (Takeshita et al., 2009). The leaves of the wild blueberry have high contents of polyphenols and proanthocyanidins (Percival & MacKenzie, 2007; Riihinen et al., 2008); and proanthocyanidins are known to possess both antimicrobial and antioxidant activities (Heinonen, 2007). Thus, blueberry leaves could be used as an excellent source for proanthocyanidins containing products specially in cosmetic and pharmaceutical industries.

1.2.5 Propagation of blueberry

Although the popularity of lowbush blueberries is sky-rocket due to their antioxidant capacities and well-known health benefits, they are mostly produced from wild stand with minimum cultivation practice. In a naturally grown commercial field, there are many bare spots raised from herbicide application or mechanical scalping which rendered for low production. Numerous planting materials are required to cover those incomplete areas, to increase production in an established field, and to develop a new blueberry farm. Lowbush blueberries are naturally reproduced both sexually from seed and clonally through an extensive underground rhizome system. In general, they are propagated in nurseries using stem or rhizome as starting materials which is easy but time consuming for large scale multiplication. Seeds are used in limited scale for blueberry propagation, but they do not maintain trueness-to-type of their respective donor plants. Cloning by micropropagation is a more demanding and effective method for improving existing blueberry fields as well as for establishing a new farm due to its potential to produce numerous desirable new clones from a single source plant (Morrison et al., 2000). Different propagation methods are discussed briefly in the following sections.

1.2.5.1 Sexual propagation

Lowbush blueberries are generally self-incompatible, while a significantly higher incidence of self-fertility has been reported in several genotypes (Wood, 1968; Bell et al., 2012). True seeds developed from fertilized ovule in a cross-pollinated hermaphroditic flower of blueberry are used as a means of sexual propagation. Pollination of lowbush

blueberry flowers occurs mainly via insect pollinators like rented honey bees and native bees which are thought to be attracted to the plants by the vibrant color and aromatic scent of the blueberry flowers (Hicks, 2011). Genetic materials of two parents are combined in a progeny of sexual propagation having a new genetic makeup which is not identical to the mother plant. Although sexual propagation is easy and numerous seedlings can be grown from a single source plant, the seedling progenies produce <50% fruits of their parental clones (Aalders et al., 1979). In sexual propagation, lowbush blueberry plants usually flower and develop rhizomes 3 - 4 years after seed germination.

1.2.5.2 Asexual propagation

Generally asexual reproduction occurs in blueberry when the rhizomes are cut or killed by fire, shading, burrowing, or frost action. Asexual propagation of blueberries is carried out through vegetative propagation with stem or root cuttings and micropropagation.

1.2.5.2.1 Propagation by stem cutting

Vegetative propagation of blueberry has long been successfully practiced using the nodal segments of softwood, semi-hardwood, hardwood stems, single node, division of sub-terrestrial rhizomes or even leaf-bud cuttings as propagules to reproduce genetically identical plants (clones) which preserve the genetic structure and uniformity of source plant. The most widespread practice is softwood cutting using young shoots or shoot tips containing meristem (**Figure 1.2**). About 4 - 6 cm long shoot tips are clipped from mother plant and planted in potting soil could be supplemented with growth hormones or in field

directly (Debnath, 2006). The stem cuttings grow shoots and develop adventitious roots

Figure 1.2 Conventional propagation of lowbush blueberry using softwood cutting (Debnath, 2007b)

within several weeks with maintenance of proper soil fertility, temperature, humidity, and light intensity and duration. The alternative to softwood cuttings is hardwood cuttings, which refers to cuttings taken once the plant tissue becomes woody, typically at the dormant stage of plants. Semi-hardwood and rhizome segments are clipped from the matured plants and place in soil media for rooting. Stem cutting propagation is time consuming for large scale multiplication of lowbush blueberry, since limited number of propagules can be prepared from a single source plant. Another difficulty of conventional propagation is that stem cuttings have limited potentiality to develop new and subsequent rhizomes which slow down the spreading tendency, and they commonly face challenges in rooting capacity (Meiners et al., 2007; Litwińczuk, 2013). Since the *V. angustifolium* is a heterogeneous species due to inclusion of numerous wild clones with divergent clonal characteristics, it is a crucial problem for commercial propagation and establishment of selected clones. As demand increases for blueberry fruits from industry and global

consumers, the importance of commercial propagation increases as well. The shortcomings of stem cutting can be overcome by using *in vitro* propagation techniques which fulfill the world demand of blueberry supply.

1.2.5.2.2 *In vitro* propagation or micropropagation

In vitro is a Latin word which means 'test tube, culture dish or glass' (Basu, 2017). It is an artificial environment created outside the living organism. *In vitro* propagation, also called micropropagation, is carried out in control environments using cells, tissues or organs of a plant as explants. The explants are grown on an artificial medium consisting of water, macronutrients and micronutrients, some carbon source (usually carbohydrates in the form of sucrose or glucose), vitamins, growth regulators (auxins, cytokinins and gibberellins) and a chelating agent (in the case of solid medium). Under aseptic conditions, all those media components act together to supply optimum nutrients that allow plant growth (Debnath & McRae, 2001a). The entire procedure is carried out in aseptic condition and growth media are changed regularly to replenish elements to continue tissue growth. *In vitro* propagation is operated based on enhanced axillary bud proliferation and on the ability of plant cells to differentiate and develop new meristematic centres that are capable of regenerating fully normal plants (Debnath et al., 2012b). Regeneration of meristem or shoot or root is carried out through two morphogenic pathways: (1) organogenesis - the formation of unipolar organs, and (2) somatic embryogenesis - the production of bipolar structures, somatic embryos with both root and shoot meristems (Steward et al., 1970). The choice of starting material or explant in tissue culture determines the path through which

the explant will go to produce new shoots and plants.

Plant regeneration through tissue culture relies on two basic concepts: totipotency and developmental plasticity. Totipotency is the ability of a cell to differentiate, proliferate, and subsequently regenerate into a mature plant under appropriate culture conditions in a hormone-dependent manner (Skoog & Miller, 1957). Although, a whole plant could be regenerated solely from one cell, practically it is a challenging process. In general, totipotency is a characteristic of the cells in young tissues and meristems, but it can also be exhibited by some differentiated cells (Debnath, 2007c). When an explant is provided with correct stimulus hormone(s) and appropriate environments, it develops into a plant identical to the source plant and it is called clone. Plasticity is the ability of the plant tissues to alter their metabolism, growth and development to the best suits their environment. Tissue culture can rapidly and aseptically produce large amount of plant material, while selecting for and cloning superior germplasms that are disease-resistant and produce elevated levels of vegetative growth. The tissue culture technique is a very efficient propagation method for economically important plants.

Plant tissue culture started early 19th century when Haberlandt (1902) first explored plant cell culture using isolated bract cells of red dead-nettle (*Lamium purpureum* L.) in Knop's solution (Knop, 1865) supplemented with 1 or 5% (w/v) sucrose (Preil, 2005). Those cells were alive for more than a month without any cell division. After about 60 years after Haberlandt's first experiments, Kohlenbach (1959) reported differentiated mesophyll cells of plume poppy (*Macleaya cordata*) which developed into cell clusters and calli forming

organs and somatic embryo. Thus, the totipotency concept of differentiated cells predicted by Haberlandt in 1902 had been confirmed. Skoog and Miller (1957) coined the auxin-cytokinin hypothesis of plant morphogenesis. They reported that the differentiation of roots and shoots in tobacco pith tissue cultures was a function of the auxin/cytokinin ratio. High auxin/cytokinin ratios promote root formation, high cytokinin/auxin ratios lead to shoot initiation. While at equal concentrations of auxin and cytokinin, the tissue tended to grow in an unorganized fashion. Murashige & Skoog (1962) optimized the medium composition for tobbaco tissue culture using different concentrations of macro and micro nutrients and organic growth factors.

In vitro culture of blueberries was initiated in early 70's by Barker and Collins (1963) who grew rhizome pieces on White's medium (White, 1943) without adding growth regulators. Boxus (1974) and Anderson (1975) were the founders for commercial micropropagation of berry crops. Although tissue culture for highbush and half-high blueberries has been routinely used for more than thirty years (Cohen & Elliott, 1979; Grout et al., 1986), micropropagation for lowbush blueberry is in developing stages. The first callus formation was induced *in vitro* in lowbush blueberry using stem internodes by Nickerson and Hall (1976) on Murashige and Skoog medium (Murashige & Skoog, 1962) supplemented with growth hormone 2,4-dichlorophenoxyacetic acid (2,4-D) (**Table 1.2**). After two years, Nickerson (1978a) induced shoots from blueberry seedling explants and the author developed callus in same genotypes using fruit explant (Nickerson, 1978b). Nowadays tissue culture techniques have been practised through axillary shoot proliferation and/or adventitious shoot generation on semisolid media for lowbush (Lloyd & McCown, 1980;

Frett & Smagula, 1983; Debnath, 2004, 2009a), rabbiteye (Hung et al., 2016a) and half-high (Grout et al., 1986; Read et al., 1989) blueberries. Most recent forms of micropropagation for lowbush blueberry involves an automated bioreactor system with liquid media for multiplication of micropropagules derived through either shoot proliferation or adventitious shoot regeneration which may be cost effective for commercial propagation (Debnath, 2009b; 2011; 2017). However, liquid culture is generally limited by low oxygen content (Smith & Spomer, 1995) and hyperhydricity in regenerants (Debnath, 2009b). Another problem in micropropagation for blueberry with shoot explant is the formation of unwanted callus at the base of the explants and the occurrence of spontaneous adventitious shoots (Zimmerman & Broome, 1980; Litwińczuk & Wadas, 2008). Appropriate growth hormone specially auxin and optimum auxin cytokinin ratio help to overcome this problem. Litwińczuk and Wadas (2008) reported that uising indole-3-butyric acid (IBA) instead of indolyl-3-acetic acid (IAA) and lowering N^6-(2-isopentenyl) adenine (2iP) concentration enhanced healthy axillary shoot with relative long internodes and rigid, well-developed leaves in highbush blueberry cv. 'Herbert' (*Vaccinium* × *covilleanum* But. et Pl.) and supressed base-adjoin unexpected shoots which were thin and fragile, mostly vitrified with short internodes, smaller and unfolded leaves.

A complete plant can be regenerated from *in vitro* culture in three different pathways (Debnath, 2007c): i) axillary shoot proliferation from pre-existing apical or axillary buds, ii) organogenesis through adventitious shoot regeneration, and iii) somatic embryogenesis through development of embryos directly.

Table 1.2 Examples of *in vitro* propagation of blueberries using different basal media[z] and explants

Species	Media types	Micropropagation via	Explants used	Rooting *in vitro*/*ex vitro*	Reference
Vaccinium angustifolium wild clones	MBM-C	shoot proliferation	single nodes, axillary buds	*ex vitro*	(Debnath, 2004)
V. angustifolium cv. 'Fundy' and wild clones	MBM-C	shoot proliferation	shoot tip and segments	*ex vitro*	(Debnath, 2006, 2009a)
V. angustifolium wild clones	MBM-C	shoot regeneration	leaf segments	*ex vitro*	(Debnath, 2009a; 2011)
V. angustifolium	WPM	shoot proliferation	single node	N/R	(Kaldmäe et al., 2006)
V. angustifolium	ANM	shoot regeneration	hypocotyl and cotyledons	N/R	(Nickerson, 1978a)

Table 1.2 cont'd

V. angustifolium	MSM	callus formation	internodes and fruits	N/R	(Nickerson & Hall, 1976; Nickerson, 1978b)
V. angustifolium	ZBM	shoot proliferation	shoot	*ex vitro*	(Frett & Smagula, 1983)
V. angustifolium	ZBM	shoot proliferation	young shoot	*ex vitro*	(Dweikat & Lyrene, 1988; Brissette et al., 1990)
V. angustifolium	ZBM	shoot regeneration	leaf	*ex vitro*	(Dweikat & Lyrene, 1988)
V. angustifolium cv. 'Dwarf Tophat'	WPM	shoot proliferation	single node	*in vitro* in WPM	(Georgieva, 2013)
V. angustifolium	ZBM	shoot regeneration	internodes	N/R	(Hruskoci & Read, 1993)
V. ashei cv. 'Titan'	MSM+WPM	shoot proliferation	multiple shoots	*ex vitro*	(Hung et al., 2016a)
V. corymbosum cv. 'Polaris', 'St. Cloud'	MBM-C	shoot proliferation	axillary shoots	*ex vitro*	(Debnath, 2017)

Table 1.2 cont'd

V. corymbosum cv. 'Huron'	MSM+WPM	shoot proliferation	nodal segments	*ex vitro*	(Hung et al., 2016b)
V. corymbosum cv. 'Huron'	MSM+WPM	shoot proliferation	nodal segments	*ex vitro*	(Hung et al., 2016b)
Hybrid of *V. corymbosum* 'Spartan' × *V. bracteatum*	MSM+WPM	shoot proliferation	axillary buds	*in vitro*	(Tsuda et al., 2014)
V. corymbosum cv. 'Berkeley', 'Bluecrop' and 'Goldtraube'	MSM, ANM	shoot multiplication	shoots	*in vitro* on ANM	(Ružić et al., 2012)
V. corymbosum cv. 'Elliot'	WPM	shoot regeneration and proliferation	buds, leaves microshoots,	*ex vitro*	(Vescan et al., 2012)
V. corymbosum cv. 'Bluecrop' 'Berkeley', 'Earliblue'	MSM, WPM, MSM+WPM	shoot proliferation	nodal segments	*in vitro*	(Tetsumura et al., 2008)
V. corymbosum × *V. angustifolium* cv. 'Northland'	WPM	shoot regeneration	nodal and leaf segments	*in vitro*	(Zhao et al., 2011)
Interspecific hybrids of *Vaccinium* spp.	MSM, ZBM	shoot regeneration	ovule	*ex vitro*	(Pathirana et al., 2015)

Table 1.2 cont'd

V. corymbosum cv. 'Ozarkblue'	WPM	shoot proliferation and regeneration	node and leaf segments	*in vitro*, *ex vitro*	(Meiners et al., 2007)
V. corymbosum cv. 'Bluecrop', 'Duke', and 'Sunrise'.	WPM	adventitious shoot regeneration	leaf	*ex vitro*	(Rowland & Ogden, 1992; Cao & Hammerschlag, 2000)
V. corymbosum cv. 'Bluecrop'	WPM	shoot regeneration	leaf	*ex vitro*	(Cao et al., 2002)
V. virgatum cv. 'Kunisato 35 Gou'	MSM+ WPM	shoot multiplication	nodal segments	*in vitro*	(Tetsumura et al., 2012)
V. corymbosum cv. 'Berkeley'	WPM	shoot proliferation	nodal segments	*ex vitro*	(Gonzalez et al., 2000)
V. corymbosum cv. 'Herbert'	ZBM	shoot proliferation and regeneration	nodal segments	*in vivo*	(Litwińczuk & Wadas, 2008)
V. corymbosum	WPM	shoot proliferation	single node	N/R	(Reed & Abdelnouresquivel, 1991)

Table 1.2 cont'd

V. corymbosum × *V. angustifolium* cv. 'Northblue'	ZBM	shoot proliferation	shoot tips	*ex vitro*	(Cohen, 1980; Grout et al., 1986; Read et al., 1989)
V. corymbosum × *V. angustifolium* cv. 'North Country'	WPM	shoot proliferation and regeneration	leaf segments	N/R	(Graham et al., 1996)
V. corymbosum (southern highbush)	MSM + WPM	shoot regeneration	leaf segments	*ex vitro*	(Liu et al., 2010)

[z]Media: MBM-C = Modified basal medium for cranberry (Debnath & McRae, 2001a); MSM = Murashige and Skoog medium (Murashige & Skoog, 1962); WPM = Woody plant medium (Lloyd & McCown, 1980); MSM+WPM = 50% MSM and 50% WPM; ZBM = Zimmerman and Broome medium (Zimmerman & Broome, 1980); ANM = Anderson's Rhododendron medium (Anderson, 1975); N/R = not reported.

1.2.5.2.2.1 Propagation via axillary shoot proliferation

The simplest type of *in vitro* propagation is the stimulation of axillary bud development. Shoots are grown from cultured explants like shoot tips containing meristem or nodal segments having axillary buds. Generally, those axillary buds are dormant or inactive because of hormonal and genetic interactions, and due to the signals raised from the active apical meristem to inhibit lateral bud activation (Wolpert, 2002). In a culture medium

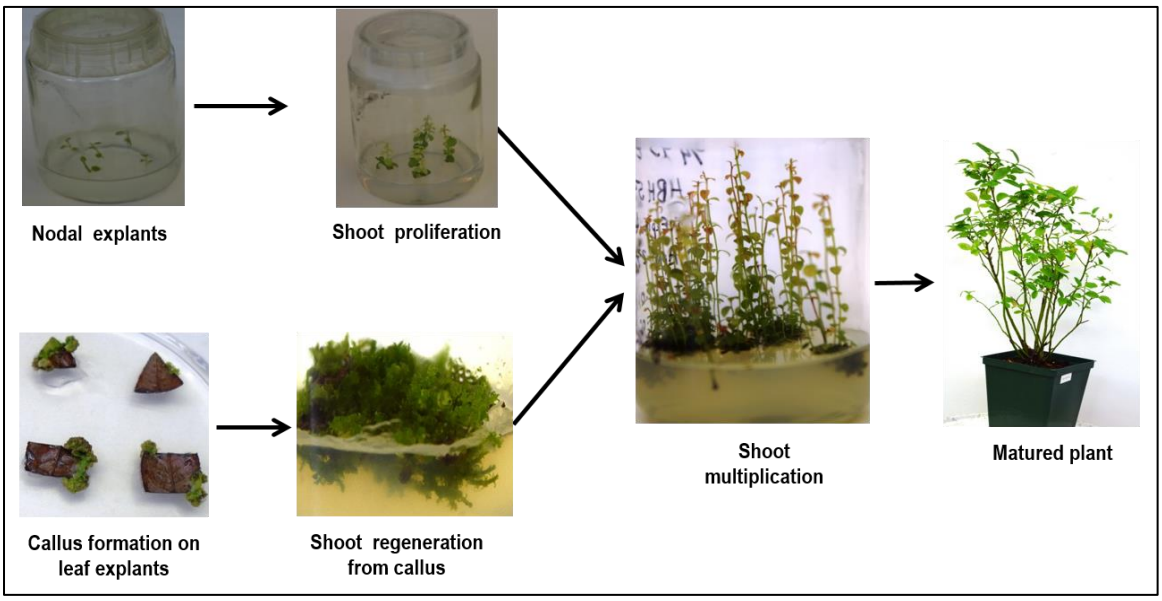

Figure 1.3 *In vitro* propagation of lowbush blueberry on modified basal medium (Debnath, 2007b, 2009b)

containing no or low levels of auxins and higher levels of cytokinins, the dormant axillar buds at leaf axis are activated to develop shoots (**Figure 1.3**). Additional shoots are produced through further axillary bud growth (Debnath et al., 2012b). This technique exploits the normal ontogenetic path for branch development by lateral meristems. Explants from juvenile stocks are more suitable for shoot proliferation than those from

non-juvenile stages (Lyrene, 1980). Plants propagated through axillary shoot proliferation were reported in lowbush (Frett & Smagula, 1983; Morrison & Smagula, 1986; Brissette et al., 1990; Debnath, 2004, 2007b, 2009b; Georgieva, 2013) and highbush blueberries (Gonzalez et al., 2000; Litwińczuk & Wadas, 2008) (**Table 1.2**). Plants produced by axillary shoot proliferation preserve the genetic makeup of the mother plant and this method is mostly applied as a reliable method for the commercial mass production of true-to-type blueberry plants.

1.2.5.2.2.2 Adventitious shoot regeneration

Plant regeneration from cultured tissue can be achieved by culturing tissue section lacking a preformed meristem (adventitious origin) or from callus and cell cultures (de novo origin). In contrast with axillary shoot proliferation, adventitious shoot regeneration occurs at unusual sites of a cultured tissue such as the internode, leaf blade and cotyledon or root elongation zone, where meristem is naturally absent (George, 2008; Vooková & Gajdošová, 1992). The pathway of regeneration undergoes through de-differentiation of plant tissue followed by re-differentiation and organization of cells into meristematic centres (Debnath & McRae, 2002). The organization into morphogenetic forms can take place directly on the isolated explant or can be expressed only after callus formation, which is called indirect morphogenesis (**Figure 1.3**). Development of shoots directly on leaf or stem explants is referred to direct morphogenesis. Shoot regeneration technique in blueberries are divided into the following steps: (1) formation of viable adventitious buds on the explant, (2) elongation of the buds into shoots, and (3) rooting of the shoots to form

complete plants (Qu et al., 2000). The requirement of exogenous auxin and cytokinin for this process depends on the endogenous levels of hormones present in the explants and hence varies with the tissue culture system (Davey & Anthony, 2010). In lowbush blueberry, the successful regeneration of adventitious shoots was first reported by Nickerson (1978a) who produced shoots from callus developed on hypocotyl and excised cotyledon of lowbush blueberry seedling on Anderson's (1975) medium containing 23 μM IAA and 75 μM 2iP (**Table 1.2**). Viable blueberry shoot regeneration directly from young internode and indirectly from callus developed on shoot internode segments was regenerated by Hruskoci and Read (1993) on Zimmerman and Broom medium (Zimmerman & Broome, 1980) with zeatin supplement. An efficient *in vitro* system to regenerate adventitious shoots on excised leaves of wild lowbush blueberry was developed by Debnath (2009a). Leaf cultures produced multiple buds and shoots with or without an intermediary callus phase on modified cranberry gelled-medium (Debnath & McRae, 2001a) supplemented with thidiazuron (TDZ). Subsequently, Debnath (2011) reported success in adventitious bud and shoot formation from blueberry leaves on liquid media using bioreactor system. Adventitious shoot regeneration also reported for highbush blueberries (Rowland & Ogden, 1992; Cao et al., 2002; Meiners et al., 2007), half-high blueberries (Graham et al., 1996), southern highbush blueberries (Liu et al., 2010) and rabbiteye blueberries (Yadong et al., 2003). Shoot regeneration system of plants can be used to identify and/or induce somaclonal variation, to develop transgenic plants following genetic transformation of plant cells and new cultivars with desired characteristics.

1.2.5.2.2.3 Somatic embryogenesis

Somatic embryogenesis is an asexual form of plant reproduction by which differentiated, and mitotically quiescent somatic cell can recover embryogenic potential and differentiate a new viable bipolar structure of embryo in *in vitro* condition without fusion of gametes (Thorpe, 1993; Vidal et al., 2008). A somatic embryo can be originated either directly from an explant without callus formation (Wang et al., 1994a), or it can be developed indirectly from proliferated cell or callus (Konar et al., 1972). The plantlets are regenerated in several small berry crops such as strawberry and grape through somatic embryogenesis using meristem, leaf, anther and ovary as explants (Vidal et al., 2008; Biswas et al., 2009). This regeneration process has not been reported in blueberry.

1.2.5.2.3 Advantages of micropropagation

Micropropagation has some major advantages over conventional methods of plant propagation (Rani & Raina, 2000): (i) it is an invaluable aid in the multiplication of elite clones of intractable/recalcitrant species; (ii) production can be continued all the year round due to independence on seasonal changes (iii) it is possible to generate disease and pathogen-free plants, even from explants collected from infected mother plants (meristem culture); (iv) plant materials such as male sterile, fertility maintainer and restorer lines can be cloned; and (v) it enables the production of a large number of genetically identical plants in a short time from a selected number of genotypes, where the traditional methods of multiplication are either not available or are ineffective in large scale multiplication systems. In breeding programs for perennials, micropropagation can accelerate the

breeding process by *in vitro* selection and facilitate the mass production for analysis in a replicated trial of new releases (Debnath, 2007c). *In vitro* technology also offers several advantages over naturally grown plants in producing bioactive compounds (Chattopadhyay et al., 2002) such as, (i) production conditions can be optimized and controlled to get desired content of pure product, (ii) shorter and more flexible production cycles, (iii) novel products not found in nature can be produced, and (iv) production of phytochemicals is not dependent on climatic and geographic conditions.

1.2.5.2.4 Disadvantages of micropropagation

Micropropagation has some limitations. This technique is a complex procedure and requires sophisticated facilities which involve expensive machinery and reagents. It demands highly trained and skilled labours in handling and maintenance of cultures compare to conventional propagation. Tissue culture procedure, media composition and growth regulator are varied depending on the plant species and even on different genotypes of the same species (Debnath, 2007a), which also increases the expense of the method. Rooting of microcuttings *in vitro* is expensive and can even double the price of the cutting (Zimmerman, 1988; De Klerk, 2002). Sometimes plants do not produce true-to-type regenerants which limit the goal of micropropagation. For example, average of 8.4% strawberry clones developed through tissue culture exhibited morphological variation (Biswas et al., 2009). From the point of commercial micropropagation, variation of any kind especially genetic variations may be considered obstructive and worthless.

1.2.6 Micropropagation and somaclonal variation in berry crop improvement

Tissue culture is an essential part of plant biotechnology that facilitates the production of somaclonal variants and genetically engineered plants. The term 'somaclonal variation' refers to tissue culture induced stable phenotypic variation displayed among somaclones, the plants derived from any form of tissue culture using somatic cells from a single donor plant (Larkin & Scowcroft, 1981; Schaffer, 1990; Skirvin et al., 1993). It can be originated from genetic (heritable) or epigenetic (non-heritable) changes or a combination of both. The genetic mechanism of somaclonal variation may include number and structural changes in chromosome, mutation in gene, exchange between sister chromatids or somatic crossing-over and changes in organelle DNA (Jain, 2001; Predieri, 2001; Bairu et al., 2011b; Krishna et al., 2016). Epigenetic bases of somaclonal variation involve insertion, excision or activation of transposable elements, DNA methylation, and segregation of pre-existing chimera tissue (Brar & Jain, 1998; Guo et al., 2007; Linacero et al., 2011; Sato et al., 2011). Somaclonal variation is usually observed when plants are regenerated from cultured somatic cells, mostly during callus formation and suspension culture (Biswas et al., 2009; Ge et al., 2015). Adventitious shoot regeneration system is more vulnerable to develop somaclonal variation than shoot proliferation system.

Although the occurrence of subtle somaclonal variation is a drawback for both *in vitro* cloning as well as germplasm preservation, it may provide a new or alternative means to the breeders to obtain genetic variability in the species which are either difficult to breed or have narrow genetic base (Krishna et al., 2016). Somaclonal variants have been reported

in several horticultural crops with increased resistance to pests, diseases, and herbicides (Brar & Jain, 1998; Ge et al., 2015) and thus it can be considered in berry improvement program (Bouharmont, 1994; Hammerschlag et al., 1995). Biswas et al. (2009) reported that somatic embryogenesis through meristem culture was the most effective way to induce somaclonal variation in strawberry regenerants. The authors selected three putative somaclones with high fruit quality and improved horticultural characters which were adopted in Bangladesh. In other studies, strawberry somaclones have also been demonstrated morphological variations with respect to hyper-flowering habit, calyx separation, earliness and abnormal fruit setting, rate of ripening and yield variation and resistance to various fungal species (Simon et al., 1987; Toyoda et al., 1991; Orlando et al., 1997; Popescu et al., 1997). Several agro-morphological traits and disease tolerance in berry crops have been improved by developing somaclonal variation.

Somaclonal variations have been reported in transformed plants of several blueberry cultivars. Ploidy doubling was achieved in diploid (Lyrene & Perry, 1983; Perry & Lyrene, 1984) and tetraploid (Goldy & Lyrene, 1984) blueberry clones. Graham et al. (1996) described transformation and regeneration of half-high blueberry cultivar 'Northcountry'. Callus lines of highbush blueberry were selected on media with salt tolerance (Muralitharan et al., 1992). Hruskoci and Read (1993) developed shoot regeneration protocol for *Vaccinium* spp. to select somaclones which were tolerant to high pH condition in media. High-level tolerance to the herbicide was observed in the *bar*-expressing greenhouse grown blueberry plants (Song et al., 2007; Song & Hancock, 2012). Although novel traits or new varieties have been developed by somaclonal variation, most of the

cases of improved variants have not been selected (Karp, 1995; Biswas et al., 2009) because (i) most of the variants are inferior to the original cultivar from which they are derived, (ii) positive changes are also altered in negative ways, (iii) the changes are not novel, or (iv) the changes are not stable after crossing, self-fertilization or even after several generations of vegetative propagation.

1.2.7 Morphological characteristics of blueberry plants propagated by different methods

1.2.7.1 Characteristics of bush or plant stature

Lowbush blueberry is prostrate, spreading shrubs visually evident as distinct 'patches'. Lowbush blueberry produces two types of stems: (i) regular/aerial stem which emerges from soil after seed germination or from bud on leaf axis and (ii) rhizome which typically grows horizontally below the soil surface and occasionally forms a leafy shoot (areal stem) either by converting the apical meristem or from a bud on the axis (Barker & Collins, 1963). Rhizome formation potential of blueberry is varied under different propagation methods. The plants propagated by stem cuttings have an upright growth habit with no or sometimes one or two developed rhizomes. Plants propagated from seeds have higher number of rhizomes compare to stem cutting (Morrison & Smagula, 1986; Morrison et al., 2000). Micropropagated blueberry plants have many similar characteristics like seedlings such as more vegetative growth with higher number of vegetative buds along stems, and a high degree of branching at the plant's base, but they produce rhizomes rapidly and more consistently than stem cuttings or seedlings do. Enhanced rhizome production of the

micropropagated lowbush blueberry plants was reported by Jamieson and Nickerson (2003). Morrison et al. (2000) observed that micropropagated lowbush blueberry plants produced ten-fold more rhizomes than those of stem cuttings. Debnath (2007b) reported that tissue culture derived plants produced longer and more number of stems than the conventional cuttings. Three times higher vegetative growth rate and 2 - 3 times more lateral branches was reported by Grout et al. (1986) for young micropropagated half-high blueberry plants than the rate of stem cutting plants. El-Shiekh et al. (1996) reported increased vigour, and more spreading growth habit for tissue cultured half-high blueberry plants compared to softwood cuttings, though plant height was not varied significantly. Litwińczuk et al. (2005) observed that conventionally propagated plants of highbush blueberry grew slowly, produced significantly less and shorter shoots in field conditions compared to the plants obtained through either axillary shoot proliferation or adventitious shoot regeneration process. Total shoot number per plant was greater for TC derived southern highbush blueberry plants compared to stem cuttings (Marino et al., 2014). *In vitro* derived shoots rooted much faster in field condition than cuttings from seedlings and naturally field-grown plants (Lyrene, 1981). Thus, micropropagation enhances the vegetative growth in blueberries.

1.2.7.2 Characteristics of leaf

Propagation methods affect size, area and number of leaves in different plant species. The concentration of growth hormones, inorganic salts in culture media and the interaction between these two components have significant effects on the length, width, and surface

area of leaf. The shoots established in the media with IBA had larger leaf in agave (*Agave potatorum* Zucc.) plants than in media without IBA (Enríquez-del Valle et al., 2016). Smaller leaf and shorter leaf petiole were reported in tissue culture derived plants compare to the conventionally propagated plants in taro (Johnston et al., 1997) and soybean (Radhakrishnan & Ranjitha Kumari, 2008). Micropropagated lingonberry plants bore smaller leaves than stem cutting ones (Debnath, 2006). Whereas, Litwińczuk et al. (2005) reported wider leaves on micropropagated highush blueberry plants compared to softwood cutting platns. Debnath (2007b) reported higher number of leaves per stem of micropropagated blueberry plant than the conventional softwood cuttings. Conversely, Radhakrishnan and Ranjitha Kumar (2008) found less leaves on micropropagated soybean plants than their parent plants grown from seed. However, the tissue culture originated plants had a similar number and turnover of leaves as the conventionally propagated plants did in taro (Johnston et al., 1997). Micropropagation has genotype specific effect on the leaf characters.

1.2.7.3 Characteristics of flower and fruit

Propagation methods significantly influence the flower bearing and fruit characteristics of blueberries grown both in greenhouse and field conditions. In highbush blueberries, Litwińczuk et al. (2005) reported that softwood cutting plants developed flowers one year earlier than tissue culture derived counterparts. Micropropagated half-high blueberry plants had similar numbers of flower buds per branch as softwood cutting plants did (Grout et al., 1986). Read et al. (1989) obtained more flower buds and higher yields on micropropagated

blueberry cultivars, 'Northblue' though average berry weight or quality did not differ significantly. However, significantly larger berries were reported in stem cutting plants by Litwińczuk et al. (2005). El-Shiekh et al. (1996) determined significantly higher fruit yields of micropropagated plants. They proposed greater number of flower buds and better yield of tissue culture derived blueberry plants due to their bushier and more spreading growth habit. In lowbush blueberry, fruit and flower characteristics are also varied with respect to propagation methods. Micropropagated lowbush blueberry plants produced fewer flower buds than did conventional softwood cuttings (Morrison & Smagula, 1986; Morrison et al., 2000; Jamieson & Nickerson, 2003). Although highbush blueberry production is influenced by *in vitro* propagation, fruit yield is less in tissue culture plants than in cutting propagated plants in lowbush blueberries.

1.2.8 Plant phenolics

Blueberries are mostly popular for their antioxidant phytochemicals especially phenolic metabolites which provide significant health benefits other than the basic nutrient such as carbohydrates, proteins, fats, minerals and vitamins. Phenolic compounds are the largest category of phytochemicals and the most widely distributed in fruits, vegetables, leaves, nuts, seeds, flowers, and even barks. Their structures range from simple moieties containing a single hydroxylated aromatic ring to highly complex polymeric compounds. The most of plant phytochemicals are classified into flavonoids and non-flavonoids (Działo et al., 2016). The chemical structure of flavonoid compounds is based on two aromatic benzoic rings commonly denoted as A and B, which are connected by an oxygen containing

three carbon pyrene ring C (Nichenametla et al., 2006). Flavonoids are compounds of low molecular weight having a common carbon skeleton based on flavan system (C_6-C_3-C_6). Due to the differences in the structure of flavonoid compounds, flavonoids are classified into flavanols, flavanones, flavonols, isoflavones, flavones and anthocyanins (Brodowska, 2017). Anthocyanins are red, blue and purple pigment molecules, and whereas flavonols, flavones, flavanols and isoflavones are colorless or white to yellow molecules (King & Young, 1999). Non-flavonoids include phenolic acids (hydroxybenzoic C_6-C_1 and hydroxycinnamic C_6-C_3 acids), lignans $(C_6$-$C_3)_2$ and stilbenes (C_6-C_2-C_6). Phenolic acids and flavonoids account for 60% and 30% of total dietary plant phytochemicals, respectively (Strack, 1997; Nichenametla et al., 2006). Other two non-flavonoid subclasses are tannins and lignins which are the polymers of particular phenolic compound and have high molecular weight and unique structure (Działo et al., 2016). Condensed tannins, a subclass of flavonoids, are polymers of catechins and epicatechins and found mainly in fruits, grains and legumes.

1.2.8.1 Biosynthesis of phenolic compounds

The biosynthetic pathways of phenolic substances in plants are predominantly controlled by endogenous processes during developmental differentiation (Weidner et al., 2000). Plant phenolics are synthesized from a limited pool of biosynthetic precursors such as pyruvate, acetate, acetyl coenzyme A (CoA), malonyl CoA and a few amino acids (Robards et al., 1999) following pentose phosphate, shikimate and phenylpropanoid metabolism pathways (Ryan & Robards, 1998; Randhir et al., 2004). The representative biosynthetic

pathways of some phenolic acids and flavonoids are outlined in **Figure 1.4**, but substituents can vary widely among plant species (Dixon & Paiva, 1995; Strack, 1997). Phenylalanine, produced in plants via the shikimate pathway, is a common precursor for most of the phenolic and flavonoid compounds in higher plants (Dixon & Paiva, 1995). The enzymes catalysing the individual steps in general phenylpropanoid metabolism are phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (CA4H) and 4-coumerate: CoA ligase (4CL) (Macheix et al., 1990; Strack, 1997). These three steps are necessary for the biosynthesis of phenolic compounds.

Entry into the flavonoid pathway from general phenylpropanoid metabolism is controlled by chalcone synthase (CHS), which condenses p-coumaroyl-CoA and malonyl-CoA into a chalcone, followed by isomerization by chalcone isomerase (CHI) to form a flavanone (Zifkin et al., 2012) (**Figure 1.4**). Flavanone-3β-hydroxylase (F3H) hydroxylates flavanone to dihydroflavanol. Dihydroflavanol is further reduced by dihydroflavonol reductase (DFR) to flavanol which is a key intermediate for anthocyanidins and proanthocyanidins. The anthocyanidins are synthesized by anthocyanidin synthase (ANS) and then glycosylated by UDP-glucose flavonol-3-*O*-glucosyl transferase (UFGT) to anthocyanins. Anthocyanidins can be diverted into proanthocyanidins via anthocyanidin reductase (ANR) which produces flavan-3-ols (**Figure 1.4**).

1.2.8.2 Function of phenolics and flavonoids in plants

An important function of flavonoids, especially anthocyanins together with flavones and flavonols, is pigmentation of flowers and fruits (Harborne, 1994) which attract insects and

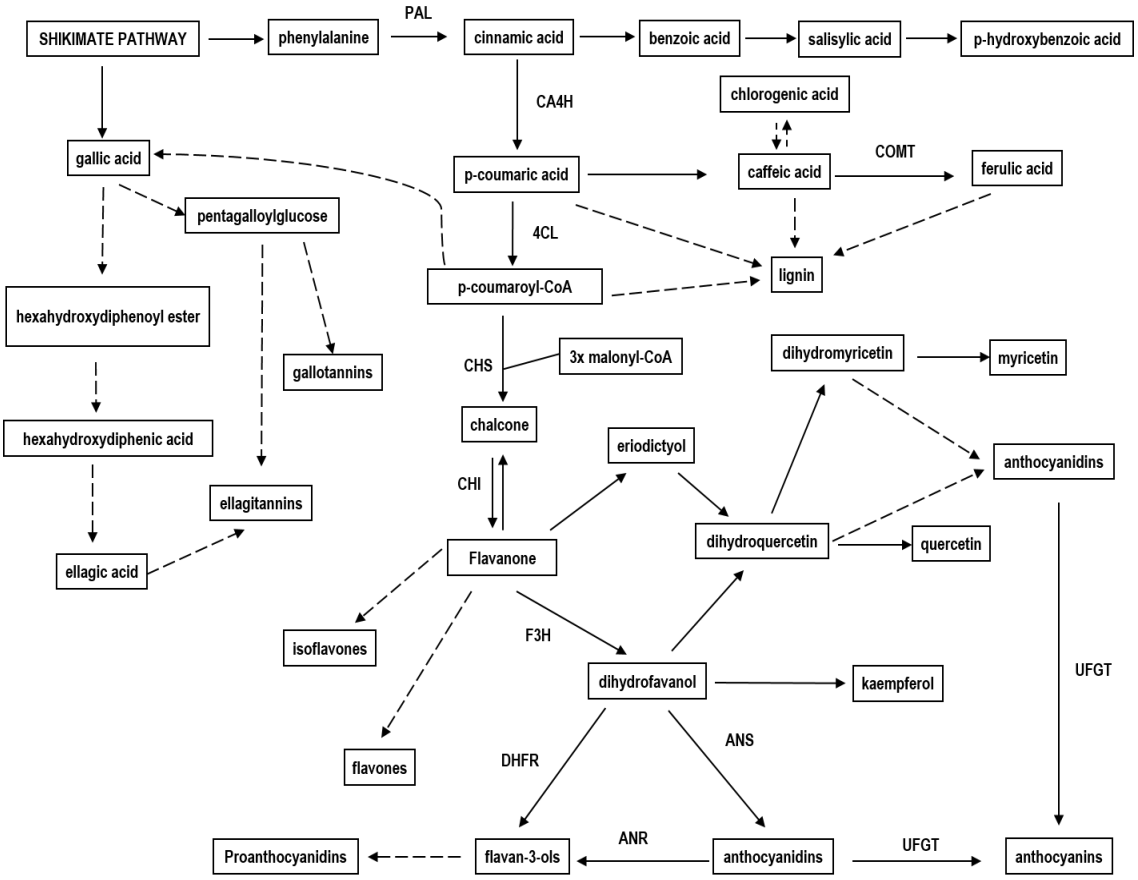

Figure 1.4 Biosynthesis pathways of phenolic and flavonoid compounds (Dixon & Paiva, 1995; Strack, 1997; Zifkin et al., 2012). Solid arrows represent reactions catalyzed by well-characterised enzyme(s). Dashed lines represent transformations that require multiple enzymes that are less characterised, or vary among plant species. Enzymes: 4CL = 4-coumarate: coenzyme A ligase; ANR = anthocyanidin reductase; ANS = anthocyanidin synthase; CA4H = cinnamic acid 4-hydroxylase; CHI = chalcone isomerase; CHS = chalcone synthase; COMT = caffeic acid *O*-methyltransferase; DHFR = dihydroflavonol reductase; F3H = flavanone-3β-hydroxylase; PAL = phenylalanine ammonia lyase; UFGT = UDP-glucose flavonol-3-*O*-glucosyl transferase.

birds to the plant for pollination and seed dispersal (Hicks, 2011). Tannins and flavonoids are responsible for fruit odour, flavour, bitterness and astringency. The phenolic compounds such as lignin, cutin, suberin are the integral parts of the cell-wall of plants serving as mechanical support (Wallace & Fry, 1994). They can act as signal molecules in the interaction between the plant and the nitrogen-fixing bacteria in certain leguminous plants (Strack, 1997). Phenolic and flavonoid compounds have significant contribution in plant defence mechanisms. Those metabolites are accumulated to defend plants against infection (Beckman, 2000), mechanical wounding (Hahlbrock & Scheel, 1989), nutritional stresses (Graham, 1991), cold stress (Christie et al., 1994; Taulavuori et al., 2004), light and heat stresses (Dixon & Paiva, 1995). Furthermore, deficiency of iron, phosphorus and nitrogen in soil, drought conditions, over application of herbicides can also trigger the production of phenolic compounds in plants as a means of tolerance (Dixon & Paiva, 1995; Solecka, 1997; Cardeñosa et al., 2016). Phenolic substances influence the competitive phenomenon called 'allelopathy' among the plants. Besides the familiar volatile terpenoids, simple phenols, toxic water-soluble hydroxybenzoic acids and hydroxycinnamic acids can affect the growth and development of agricultural and biological system (Strack, 1997). For example, among the phenolics produced in Olive (*Olea europaea* L.), low molecular weight phenols such as hydroxytyrosol and catechol have allelopathic properties on seed germination and seedling growth of radish and wheat (Scognamiglio et al., 2013). The phenolic and flavonoid compounds have significant role in physiological process and defence mechanism of plants.

1.2.8.3 Phenolic content and antioxidant activity in blueberries

Phenolic acids such as chlorogenic acid, caffeic acid, p-coumaric acid, ellagic acid and vanillic acid derived from hydroxybenzoic or hydroxycinnamic acids are widely distributed in blueberry leaves and fruits as natural antioxidants (Harris et al., 2007; Riihinen et al., 2008; Correa-Betanzo et al., 2014; Cardeñosa et al., 2016). Important group of flavonoids found in blueberries are flavonols (quercetin derivatives), anthocyanidins, proanthocyanidins, catechins and their glycosides (Häkkinen et al., 1999; Gavrilova et al., 2011; Giovanelli et al., 2013). Among over 300 different anthocyanidins found in plants, cyanidin, delphinidin, petunidin, peonidin and malvidin derivatives are most common in blueberries (Gao & Mazza, 1994; Giovanelli & Buratti, 2009; Cardeñosa et al., 2016). Anthocyanins, glycosidic forms of anthocyanidins, are major pigments in dark and bright color fruits such as blueberries, bilberry, cranberries and lingonberry. Proanthocyanidins, which differ from other phenolic compounds by their polymeric structure, are widely distributed in berries (Zifkin et al., 2012). Proanthocyanidins can bind strongly with carbohydrates and proteins and act as strong free radical scavengers (Kähkönen et al., 2001). Those are believed to be at least 15 to 25 times stronger in antioxidant capacity compare to vitamin E, and demonstrate a wide range of pharmacological activity.

Blueberries have received much attention due to their high antioxidant activities. The antioxidant capacity of blueberries depends on their phytochemical complex, mainly polyphenolic and flavonoid compounds, their structures and redox potential (Prior et al., 1998; Wang, 2007). Phenolic compounds possess one or more aromatic rings with a

conjugated aromatic system and one or more hydroxyl groups. Phenolics donate an electron or a hydrogen atom to a free radical, convert it into a neutralized inoffensive molecule and thus they act as antioxidant molecules *in vitro* and *in vivo* (Skrovankova et al., 2015). They also quench singlet and triplet oxygen or decompose peroxides (Larson, 1988). It is well established that a strong and positive relationship exists between total phenolic and anthocyanin content and antioxidant activity (Moyer et al., 2002; Sellappan et al., 2002; Ehala et al., 2005; Giovanelli et al., 2013). The correlation between total phenolic content and antioxidant capacity in blueberries is higher than the relationship between anthocyanin and antioxidant activity (Moyer et al., 2002). However, the overall antioxidant activity may be elucidated by the linkage of different phytochemicals, working additively or synergistically in relation to the total antioxidant capacity.

The phenolic compounds and their antioxidant activities in berries are variable depending on the species, cultivars and varieties, degree of maturity, plant tissues, growing seasons and locations, environmental conditions and time of harvest as well as postharvest conditions. The effects of those internal and external factors on phenolic and flavonoid content and antioxidant activities have been discussed.

1.2.8.3.1 Species, cultivars and selections of blueberries

It is well-known that species, variety, cultivar and genotype of blueberries are varied in the content of phenolics and their profiles. There are substantial variations among commercial and non-commercial blueberry species in the content of antioxidant phenolics (Moyer et al., 2002; Cardeñosa et al., 2016). Rabbiteye blueberries have higher average polyphenolic

concentration compare to northern and southern highbush blueberries (Sellappan et al., 2002; Wang et al., 2015). Kalt et al. (2001a) and Koca and Karadeniz (2009) found that lowbush blueberries were consistently higher in total phenolic content compared with highbush blueberries and blackberries. Wild blueberries have much higher concentrations of total phenolics ranging from 299 to 600 mg/100 g compare to cultivated highbush blueberries which are ranged from 181 to 390 mg/100 g (Prior et al., 1998; Giovanelli & Buratti, 2009). Total phenolic content was varied 2.1-fold (Prior et al., 1998), 3.4-fold (Ehlenfeldt & Prior, 2001), 2.4-fold (Howard et al., 2003) among highbush blueberry cultivars and 3.4-fold among rabbiteye and 2.2-fold among southern highbush blueberries cultivars (Sellappan et al., 2002). The levels of phenolic compounds in blueberries vary significantly not only with inter-species variability but also with intra-species variations.

Generally lowbush blueberries contained higher level of especially anthocyanins compared with highbush blueberries (Kalt et al., 2001a; Vendrame et al., 2016). Half-high blueberry cultivars had higher concentrations and proportions of anthocyanidins than highbush cultivars (Li et al., 2017). Sellappan et al. (2002) reported the average anthocyanin content in rabbiteye blueberries (113.5 mg/100 g F.F.) was higher than southern highbush blueberries (84.1 mg/100 g F.F.). Different cultivars of same blueberry species also contain various levels of anthocyanins. The differences in anthocyanin content among highbush blueberry cultivars varied 5-fold (Howard et al., 2003), 2.2-fold (Ehlenfeldt & Prior, 2001) and 3.7-fold (Sellappan et al., 2002), and among rabbiteye blueberry cultivars varied around 9-fold (Sellappan et al., 2002). Total anthocyanin was ranged from 110 to 260 mg/100g F.F. among ten cultivars and hybrids of lowbush blueberries (Gao & Mazza,

1994). Kalt and McDonald (1996) reported that ripe fruit of 'Fundy' had about 40% higher anthocyanin than of 'Blomidon'. Variation in the content of phenolic and anthocyanin compounds within same species is mainly due to differences in the berry genotypes, or differences in the growth and maturity stages of fruits, or in growth conditions of plants.

Several studies showed significant differences in antioxidant activity among various types, species, cultivars and genotypes of blueberries (Kalt et al., 1999a; Castrejón et al., 2008; Cardeñosa et al., 2016). Lowbush blueberries have significantly higher antioxidant activities compared to highbush, rabbiteye and southern highbush blueberries (Prior et al., 1998; Kalt et al., 2001a; Sellappan et al., 2002; Ehala et al., 2005). Moyer et al. (2002) and Wang et al. (2015) reported that rabbiteye blueberries had the highest antioxidant capacity among the genotypes of nine *Vaccinium* species, followed by *V. angustifolium, V. ovatum* and *V. parvifolium*. This might be due to thicker skin of rabbiteye blueberry having higher concentrations of anthocyanins (Skrovankova et al., 2015). Antioxidant activity ranged from 19.7 to 38.3 µmol trolox equivalent (TE)/g F.F. and from 8.11 to 26.5 TE/g F.F. among the Georgia-grown rabbiteye and southern highbush blueberry cultivars, respectively (Sellappan et al., 2002). Prior et al. (1998), reported an overall range in antioxidant activities from 13.9 to 45.9 µmol TE/g F.F. in their study of northern and southern highbush, rabbiteye, and lowbush blueberry genotypes harvested in a single year, with considerable overlap in antioxidant activity values among the genotypes of different species. Howard et al. (2003) found that antioxidant activity determined as oxygen radical scavenging capacity (ORAC) of blueberry genotypes ranged from a low of 20.5 mmol TE/kg F.F. to a high of 60.3 mmol TE/kg F.F. reflecting a 2.9-fold difference. In other

studies, antioxidant activity was varied 1.8-fold (Kalt et al., 1999a), 2.5-fold (Prior et al., 1998), 3.3-fold (Sellappan et al., 2002), 4.7-fold (Connor et al., 2002a), 5.2-fold (Moyer et al., 2002), and 6.8-fold (Ehlenfeldt & Prior, 2001) among blueberry cultivars and wild clones. The variances in total phenolic and anthocyanin content between cultivars and maturity stages are pertinent for the changes in the antioxidant activity of blueberry.

1.2.8.3.2 Growth and maturity stages of blueberries

The synthesis of phenolic and flavonoid compounds varies significantly in relation to the physiological state of fruits, being a result of equilibrium between biosynthesis and further metabolism. Most important control mechanisms in the phenolic metabolism include synthesis and activities of enzymes, location of enzymes, accessibility of precursors and intermediates, and integration in the differentiation and development programs (Macheix et al., 1990; Harborne, 1994). A number of studies have confirmed that concentration of phenolic compounds is usually higher in young fruit tissues which drop steadily with the advancement of maturity stages in some species of white-colored fruits such as white grape, mango and banana (Macheix et al., 1990). However, it rises at the end of maturation in most of the red, purple or blue fruits such as raspberries, lingonberries, cranberries and blueberries in which anthocyanins are noticeable flavonoids (Macheix et al., 1990; Wang & Lin, 2000; Ribera et al., 2010). Thus, accumulation of phytochemical pigment plays an important role during maturity or ripening of berries.

The total phenolic content in unripe and fully ripe fruits was high and similar in highbush blueberry and black raspberry cultivars, whereas the lowest level of phenolic compounds

was found in the fruits at intermediate maturity stage (Wang & Lin, 2000; Ribera et al., 2010; Forney et al., 2012). Connor et al. (2002a) reported that increased maturity at harvest increased the total phenolic content in highbush blueberries. Although phenolic content increases linearly in blueberry skin extracts with increasing fruit maturity (Ribera et al., 2010), it reached the lowest level in whole berries and pulp at fully maturation stage (Kalt et al., 2003; Castrejón et al., 2008; Ribera et al., 2010). Substantially higher levels of total phenolics were reported in fully ripe fruits, compared to semi-ripe and unripe-green fruits of red raspberries, strawberries and mulberries (Wang & Lin, 2000; Mahmood et al., 2012). Unripe green lowbush blueberries have the same concentration of most common phenolic compound chlorogenic acid as fully ripe and overripe fruit. Whereas, the other phenolic compounds, such as cinnamic acid, p-coumaric acid, ellagic acid and their derivatives are generally high in young fruits of blueberries, strawberries and raspberries which fall slowly during maturation (Maas et al., 1991; Kosar et al., 2004; Castrejón et al., 2008). It is suggested that during ripening there is phenolic conversion toward anthocyanin synthesis that results in changing overall phenolic content.

The three common types of flavonoids (flavonols, anthocyanins and proanthocyanidins) that accumulate in blueberry fruits (Prior et al., 2001; Zifkin et al., 2012) vary in their concentration at different fruit maturation stages. In general, immature fruits have less anthocyanin content than fully ripe red, blue and black fruits. Following the green stage of fruits, anthocyanins are increasingly synthesized in parallel with the overall development and maturation of fruits whereas flavonols synthesis is decreased (Castrejón et al., 2008). Blueberries harvested at the ripe stage consistently yield higher anthocyanin than those

harvested during the green or semi-ripe (immediately after turning blue) stages (Connor et al., 2002a). Kalt et al. (2003), Castrejón et al. (2008) and Forney et al. (2012) reported that green fruits of highbush blueberry and cranberry contained very low or undetectable levels of anthocyanins which increased strongly with the progress of fruit maturation. Anthocyanin content was highly increased from unripe berries to ripe berries, but it was decreased in overripe fruits of lowbush blueberries (Kalt & McDonald, 1996). Prior et al. (1998) reported that total anthocyanin content of blueberries and bilberries were present mostly in skins and they were substantially higher in fruit of more advanced stages of ripeness. However, the other common types of flavonoids proanthocyanidins and flavonols localized predominantly to the inner fruit tissue containing the pulp, seeds and placentae are mostly accumulated in young stage and decreased from unripe green to ripe blue stage of ripening (Castrejón et al., 2008; Zifkin et al., 2012). Synthesis of different flavonoids varied with maturity stages.

Maturation of leaf tissue plays an important role in the phytochemical composition of blueberry species. Riihinen et al. (2008) reported that the red leaves of highbush blueberry contain higher amounts of certain phenolics such as *p*-coumaric, caffeic, ferulic acids than the green leaves. They contain very low level of anthocyanins, which are absent from the green (Ferlemi et al., 2016). Percival and MacKenzie (2007) reported that substantially higher levels of polyphenolic compounds and anthocyanins were found in the red leaves compared to young green leaves of lowbush blueberries. However, the leaves become older, the total phenolic content decreased in some cultivars of blackberry, strawberry and raspberry (Wang & Lin, 2000). Not only individual phenolic compounds in leaves vary

with the progress of leaf maturity, but also the total phenolic content increased in red leaves.

Antioxidant activity of blueberries generally varies as per progress in fruit maturation which is genotype specific. In highbush blueberries, Connor et al. (2002a) reported that the levels of fruit maturity and the interaction between bush ripeness and fruit maturity had significant effect on their antioxidant activity. Berries with 100% blue coloration (ripe) harvested from the bush with 60–80% matured fruits exhibited a significant increase in antioxidant activity over the berries with 50% and 75% blue coloration (semi-ripe). Prior et al. (1998) and Kalt et al. (2003) showed that antioxidant capacity of blueberry and cranberry increased linearly at increasing maturity stages. Level of antioxidant activity also diverged in whole fruits and its individual tissues (skin, pulp or seed) in respect to maturity stages. Ribera et al. (2010) reported that in the late stages of maturity such as >75% red, 100% red and 100% blue coloration (ripe), DPPH (1,1-diphenyl-2- picrylhydrazyl) radical scavenging capacity increased steadily in whole blueberry and its skin but it decreased in the pulp with the advance in fruit maturity. However, total antioxidant activity of whole fruits and pulp decreased from unripe green stage to red colored fruits (≤75% red coloration). The antioxidant capacity of fruit was highest in the least mature fruit of blueberry, strawberry and blackberry which was declined as fruit matured (Wang & Lin, 2000; Castrejón et al., 2008; Forney et al., 2012). Similar trend in antioxidant activity was reported in leaf of blackberry, raspberry and strawberry cultivars, as the leaves become older, the antioxidant values decreased (Wang & Lin, 2000). The high antioxidant capacity in green stage of fruits may be due to high phenolic content in immature fruits and high

capacity in ripe fruit may be because of high anthocyanin in mature berries.

1.2.8.3.3 Different tissues of blueberry plants

Phenolic compounds are not evenly distributed in leaf, flower, fruit or even in the different fruit parts of blueberry, lingonberry, cranberry and strawberry. Those compounds vary considerably from one tissue to another. Compare with fruits, leaves of blueberries contain significantly higher phenolic content (Ehlenfeldt & Prior, 2001; Percival & MacKenzie, 2007; Wang et al., 2015), although anthocyanin content is highest in the ripe berries. Harris et al. (2007) reported that the phenolic profile of lowbush blueberry leaves was highly similar to that of highbush blueberry counterparts. Chlorogenic acid was the most abundant phenolic and it was 30 times more concentrated in the leaf extract than in the respective fruit and over 100 times more concentrated than in the stem or root extracts, respectively (Harris et al., 2007). In strawberry, concentrations of phenolic compounds in leaf extracts were found at least five times higher than in fruit extract (Yildirim & Turker, 2014). Higher concentration of phenolics, flavonoids, anthocyanins and condensed tannins were reported in leaves of wild blueberries, lingonberries, cranberries and bilberries compared to their fruits (Vyas et al., 2013a; Vyas et al., 2013b; Teleszko & Wojdyło, 2015). Conversely, Alam et al. (2016) reported that mean phenolic content was higher in fruit than in leaves of wild lingonberry populations across Newfoundland and Labrador, Canada.

Antioxidant activities of *Vaccinium* species differ significantly in various plant tissues. Vyas et al. (2013a; 2013b) reported that antioxidant activities measured as DPPH radical scavenging capacity and reducing power of ferric ions were much higher in the leaves of

Newfoundland grown lowbush blueberries and lingonberries compared to their fruits. Naczk et al. (2003) also found a high level of DPPH radical scavenging activity and reducing power in wild blueberry leaves collected from Antigonish county, Nova Scotia, Canada. Antioxidant capacity was significantly higher in the leaf tissues of 87 highbush and half-high blueberry cultivars than in fruits of respective genotypes (Ehlenfeldt & Prior, 2001). Antioxidant capacity of leaves was 6-8-times of fruits in strawberry and blackberry (Wang & Lin, 2000). Comparing with root, the leaves of two highbush blueberry cultivars 'Legacy' and 'Bluegold' have more than double DPPH radical scavenging capacity (Reyes-Díaz et al., 2010). Higher antioxidant capacity in leaves compared to fruits due to the high levels of phenolic and flavonoid content in leaves.

Accumulation of phenolic compounds and their antioxidant activities vary with different parts of an individual fruit. Soluble polyphenolics are in higher level in the external tissues (epidermal and sub-epidermal layers) of small fleshy berries than in the internal tissues (mesocarp, pulp or seed) (Macheix et al., 1990). Since the phenolic especially anthocyanin synthesis depends on light, they are mainly found in the skins of berries (Zoratti et al., 2015). Ribera et al. (2010) reported that phenolic and anthocyanin content was significantly higher in the fruit skin of ripe blueberries compare with those found in pulp and whole fruit extracts. Total antioxidant activity in skin of ripe blueberry was around 7 and 192 times higher than in whole fruit and its pulp, respectively (Ribera et al., 2010). Mainland et al. (2002) reported that antioxidant activity in skin of ripe blueberries was 4-fold higher than that found in whole fruits. Overall blueberry skin has higher antioxidant capacity compare to pulp.

Smaller fruits have more epidermal tissue or skin per unit volume than larger fruits and thus small-size blueberries typically have higher phenolic and anthocyanin content and antioxidant activity (Connor et al., 2002c; Howard et al., 2003). Significant inverse relationships between berry weight and/or size especially fruit diameter and the level of polyphenolics, anthocyanins and their antioxidant activities were reported in several blueberry species (Prior et al., 1998; Ehlenfeldt & Prior, 2001; Connor et al., 2002c). Wang and Lin (2000) also reported that small green strawberry had the highest total phenolic content which steadily decreased with the increase in size and maturity stages. Small berries having higher skin pulp ratio confirm higher antioxidant capacity.

1.2.8.3.4 Growing seasons and locations

Plant phenolics respond to the physical environment such as light, temperature, humidity, precipitation (Hansen et al., 2006; Zoratti et al., 2015; Alam et al., 2016). Higher temperature results in significantly higher flavonoids content in strawberry fruit compared to fruit produced in cool day and night temperatures (Wang & Zheng, 2001). Hansen et al. (2006) reported that the concentration of condensed tannins (proanthocyanidins) in leaves of lingonberry increased with the increase in shading and temperature, whereas the levels of flavonols and other low molecular weight phenolics in leaves of arctic-alpine (*Salix herbacea* × *polaris*) were decreased under the same conditions.

Variations in phytochemical content in blueberries are affected by growing season, ecoregion, soil fertility, soil pH and cultivation practice. The effect of the cultivation location on phenolic and flavonoid synthesis was reported in the lowbush, highbush and

interspecific hybrid blueberry cultivars. Connor et al. (2002c) reported that the antioxidant metabolite content in highbush blueberries harvested from three sites in Michigan, Minnesota and Oregon in a single year was varied significantly across locations. Whereas, Prior et al. (1998) found no substantial difference in antioxidant metabolite content in 'Jersey' cultivar grown in those locations. Häkkinen and Törrönen (2000) reported differences in phenolic content among blueberry cultivars grown in two different parts of Finland. The total phenolic content was higher in 'Northcountry' and 'Northblue' cultivars grown in Piikkio, in southwestern Finland (5.0 and 6.3 mg/l00 g F.F., respectively) compared to the same cultivars in Kuopio, in eastern Finland (4.4 and 4.7 mg/l00 g F.F.). Andreotti et al. (2014) reported that highbush blueberry cultivars ('Berkeley', 'Bluecrop', 'Blueray', 'Bluetta', 'Brigitta' and 'Toro') produced more anthocyanins when cultivated under open-field conditions at higher altitudes in Trento and Cuneo districts in Italy. This may reflect differences in climate and cultural practices among locations, including differences in sunlight or radiation, temperature, water stress, and mineral nutrient availability.

The growing season has strong influence on the phenolic accumulation in blueberry species. Biotic and abiotic factors in growing conditions varied remarkably from year to year which affect the content of phenolic compounds in fruits. Significant main effects of growing season and genotype × growing season have been reported for phenolic and anthocyanin content in blueberries (Connor et al., 2002b; Howard et al., 2003). The phenolic content of several highbush and half-high blueberry cultivars grown at three locations varied considerably (-24 to 56%) over two growing seasons (Connor et al., 2002b;

2002c; Howard et al., 2003). Kalt and McDonald (1996) and Percival and MacKenzie (2007) found that seasonal variations on phenolic and anthocyanin content among lowbush blueberry cultivars was quite remarkable in fruit harvested from the same site. Anthocyanin levels varied by up to 2.4-fold for 'Blomidon', 1.8-fold for 'Cumberland' and 2.0-fold for 'Fundy' cultivars of lowbush blueberries over seven growing seasons (Wang, 2007). Kalt et al. (1999b) reported that anthocyanin content varied up to 30% between two growing seasons, whereas Connor et al. (2002c) found several highbush and inter-specific hybrid blueberry cultivars varied from -35% to 40% over two growing seasons for anthocyanin content. Another common berry flavonoid condensed tannin exhibited a pronounced seasonal variation. The peak proanthocyanidin concentration in leaf of *S. herbacea* × *polaris* was in mid-summer and in leaf of *V. vitis-idaea* was in late-summer which was lowest in early summer for both species (Hansen et al., 2006). The effect of seasonal variation is significant on the content of phenolics and flavonoids in blueberries.

Antioxidant activity of blueberries varies from location to location and from year to year, but this variation is genotype specific. Howard et al. (2003) compared the antioxidant activity for 18 blueberry genotypes in two growing seasons and found that 7 genotypes had higher antioxidant capacity in one season, 4 had higher capacity in another season and 7 had similar potentiality over the two growing seasons. The differences in antioxidant values between the two growing seasons were more than 60% within some genotypes. Connor et al. (2002c) reported that the antioxidant capacity varied considerably in highbush blueberry cultivars 'Jersey' 'Bluegold' and 'Northland' grown at Michigan, Minnesota and Oregon in USA over two growing seasons. The average antioxidant activities of nine highbush

blueberry cultivars were substantially lower in Michigan than in Minnesota and Oregon. Whereas, the growing location (Oregon vs Michigan vs New Jersey) did not affect oxygen radical scavenging capacity values of 'Jersey' in Prior et al. (1998) study. It is noticeable that blueberry fruits grown in southern Chile have exceptionally higher antioxidant activities compared with those cultivated in the northern hemisphere (Ribera et al., 2010). The effects of production year and location on phytochemical content and antioxidant activity are dominant and genotype specific.

1.2.8.3.5 Different propagation methods

There are numerous reports on the advantages of micropropagation in the production of the antioxidant phytochemicals from many medicinal plants to fulfill the high pharmaceutical demands and several reviews that compile many of this information (Chattopadhyay et al., 2002; Karuppusamy, 2009; Dias et al., 2016; Giri & Zaheer, 2016). Although blueberry is one of the highest phenolic containing fruits, application of tissue culture to enhance the antioxidant quality of fruit is rare. Georgieva et al. (2016) reported that the phenolic content was higher in fruit extract of strawberry (*Fragaria vesca* L.), raspberry (*Rubus idaeus* L.), bilberry (*V. myrtillus* L.) and lingonberry (*V. vitis-idaea* L.) propagated *in vitro* compare to the plants grown *ex vivo*. Debnath (2009c) reported more anthocyanin content in tissue culture derived strawberries than conventionally propagated plants. The content of soluble phenolics, flavonoids and proanthocyanidins was augmented in fruits of *in vitro*-propagated lingonberry cultivars (Vyas et al., 2013a). Micropropagation enhances phytochemical synthesis in berry of different plant species.

Leaf tissue responds in separate way to the propagation techniques for their phenolic content. Concentrations of phenolic compounds in leaf extracts of field-grown woodland strawberry were much higher than in extracts of *in vitro*-grown leaves and callus (Yildirim & Turker, 2014). Micropropagated lingonberry leaves contain less phenolics, anthocyanins and proanthocyanidins compare to conventional stem cutting derived plants (Vyas et al., 2013a). However, flavonoid content was higher in micropropagated leaf tissues than in those of stem cuttings.

Propagation techniques affect the antioxidant activity in berries and leaves. Georgieva et al. (2016) reported that the total antioxidant activity was higher in fruit of *in vitro* propagated plants of strawberry, raspberry, bilberry and lingonberry compare to the plants grown *ex vivo*. The total DPPH radical scavenging capacity was higher in fruit extract of three lingonberry cultivars 'Regal', 'Erntedank' and 'Splendor' derived through node and leaf cultures compared to conventional stem cutting plants (Vyas et al., 2013a). Debnath (2009c) also reported that micropropagated strawberries exhibited higher antioxidant activities than those produced by the runner cuttings. Whereas, higher antioxidant potential was reported in the leaf extract of conventionally propagated plant of sweet passion fruit (*Passiflora alata*) compared to *in vitro* derived plants (Lugato et al., 2014). Vyas et al. (2013a) reported that antioxidant capacity of leaf tissue of lingonberry was not affected by propagation methods.

1.2.9 Genetic fidelity of micropropagated blueberry

In tissue culture system, the cultured explants reset its genetic and epigenetic program to

endure the stress in the artificial media and other physical environmental conditions which determine its fitness and adaptability *in vitro*. Due to these dynamic processes composed at the molecular level, off-types or somaclonal variants are often identified among the tissue culture derived progenies (Neelakandan & Wang, 2012; Landey et al., 2015) and this is considered a major problem in commercial micropropagation wherein the regenerant population is expected to be homogenous. Alterations in genetic and molecular levels which cause somaclonal variation or loss of genetic integrity in tissue culture regenerants, are controlled by several factors including genotype, presence of chimera tissue, degree of deviation from organized meristematic growth, ploidy level and age of donor plant, type and source of explant, cultural environment (temperature, light, etc.), types and concentrations of endogenous and exogenous plant growth regulators particularly auxin-cytokinin balance in media, duration and number of subcultures (Gaj, 2004; Landey et al., 2015). Higher concentration of growth regulators in tissue culture media accumulated much genetic variation which cause the morphological changes such as curled shoots, hyperhydricity and undifferentiated shoots in somaclones derived in micropropagated shoot tip culture of banana (Bidabadi et al., 2010). Since somaclonal variation due to genomic alteration is risk in *in vitro* propagation, the magnitude of which needs to be quantitatively determined (Neelakandan & Wang, 2012). Several techniques used to determine the genetic variations among the regenerants or somaclones consist of morphological markers, biochemical markers including secondary metabolites and macromolecules (proteins), and molecular markers that allow the detection of specific DNA sequence differences between two or more individuals.

1.2.9.1 Isozyme and metabolite marker systems

Isozymes are characterized by similar chemical structure and catalytic properties but products of different genes or different alleles at the same locus (called allozymes) (Hunter & Markert, 1957). The protein coding technique using isozyme (or allozyme) markers has been employed for long time as one of the best markers close to DNA level to assess genetic similarities among plant genotypes and between clones or progenies and parents (Sulkowska, 2012). Isozyme analysis method enables to assess the variability of isozymes in individuals at different level such as within species, within population and among populations within species (Hokanson & Hancock, 1998), and in different types of tissue such as young leaves, buds, pollen and seeds (Sulkowska, 2012). Isozymes were found to be useful markers for somaclonal variation. Martelli et al. (1998) reported isozyme polymorphism among the regenerants derived from leaf culture of apple, and they could distinguish rootstocks from regenrants based on polymorphism in isozyme banding patterns. In higher plants, major portion of the genome (nearly 90%) are not expressed at the phenotypic level (Dahlberg, 2000). These markers are not neutral to environmental effects or management practices. The inadequacies of the biochemical markers resulted in the development of DNA-based markers (Kan & Dozy, 1978) which detect nucleotide sequence variation at a particular location in the genome and can be used for clonal fidelity study in micropropagated berry plats.

1.2.9.2 DNA markers

The introduction of molecular markers to investigate polymorphism among genotypes is

one of the most critical developments in molecular biology. DNA markers are scattered throughout the plant genome. Since the noncoding (unexpressed) as well as coding (expressed) regions of genome are accessible to DNA markers to sort out the relationship among plant species, they are capable to differentiate very closely related genotypes such as tissue culture regenerants, somaclones which may not be possible in phenotypic analysis. Molecular markers are universal to most of the living organisms and they are powerful tools to determine precisely the origin of plants from different tissue culture systems such as microspore or another culture, protoplast fusion, node and leaf cultures tissue culture studies where this information is important (Cloutier & Landry, 1994; Weising et al., 1995). They are not influenced by environmental factors as in phenotypic and karyologic analysis.

DNA markers are categorized as hybridization-based markers, polymerase chain reaction (PCR)-based markers and DNA chip and sequence based markers (Debnath, 2016). Restriction fragment length polymorphism (RFLP) is hybridization-based markers. PCR-based markers are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR) and simple sequence repeat (SSR). DNA markers are further classified as dominant markers such as RAPD, AFLP, ISSR and co-dominant markers like RFLP, SSR and expressed sequence tag (EST)-PCR. A number of molecular markers mostly PCR-based are available for genetic analysis of tissue culture-raised plants (Debnath, 2014a). PCR development has set the stage to overcome many of the shortfalls in the Southern blotting RFLP technique (Saiki et al., 1985). PCR-based DNA marker systems can be divided into two basic classes; those that

use primers designed from arbitrary or non-specific sequences such as RAPD and AFLP, and those that use primers designed from known sequence for targeting a single specific locus such as SSRs. Each marker system has its own strengths and limitations which are considered to choose a DNA marker for the assessments of genetic integrity or DNA fingerprinting.

1.2.9.2.1 Restriction fragment length polymorphism (RFLP)

RFLP technique was applied first as a tool for genetic analysis to find out the linkage of temperature-sensitive mutations in adenovirus in 1974 (Grodzicker et al., 1974). Since then it has been widely used in mapping of genome, identification of species, evaluation of genetic diversity, paternity and localization of genes for genetic disorder in different plant species (Debnath, 2008; Debnath et al., 2012a). Although RFLP is unlimited, they require extensive laboratory techniques, including the development of specific probe libraries, use of autoradiography and Southern blot hybridizations (Williams et al., 1990; Kesseli et al., 1994). RFLP are laborious, time consuming, costly and incompatible with the high analytical throughput required for many applications.

1.2.9.2.2 Random amplified polymorphic DNA (RAPD)

The RAPD marker was introduced by Williams et al. (1990). The basis of RAPD technique is the PCR-based random amplification of genomic DNA using the short (10-base pair) primers of arbitrary nucleotide sequence. RAPD markers are proved to be one of the simple and efficient techniques for identifying the cultivars and clones of blueberry and

lingonberry, which could easily be distinguished by their characteristic polymorphic banding patterns. Although Gajdošová et al. (2006) distinguished the cultivars of *Vaccinium* sp. from each other, they found no differences in the amplified DNA profiles of the mother plants and any of the clones derived from either axillary shoot proliferation or from adventitious organogenesis using isolated meristem. RAPD markers also detected genetic integrity among the mother plant and *in vitro* derived progenies in of grape (Khawale et al., 2006; Alizadeh & Singh, 2009), banana (Lakshmanan et al., 2007), apple (Modgil et al., 2005; Gupta et al., 2009) and pineapple (Feuser et al., 2003). However, Biswas et al. (2009) detected polymorphism in RAPD profile among the regenerants in strawberry. They proposed the phenotypic changes in somaclones were due to genetic variation. In garlic, 0.35-8% of somaclonal variation was detected by using RADP (Al-Zahim et al., 1999). The advantages of RAPD are: (i) those are simple, quick and little amount of DNA is required to generate significant polymorphisms, (ii) these markers can be used to detect polymorphisms in the absence of specific nucleotide sequence information, (iii) radioactive probe like in RFLP technique is not essential (Debnath et al., 2012b). However, RAPD technology has some drawbacks such as problematic reproducibility among laboratories, markers with dominant inheritance and less informative in some genetic studies than co-dominant markers (Jones et al., 1997). This analysis is not sufficiently sensitive for the detection of somaclonal variation in the plants of *Begonia* × *hiemalis* (Fotsch.) cv. Schwabenland Red (Bouman & De Klerk, 2001). However, this technique is easier to use than RFLP method.

1.2.9.2.3 Amplified fragment length polymorphism (AFLP)

AFLP technique has been introduced by Vos et al. (1995) in which the reliability of the RFLP technique (Botstein et al., 1980) is combined with the power of the PCR technique (Mullis & Faloona, 1987). In RAPD and arbitrarily primed-PCR methods, DNA fragments are amplified without prior knowledge in DNA sequence, and the produced fragment patterns depend on the primer sequence (Welsh & McClelland, 1990). In these techniques, the primers may anneal to multiple loci in the DNA at low annealing temperatures, and fragments are produced when primer binding sites are within a distance that allows amplification. Whereas, in AFLP technique, PCR amplification of restriction fragments is accomplished by using the oligonucleotide adapter which serves as target sequence for primer annealing. The selective amplification is achieved by using the primers, which are complementary to the known combined sequence of adapter, the restriction site and a few extra nucleotides, that extend into the restriction fragments (Vos et al., 1995). The AFLP technique provides a novel and very powerful tool for DNA fingerprinting in cranberry and blueberry (Polashock & Vorsa, 1997). This technique allows the specific co-amplification of high numbers of restriction fragments. Less reproducibility problem, higher speed and accuracy of detection were reported in AFLP compared to in RAPD (Vos et al., 1995; Polashock & Vorsa, 1997). A major drawback for AFLP is that they are dominant markers, like RAPD; therefore, heterozygotes cannot be distinguished from homozygotes (Nybom, 2004). AFLP is robust and reliable because it employs longer primers and higher annealing temperatures or stringent reaction conditions, but needs more steps, and the cost is higher

than RAPD. However, since more polymorphic information can be detected by a single AFLP reaction, the relative cost is less.

1.2.9.2.4 Inter simple sequence repeat (ISSR)

Inter simple sequence repeat (ISSR) is a simple and quick method that combines most of the advantages of microsatellites or simple sequence repeats (SSRs) and AFLP to the universality RAPD (Reddy et al., 2002). In ISSR technique, SSRs are used as primers to amplify mainly the inter SSR regions at an amplifiable distance between two identical microsatellites oriented in opposite direction. The primers used can be either anchored at 3′ or 5′ termini with 1 to 4 degenerate nucleotide bases extended into the flanking sequences (Zietkiewicz et al., 1994), or unanchored (Gupta et al., 1994; Wu et al., 1994). They revealed higher levels of polymorphism than RAPD due to longer primer sequences (16 - 25 bp) and the higher annealing temperature (45 - 60 °C) leading to higher stringency (Qian et al., 2001). These markers are cost effective, easy to use and have high reproducibility (Lakshmanan et al., 2007). They do not require prior knowledge of flanking sequences like RAPD and SSRs (Reddy et al., 2002). However, ISSR markers segregate mostly as dominant markers following simple Mendelian inheritance (Gupta et al., 1994). Their co-dominant segregation also reported in some cases thus enabling distinction between homozygotes and heterozygotes (Wu et al., 1994). Compared with RAPD and AFLP, ISSR exaggerates differences between closely related populations and thus can now be used in micropropagated berry plants to verify clonal fidelity. ISSR makers have been used to assess the genetic fidelity of different small fruit crops. Debnath (2009c) used ISSR

technique to assess the genetic fidelity of strawberry plantlets obtained through adventitious shoot regeneration using sepals, leaf disks, and petiole halves explants and found homogenous amplification profile in the tissue culture progenies and donor plants confirming the clonal fidelity of micropropagated strawberry. ISSR markers were also used in other fruit species such as grape, apple, banana (Lakshmanan et al., 2007; Alizadeh & Singh, 2009; Nookaraju & Agrawal, 2011; Pathak & Dhawan, 2012) to evaluate genetic integrity.

1.2.9.2.5 Simple sequence repeats (SSR) and expressed sequence tag (EST)-SSR markers

Simple (short) sequence repeats (SSRs) or microsatellites are short tandem repeats or variable number of tandem repeats ranging from mononucleotide up to penta-nucleotide (Weber & May, 1989) and dispersed universally throughout the plant genomes covering its' significant portion (Morgante & Olivieri, 1993; Wang et al., 1994b). Those are multi-allelic, highly polymorphic and simple to detect by PCR using the locus-specific markers that flank the microsatellite motifs termed sequence tagged microsatellite site (STMS) (Senthilvel et al., 2008; Debnath et al., 2012a). SSRs typically provide single-locus markers which are often co-dominantly inherited and are characterized by hypervariability and reproducibility.

Most of the molecular markers including AFLP, RFLP, RAPD, ISSR, SSR are related to genomic DNA. They could belong to either the transcribed region or the non-transcribed region of the genome, and so they have been described as random DNA markers (RDMs)

by Andersen and Lubberstedt (2003). Recently, molecular marker technology in higher plants has witnessed a shift from the so-called RDMs to the molecular markers representing the transcriptomes or genes, commonly known as functional markers (Gupta et al., 2015). Functional markers are preferred over RDMs because of their complete linkage to the trait of interest and target the functional polymorphism in the gene. Recent studies have indicated large numbers of SSRs are present in coding regions, expressed sequence tags (ESTs) or in gene (Li et al., 2004) called EST-SSR markers. Identification of SSRs in gene sequences (genic SSRs or EST-SSRs) of plant species was carried out as early as 90's by Morgante and Olivieri (1993).

ESTs are unedited, automatically processed, single-read sequence (300−500 bp) produced from complementary DNA (cDNA; small DNA molecules reverse-transcribed from a cellular mRNA population). ESTs are originally anticipated to identify gene transcripts, but have been used to discover gene, obtain data on gene expression and regulation, sequence determination, and to develop highly valuable molecular markers, such as EST-based SSR and PCR markers (Rowland et al., 2003; Boches et al., 2005). EST-SSRs are easily transferable to closely related species (Wang et al., 2005; Aggarwal et al., 2007; Dai et al., 2013) and they facilitate the use of the candidate gene mapping approach. This marker system offers the possibility of selecting markers according to the biochemical and physiological properties of their gene products in relation to the phenotype (Chee et al., 2004). With the evolving bioinformatic tools it is now possible to identify and develop EST-SSR markers at a large scale in a time and cost-effective manner (Varshney et al., 2005). However, exploitation of the source of SSR markers is obviously limited to the

species having sufficient sequence data (for ESTs or genes) available. SSRs are present in only 2% to 5% of the unigenes examined.

EST-SSR markers are first developed by Boches et al. (2005) for blueberries from two EST libraries and from a microsatellite-enriched genomic library, constructed from *V. corymbosum* cv. 'Bluecrop' DNA. They used a total of 1305 EST sequences from both cold acclimated (CA) and non-acclimated (NA) EST libraries constructed from floral buds (Rowland et al., 2003; Dhanaraj et al., 2004) and 136 SSR-enriched genomic sequences to isolate 30 microsatellite markers. Because of lower polymorphism compared to genomic SSRs in crop plants due to greater DNA sequence conservation in transcribed regions, EST-SSRs are not as efficient as genomic SSRs for distinguishing the closely related genotypes or for clonal fidelity analysis (Russell et al., 2004; Chabane et al., 2005). Genic SSR and genomic SSR markers tend to be complementary for genetic fidelity analysis, with genic microsatellites being less polymorphic but concentrated in the gene-rich regions. EST-SSR markers have been frequently used in the genetic fingerprinting and diversity analysis of several *Vaccinium* species such as lowbush, highbush and rabbiteye blueberries (Levi & Rowland, 1997; Boches et al., 2006; Debnath, 2014b, 2016; Tailor et al., 2017) and cranberries (An et al., 2015). Genomic SSRs combined with EST-SSRs are used in clonal fidelity analysis in micropropagated berry species. Debnath (2017) used two genomic SSRs and two EST-SSRs to assess true-to-type propagules in half-high, highbush, and hybrids between half-high/highbush and lowbush blueberries produced through shoot proliferation using nodal explants in liquid and semisolid media. Those markers formed a homogenous monomorphic banding pattern in EST-SSR profile among the regenerants,

and between regenerants and donor plants proving the clonal fidelity of liquid-culture derived micropropagated plants. The plants of red raspberries derived from bioreactor-induced adventitious shoot regeneration maintained clonal fidelity which was detected using SSR markers (Debnath, 2014a). Although SSR markers are frequently used to detect genetic diversity in plant species, they are reliable to evaluate clonal fidelity in berry crops.

1.2.9.2.6 Expressed sequence tag-polymerase chain reaction (EST-PCR) markers

Rowland et al. (2003) first developed seventeen EST-PCR markers by using the CA and NA EST libraries constructed from highbush blueberry cultivar 'Bluecrop' (Levi & Rowland, 1997) to study DNA fingerprinting in blueberry. Since then those are routinely used to differentiate and evaluate genetic relationships among highbush, half-high, rabbiteye and lowbush blueberry cultivars and clones (Rowland et al., 2003; Bell et al., 2008; Bell et al., 2009; Debnath, 2014b). There are several advantages to use EST-based molecular markers (Rowland et al., 2003) such as (i) they target expressed gene, (ii) they are derived from gene coding regions, more likely to be conserved across populations and species than markers derived from noncoding or random regions of DNA, such as RAPD or AFLP markers, (iii) those have the potential for being co-dominantly inherited. There are very few reports available on the use of EST-PCR markers for monitoring genetic fidelity in blueberry plants. Debnath (2011) used first EST-PCR markers for assessing the clonal fidelity in micropropagated lowbush blueberry clone. The author used fourteen EST-PCR markers in lowbush blueberry clones regenerated through adventitious shoot multiplication and confirmed genetic integrity in *in vitro*-derived plants based on the

monomorphic amplification DNA profiles among those clones. Debnath (2017) also monitored trueness to type of micropropagules in highbush, half-high, and hybrids between high/half-high and lowbush blueberries regenerated in a bioreactor containing liquid medium combined with a gelled medium by applying EST-PCR technique. Although the markers enabled to differentiate the genotypes from each other, the propagules derived from each genotype through bioreactor systems were genetically identical.

Since the variation in somaclones or normal regenerants from donor is caused by genetic and/or epigenetic factors, one type of marker is not sufficient to detect the genetic integrity or somaclonal variation in the regenerants of tissue culture systems (Imazio et al., 2002; Landey et al., 2015). Genetic fingerprinting techniques along with analysis of DNA-base methylation could be a good choice to confirm and characterize variability (genetic and epigenetic levels) in tissue culture derived plants (Hanai et al., 2010; Landey et al., 2015). Park et al. (2009) did not find any difference in DNA profiles among somaclonal variants and normal plants of *Doritaenopsis* using even 100 RAPD primers. However, methylation sensitive amplification polymorphism (MSAP) analysis revealed significant differences in the DNA methylation patterns in the normal and variant plants which were correlated with phenotypic variation.

1.2.10 Epigenetic variation in micropropagated plants

Epigenetic variations are defined as mitotically and/or meiotically heritable and non-heritable alterations in gene function without change in the DNA sequence (Russo et al., 1996). Heritable epigenetic variation continues upto few generations in plants. In triticale

cv. ‘Bogo, for example, *in vitro* cultures induced epigenetic alteration especially methylation of genomic DNA proceeded two successive generations (Machczyńska et al., 2014). Non-heritable changes in grape, which are established by exposure to tissue culture and thermotherapy, are reverted and plants returned to epigenetic states similar to those of maternal plants once stress conditions have been discontinued (Baránek et al., 2015). While epigenetic factor affects phenotypic characteristics of *in vitro* regenerants, the epigenetic alteration has been reported in many plants, even in the absence of phenotypic variation (Valledor et al., 2007; Miguel & Marum, 2011; Smulders & Klerk, 2011). Alteration in DNA methylation (or hydroxymethylation), histone modification or simultaneously occurrence of both are the predominant epigenetic factors influencing gene expression in plant tissue culture systems (Chinnusamy & Zhu, 2009). Phenotypic variation caused by DNA methylation.

1.2.10.1 Phenotypic alteration in micropropagated plants due to epigenetic changes

Several changes in phenotypic levels, specifically vitrification (hyperhydricity), recalcitrance (absence or loss of organogenic potential) and somaclonal variation have been reported among regenerants of cell, tissue and organ cultures in several agronomic and horticultural crops which are proposed due to the epigenetic variation (Cassells & Curry, 2001; Song et al., 2008; González et al., 2013a). Aberrant morphology, typically hyperhydrated, translucent tissues, curly and undifferentiated shoots and physiological dysfunction are common in vitrified plants *in vitro* (Ziv, 1991; Bidabadi et al., 2010). The higher variation in leaf shape of begonia plants regenerated from an intermediate callus

phase than that of the plants regenerated directly from leaves *in vitro* was reported due to an epigenetic variation, especially an alteration in DNA methylation (Bouman & De Klerk, 2001). Swartz et al. (1981) and Boxus et al. (2000) proposed that changes in DNA methylation caused discrete morphological variations in micropropagated strawberry such as sporadic occurrences of abnormal fruit setting, hyper-flowering habit and development of special buds (stipular-buds) on a specific position of the leaf petiole. Kubis et al. (2003) reported that the cause of somaclonal variation among the regenerants of leaf culture in oil palm (*Elaeis guineensis*) appeared as mantled phenotype of flowers including abnormal and a few normal flowers in inflorescence leading to fruit abortion and zero yield. Those were not genetic changes rather than the variation in genome-wide distribution and structure of DNA methylation. Decreased methylation in regenerants raised numerous morphological and phenotypic abnormalities in *Arabidopsis thaliana* including decreased apical dominance, reduced plant size, modified leaf size and shape, diminished fertility, and altered flowering time (Finnegan et al., 1996). Deformed flowers including magenta pigmentation at lateral sepals, complete fusion of lateral sepals with labellum and small flowers with faintly magenta petals were postulated as a cause of changes in DNA methylation in somaclonal variants of *Doritaenopsis* (Park et al., 2009). The phenotypic changes in plants due to alteration in DNA methylation raised from tissue culture stress.

Matured plant tissues accomplish the rejuvenation in the tissue culture system and repetitive subculture is commonly used to keep the juvenility of the perennials like blueberry (Lyrene, 1981). Cassells and Curry (2001) reported that persistent juvenility of plants in tissue culture was related to DNA methylation and those plants were more

susceptible to damping-off diseases. However, the juvenile potato plants derived from tissue cultured microplants exhibited more resistance to blight disease compared to a tuber-derived plants (Cassells et al., 1991). Alterations in morphological characters associated with leaf-tip and bud necrosis causing loss of apical dominance, lower leaf number and leaf size, prolonged flowering, and yield quality especially number and size of tubers have been shown among the *in vitro* regenerants of potatoes (Cassells et al. (1999). Qureshi et al. (1992) reported that tissue culture regenerant families of spring wheat were of agronomically inferior genotypes which produced fewer, lighter kernels per spike and yielded less than donors, but they had higher-level grain protein than the control donor plants.

Although the evaluation of epigenetic variations in *in vitro* regenerants has mostly been reported on DNA methylation, a few studies have been focussed on the detection of modifications in histones and small interfering RNA (siRNA) levels which are also responsible for epigenetic variation in plant cultured *in vitro* (Miguel & Marum, 2011). Modifications in histone H3 and histone H4 have been detected in cell suspension cultures of *A. thaliana* (Berdasco et al., 2008; Tanurdzic et al., 2008) and potato (Law & Suttle, 2005) which influenced the levels of siRNA. Williams et al. (2003) detected increased levels of acetylated H3 and modification of Lys9-methylated H3 in protoplast culture of *Nicotiana tabacum*.

1.2.10.2 DNA methylation during micropropagation

DNA methylation refers to the addition of a methyl group to adenine or cytosine bases.

Although adenosine methylation has now been detected in plants (Ashapkin et al., 2002), the presence of functional quantities of adenine DNA methylation in higher eukaryotes is still argumentative (Ratel et al., 2006; Vanyushin & Ashapkin, 2011). In contrast, cytosine DNA methylation is observed in most eukaryotes, and it serves various functions in plants including the silencing of transposons, repeat elements and transcriptional genes (Suzuki & Bird, 2008; Feng et al., 2010; How-Kit et al., 2015). Thus, the term "DNA methylation" usually refers exclusively to the presence of a methyl group on carbon 5 of a cytosine base. In mammals, DNA methylation is almost completely limited to cytosine-guanine (CG) dinucleotides, although, non-CG methylation has recently been observed in embryonic stem cells (Lister et al., 2009). In higher plants, DNA methylation is appeared frequently in CG, CHG (where H is any base other than G) and CHH sequences (Gruenbaum et al., 1981; Finnegan et al., 1996). In *A. thaliana*, 55% of methylated cytosines exist in CG sites, with CHG and CHH sites accounting for 23% and 22% of methylated cytosines, respectively (Lister et al., 2008). Plants possess higher cytosine methylation compare to animals.

In micropropagation, plant cells of organized tissues undergo dedifferentiation followed by redifferentiation or directly organogenesis which is stimulated with the introduction of plant growth regulators in the culture medium. Dedifferentiation or callus formation is a massive commitment for a fully-grown plant system since during this phase, plants give up their fully established body plans and switch to new developmental program once again and turn on the callogenic gene expression process (Ikeuchi et al., 2013). The changes in DNA methylation play an important role in these transitions: from organized tissue state

to dedifferentiation or callus formation and then redifferentiation or organogenesis to develop tissue or organ from callus stage (Huang et al., 2012a). In general, hypomethylation of gene or its promoter influences explant to develop callus. Gao et al. (2014) assessed DNA methylation in hypocotyl explant of rapeseed (*Brassica napus* L.) in adventitious shoot regeneration system and found that the lowest methylation level in hypocotyls performed the highest induction rate of callus. Application of the demethylating agent such as 5-azacytidin in culture media induces hypomethylation at *Hpa*II/*Msp*I recognition sites of 5′-CCGG-3′ in explants causing callus formation and inhibits the induction of adventitious shoots in leaf culture of petunia (Prakash et al., 2003) and cell culture of carrot (LoSchiavo et al., 1989). They also noted that cytosine methylation was restored at 5′-CCGG-3′ sites when the explants were transferred from medium with methylation inhibitor to medium without inhibitor, simultaneously recovered the ability to develop adventitious shoot buds.

The modification in DNA methylation which regulates gene expression is linked with the adaptation of plants in the abiotic stresses. Wild-type tobacco plants exposed to aluminum, salt, paraquat and cold stresses showed a selective decrease of cytosine methylation at 5′-CCGG-3′ sites in the coding region of the *NtGPDL* (glycerophosphodiesterase-like protein) gene (Choi & Sano, 2006). CHG hypermethylation was took place in two heterochromatic loci when tobacco cell cultures were exposed to osmotic stress (Kovarík et al., 1997a). Higher level of methylation events at cytosine of 5′-CCGG-3′ recognition sites was reported in micropropagated banana (*Musa acuminata* cv ‘Grand Nain’) plants (Peraza-Echeverria et al., 2001) and *Jatropha curcas* (Rathore et al., 2015) than those of

conventionally propagated plants. They also found higher level of polymorphism in MSAP profiles among the tissue culture derived plants in comparison of conventionally propagated plants and it was irrespective to source of explant tissues. The differences in the DNA methylation patterns in the somaclones regenerated from tissue culture were higher than in normal plants of *Doritaenopsis* (Park et al., 2009) and potato (Harding, 1994). Ghosh et al. (2017) detected higher number of cytosine methylation events in *in vitro* developed callus compared to leaf tissues of lowbush and hybrid blueberry genotypes. Those studies indicated that changes in DNA methylation level and polymorphisms in MSAP profiles were associated with micropropagation process.

The type and function of endogenous and exogenous growth regulators are related to the DNA methylation in plant tissues during micropropagation. LoSchiavo et al. (1989) reported that a mutant carrot line with higher internal level of IAA showed stable cytosine methylation compared to wild type plants when hypomethylation induced drugs were applied in embryogenic cell culture. The increase concentration of external growth regulators such as 2,4-D, IAA and 1-naphthaleneacetic acid (NAA) in culture media increased the level of DNA methylation in those embryogenic carrot cells. Level of DNA methylation in *Malus xiaojinensis*, a woody perennial, increased with increasing 2,4-D concentrations, and decreased significantly with increasing 6-benzyladenine concentrations (Huang et al., 2012a). The callus of blueberry developed in the media with 0.5 mg/L TDZ had hypomethylation compare to in the media with 0.1 mg/L TDZ (Ghosh et al., 2017). However, Leljak-Levanić et al. (2004) observed that the high level of DNA methylation was not exclusively a consequence of application of exogenous auxin in

media.

The levels and distributions of DNA methylation varied significantly in different developmental phase and growth stage *in vitro*. Due to *de novo* methylation and passive demethylation during DNA replication, plant tissues fail to maintain their existing methylation status during the developmental process *in vitro* (Hsieh, 1999). Leljak-Levanić et al. (2004) reported that cytosine methylation plays a primary role in the control of gene expression during embryogenesis and they observed the level of DNA methylation increased in the early stage of somatic embryogenesis and decreased during embryo maturation stage. Levels of DNA methylation in adult-phase shoot apical meristems of peach (*Prunus persica* L.) were significantly higher than that of juvenile or rejuvenated meristems (Bitonti et al., 2002). The needle maturation of *Pinus radiata* which is associated with a decrease in organogenic capability, is related to increase DNA methylation in heterochromatin region (Valledor et al., 2010). Fraga et al. (2002) reported that the degree of genomic DNA methylation in needles of *P. radiata* was 35% in juvenile and 60% in adult trees, whereas differences in DNA methylation between differentiated tissues of juvenile and mature trees were very small. A gradual decrease in DNA methylation in meristematic areas demonstrated that there was strong correlation between DNA methylation and reinvigoration of plants. The changes in DNA methylation during aging and reinvigoration indicate that reinvigoration could be a consequence of epigenetic modifications opposite in direction to those that occur during aging of plants (Fraga et al., 2002; Joyce & Cassells, 2002). However, stable cytosine methylation was found in different developmental phases (explants, embryogenic callus and regenerated plantlets) of

somatic embryogenesis in *Bambusa balcooa* (Gillis et al., 2007). Variation in DNA methylation with growth stages is specific to plant species.

DNA methylation is changed in the different tissues of a plant derived *in vitro*. Song et al. (2008) found that the level of DNA methylation was higher in dry seeds of *in vitro* derived radish than in leaves. Although the hypocotyl and cotyledon of cauliflower seedlings developed *in vitro* share the same genome, the genomic DNA methylation levels and patterns at 5′-CCGG-3′ sites were different from each other (Li et al., 2014). The authors also indicated that eight out of twelve sequenced fragments showed differential expression between the hypocotyl and cotyledon, of which the expression of six sequences was identified to be negatively correlated with their DNA methylation status. Arnholdt-Schmitt et al. (1995) reported that DNA methylation of root cambium, secondary phloem and leaf petioles of regenerated carrots was strikingly different, and the methylation level of secondary phloem was independent on culture of origin and the age of the plants.

DNA methylation is also affected by the duration in and number of passages of tissue culture. Huang et al. (2012a) reported that global DNA methylation was decreased with the increasing in number of passages of subcultures in *M. xiaojinensis*. Rodríguez-López et al. (2010) reported that leaves of ‘late regenerants’ of cocoa (*Theobroma cacao*) exhibited significantly less epigenetic divergence from source leaves than those exposed to short periods of callus growth, evidencing a progressive erosion of epigenetic variation in callus-derived plants. However, the prolongation of culture time and number of subcultures caused increase in the global DNA methylation of cell lines of *Taxus media* (Fu et al.,

2012). DNA methylation decreases with the increased number of subculture, but it also increases in some plant species in similar situations.

1.2.10.2.1 Detection of DNA methylation

1.2.10.2.1.1 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a quantitative technique to study the global DNA methylation. Relative content of methylcytosine in genomic DNA to its total base composition can be quantified through fractionation of hydrolysis products (four main bases) of DNA using reversed-phase HPLC (Fraga & Esteller, 2002). Although hydrolysis of DNA can be carried out by incubating with organic or inorganic acid at elevated temperature (Catania et al., 1987), Kuo et al. (1980) reported a better option for DNA hydrolysis with Nuclease P1, DNase I and Phosphatase enzymes for quantifying the degree of DNA methylation.

HPLC technique is used to get information on global DNA methylation in plants regenerated *in vitro*. Jaligot et al. (2000) and Kubis et al. (2003) used reversed-phase HPLC technique to determine alteration in DNA methylation among regenerants of oil palm and found the variability in the percentage of methylcytosine was less in regenerants of callus culture (0.5-2.5%) than in the normal mother plant. A decrease in global DNA methylation from the donor plants was reported among regenerants of a tree species (*Cedrus* sp.) derived through shoot proliferation (Renau-Morata et al., 2005). The similar trend was also observed in herbs like triticale and barley (Machczyńska et al., 2014; Orłowska et al.,

2016), where tissue culture reduced DNA methylation of the regenerants compare to donor plants. Sianipar et al. (2008) reported that the change of methylcytosine content between plantlets and mother plant were due to hypomethylation in regenerants of somatic embryogenesis. They also used MSAP as a complementary technique to see the location of the cytosine methylation in plant genome. Both techniques were applied by Chakrabarty et al. (2003) to determine methylation levels in embryogenic and non-embryogenic callus of Siberian ginseng (*Eleuterococcus senticosus*). In qualitative HPLC separation, the global DNA methylation rates were significantly lowered in embryogenic callus than that of non-embryogenic counterparts which were in similar in MSAP analysis where 17% of 5′-CCGG-3′ sites of non-embryogenic callus were cytosine methylated and 11% sites were methylated in case of embryogenic callus tissue. However, by HPLC approach, Baurens et al. (2004) could not differentiate the methylation levels in microshoots of *Acacia mangium* regenerated from juvenile explants from those in the microshoots derived from matured explants. Whereas, they identified six age-specific MSAP markers among which three were exclusive in the juvenile plant material and three sites were exclusive to the mature source. Although the degree of global cytosine methylation can be quantified using HPLC, it requires access to sophisticated equipment that is not always available.

1.2.10.2.1.2 Sodium bisulfite modification (SBSM)

Sodium bisulfite modification (SBSM) technique was first reported by Frommer et al. (1992) and optimized by Clark et al. (1994) to detect cytosine methylation in individual DNA strands of a particular genomic sequence. SBSM assay involves denaturation of

genomic DNA, modification of DNA by sodium bisulfite, conversion of all unmethylated but not methylated cytosines (mC) bases to uracils creating non-complementary strands (i.e., uracils as opposed to guanines), and subsequent PCR amplification with primers specific for methylated versus unmethylated DNA. Following PCR amplification, the uracils are amplified as thymines, whereas mC residues are amplified as cytosines. To determine methylation status at single nucleotide of specific loci, the PCR amplicon(s) is either sequenced directly or cloned followed by sequencing (Clark et al., 2006). Krizova et al. (2009) studied the stability of the methylation patterns in cell culture and regenerated transgenic tobacco plants using SBSM technique. They observed decreased expression of transgene post-transcriptionally gene silence (PTGS) *Lo1* and partial loss of methylation in promoter region of that gene in callus compared to leaf tissue in the parent. Cells having *Lo1* gene in callus culture with no, intermediate, and high levels of methylation, demonstrated cell-to-cell methylation diversity in callus. How-Kit et al. (2015) determined CG and non-CG cytosine methylation in the promoter regions of two tomato genes (*NOR* and *CNR*) controlling fruit developmental stages during ripening using SBSM coupled with high throughput locus specific pyrosequencing and observed that at later developmental stages, the highly methylated cytosines of the *NOR* and the *CNR* promoter showed a progressive decrease in methylation during fruit development and became almost completely unmethylated at fully matured fruits. However, Lister et al. (2008) and Cokus et al. (2008) used second-generation sequencing with SBSM in the whole genome bisulfite sequencing experiments of *A. thaliana* to produce single base resolution methylome. About 78–93% of genomic cytosines were detected using this technique and more than 5% of

those were methylated, around half of which were in CG nucleotide context and the remaining half in CHG and CHH contexts (Cokus et al., 2008). Differential methylation pattern in 5′-CCGG-3′ sites of *Petunia* shoots regenerated in the methylation-inhibitor treated and control cultures was identified by Prakash et al. (2003) using SBSM and MSAP. They postulated that cytosine methylation at 5′-CCGG-3′ and 5′-CGCG-3′ sites within a *MADS-box* gene and a *CDC48* homologue showed strong positive correlation with adventitious shoot bud induction in *Petunia* leaf explants.

The methylation status at any group of CG sites within a CG island can readily be assessed by SBSM technique which is independent on the use of Methylation sensitive restriction enzymes. This technique requires only small quantity of DNA to determine the methylation state of every cytosine residue in the target sequence. Although SBSM technique is very sensitive by which even 0.1% methylated alleles of a given CG island locus can be detected, the reaction is highly single-strand specific and cannot be performed on double stranded DNA. Common problem in SBSM approach is amplification of unconverted part of genomic DNA is not possible. Incomplete denaturation of the template DNA contributes to the problematic artifacts in the plant genome, which shows methylation in any sequence (CG, CHG, CHH) context (Henderson et al., 2010). In *A. thalian*, CHG and CHH sites are on average methylated at 6.7% and 1.7%, respectively and the methylation status of adjacent sites does not show a high correlation in most cases (Cokus et al., 2008; Henderson et al., 2010). Another problem is that this method is entirely dependent on detailed knowledge of the genome sequence. Moreover, in case of the loci in which all sequence contexts are highly methylated, should be verified using alternative techniques that do not

use a bisulfite conversion step for example, Southern blotting combined with digestion using methylation sensitive restriction endonucleases (Henderson et al., 2010). Therefore, many scientists routinely use MSAP technique for global methylation in higher plants.

1.2.10.2.1.3 Methylation sensitive amplification polymorphism (MSAP)

Common methods used for studying DNA methylation patterns are based on the sensitiveness of restriction endonucleases to methylation of the target sites especially of cytosine base (Cedar et al., 1979). Restriction enzymes that recognize a sequence containing CG or CHG will usually cleave only when cytosine in those sequences is not methylated. Using this approach, MSAP techniques are developed and many isoschizomer pairs were designated for analyzing global DNA methylation in plants (Vos et al., 1995; Reyna-López et al., 1997; Bednarek et al., 2007). One of the restriction enzymes of the isoschizomer pair is able to cleave the DNA only when its recognition site is unmethylated, whereas the other is not sensitive to methylation (Fraga & Esteller, 2002). Several restriction enzymes that recognize the localization of the methyl group in plant DNA (McClelland et al., 1994). *Msp*I/*Hpa*II isoschizomer endonucleases which cleave the DNA at the 5′-CCGG-3′ targets are frequently used to analyze the tissue culture induced DNA methylation variation in higher plants. *Hpa*II is sensitive to the internal cytosine methylation whereas, *Msp*I cannot cleave when external cytosine is methylated. *Eco*RII/*Bst*NI isoschizomer are used where most of the methylated cytosines are located at 5′-CCHGG-3′ sequences (Fraga & Esteller, 2002). *Eco*RII recognizes 5′-CCHGG-3′ targets, but it only cleaves when cytosine is unmethylated, whereas *Bst*NI is insensitive to

cytosine methylation (McClelland et al., 1994; Kovarík et al., 1997b). The isoschizomeric combinations of *Acc*65I/*Mse*I and *Kpn*I/*Mse*I are also available to analyze DNA methylation variation (Bednarek et al., 2007). Among the existing isoschizomers, the most common used isoschizomer is the *Hpa*II/*Msp*I pair to assess DNA methylation.

The MSAP technique was first introduced by Reyna-López and co-workers (1997) using *Hpa*II/*Msp*I isoschizomer pair with the modification of original AFLP technique (Vos et al., 1995). In their modification, the frequent cutter *Mse*I was replaced by methylation sensitive *Msp*I and *Hpa*II restriction enzymes. The principle of MSAP technique with this isoschizomer pair was described by Fulneček and Kovařík (2014). Both endonucleases cleave the DNA at the 5′-CCGG-3′ target and produce similar DNA fragment profile, but *Hpa*II is not able to cut when the internal cytosine is methylated (5′-CmCGG-3′). In this technique, genomic DNA is digested with *Eco*RI and one of the methylation sensitive *Msp*I or *Hpa*II isoschizomers, which can cleave 5′-CCGG-3′ sequences. *Msp*I cleaves 5′-CCGG-3′ site when both cytosines are non-methylated and internal cytosine is hemi- (mC in one DNA strand only) or fully-methylated (5′-CmCGG-3′), but *Msp*I cannot cleave the recognition site if outer cytosine is hemi- or fully-methylated (5′-mCCGG-3′) (Reyna-López et al., 1997). *Hpa*II is assumed to digest only non-methylated and hemi-methylated 5′-CCGG-3′ sequences. However, electrophoretic patterns produced from two individual reaction one from *Msp*I and another from *Hpa*II are ambiguous to confirm about the situation in methylation, because MSAP pattern in plant DNA represented by the signal in the *Hpa*II is controversial (Fulneček & Kovařík, 2014). Moreover, cleavage by *Msp*I of 5′-GGCCGG-3′ sequences is also inhibited by the methylation of the C next to the CG

(Busslinger et al., 1983). To resolve this ambiguity, Fulneček and Kovařík (2014) added a combined *Hpa*II+*Msp*I digestion which assisted in the interpretation of the most controversial MSAP pattern in the *Hpa*II but not in the *Msp*I profile.

MSAP is based on the AFLP technology and therefore prior genome information other than the approximate genome size is not required in this technique. The high number of methylation events can be detected using a relatively small number of primer combinations and the additional ability to clone and characterize novel methylated sequences (Peraza-Echeverria et al., 2001). Since MSAP method is dependent on the number and specificity of the primer pairs chosen, it provides a qualitative measure of DNA methylation analysis. MSAP technique could detect cytosine methylation location of internal, external and fully methylated of genomic DNA whereas the reversed-phase HPLC analysis showed very little changes of cytosine in oil palm (*E. guineensis*) regenerants.

In fact, MSAP can investigate only a small proportion of the methylated cytosine in the genome, and is also limited by distinct scale of variation in methylation within recognition site of particular restriction endonuclease or isoschizomers (Baránek et al., 2015). They cannot provide the critical information required for a complete understanding of the role of methylcytosine in cell and molecular biology (Fraga & Esteller, 2002). Despite these circumstances, MSAP method is still frequently used in higher plants as an important technique to measure epigenetic fidelity of *in vitro* regenerants, and to determine developmental plasticity of adaptation in stress condition.

MSAP technique has been used in several plant species propagated *in vitro* (**Table 1.3**).

Peraza-Echeverria et al. (2001) used MSAP to assess the effect of propagation methods on the epigenetic variation in banana and found higher level of cytosine methylation in the genome of micropropagated banana plants compared to conventionally propagated ones. While DNA methylation events were polymorphic in plants micropropagated from the male inflorescence and sucker explants, no DNA methylation polymorphism was detected in banana plants propagated conventionally with corms. Ghosh et al. (2017) applied MSAP to determine the global DNA methylation between organized tissue and callus of blueberries and they found that number of methylated 5′-CCGG-3′ sites were varied significantly within the genotypes. Methylated sites were higher in callus (215–258) than in leaf tissues (75–100). Methylation events were more polymorphic in callus than in leaf tissues. Chakrabarty et al. (2003) reported higher cytosine methylation at 5′-CCGG-3′ sites in the genome of non-embryogenic callus of *E. senticosus* compare to those of embryogenic callus.

Several studies were carried out using MSAP technique to assess the effect of tissue culture systems on DNA methylation. Peredo et al. (2009) and Baránek et al. (2010) found hypomethylation in tissue culture regenerants derived from different regeneration systems including axillary bud proliferation, adventitious bud regeneration and somatic embryogenesis compared to normal or donor plants. Rathore and Jha (2016) reported that methylation level was higher in *in vitro* regenerants of *J. curcas* raised through direct organogenesis via enhanced axillary shoot proliferation than those of the regenerants derived via shoot regeneration system using leaf explant. Compared to the donor of *J. curcas*, regenerated plants of shoot proliferation exhibited hyper-methylation while

Table 1.3 Analysis of DNA methylation in *in vitro* regenerated plants of several species using methylation sensitive amplification polymorphism (MSAP) technique

Species	Tissue culture system	Methylation levels in regenerants	References
Jatropha curcas	shoot proliferation and regeneration from leaf	higher than mother plant lower than mother plant	(Rathore & Jha, 2016)
J. curcas	shoot culture	lower than *in vivo* grown plants	(Rathore et al., 2015)
Coffea arabica var. 'Caturra' and F1 hybrids	somatic embryogenesis	hypomethylation compare to donor	(Landey et al., 2013; Landey et al., 2015)
Solanum tuberosum	node culture	hypomethylation compare to donor	(Dann & Wilson, 2011)
Freesia hybrida	direct somatic embryogenesis	slightly lower than donor plant	(Gao et al., 2010)
Codonopsis lanceolate	adventitious bud regeneration	hypermethylation compare to donor	(Guo et al., 2007)
Doritaenopsis	micropropagation with root tip explants	12% in normal and 14-22% in regenerants	(Park et al., 2009)

Table 1.3 cont'd

Species	Tissue culture system	Methylation levels in regenerants	References
Musa AAA cv. 'Grand Naine'	micropropagation with sucker apex and male inflorescence	higher methylation than in conventionally propagated plant with corm	(Peraza-Echeverria et al., 2001)
Humulus lupulus	adventitious bud regeneration	hypomethylation compare to field grown plants	(Peredo et al., 2009)
Theobroma cacao	somatic embryogenesis	hypermethylation than leaf and hypomethylation than staminode	(Rodríguez-López et al., 2010)
Vitis vinifera	axillary shoot multiplication	hypomethylation compare to donor	(Baránek et al., 2010)
V. vinifera	somatic embryogenesis	hypermethylation compare to donor	(Schellenbaum et al., 2008)
Vaccinium angustifolium	leaf culture	higher methylation in callus than explant	(Ghosh et al., 2017)

Table 1.3 cont'd

Species	Tissue culture system	Methylation levels in regenerants	References
Eleuterococcus senticosus	somatic embryogenesis	17% for non-embryogenic callus and 11% for embryogenic callus	(Chakrabarty et al., 2003)
Rosa hybrida cv. 'Carefree Beauty'	shoot regeneration	hypomethylation compare to *in vivo* grown plants	(Xu et al., 2004)
Bambusa balcooa	somatic embryogenesis	no methylation variation between donor and regenerants	(Gillis et al., 2007)

regenerants of shoot regeneration had hypo-methylation. In *Rosa hybrida*, methylation patterns during shoot organogenesis was quite different from those somatic embryogenesis system (Xu et al., 2004). However, Gillis et al. (2007) found stable MSAP patterns in regenerants of *B. balcooa* derived through somatic embryogenesis using two different explants (pseudospikelets and shoot apical meristems).

In general, regeneration system with dedifferentiation stage showed higher variation or polymorphism in DNA methylation than the tissue culture through shoot proliferation system. Schellenbaum et al. (2008) and Rathore and Jha (2016) reported that polymorphism in methylation profiles was higher in shoot regeneration system than in shoot proliferation. Sharma et al. (2007) analyzed changes in methylation pattern in *in vitro* regenerated potato plants via somatic embryogenesis and axillary bud multiplication. They found that changes in DNA methylation occurred during somatic embryogenesis but regenerants of axillary bud proliferation was epigenetically stable. Baránek et al. (2010) reported significant differences in cytosine methylation at 5′-CCGG-3′ sites between regenerants of *Vitis vinifera* originated from single plant regeneration system with no intermediary dedifferentiation step. Guo et al. (2007) reported polymorphism in MSAP profiles of specific loci among the regenerants and/or between the regenerant(s) and the donor plant of a hardy perennials *Codonopsis lanceolata*, though their estimated total level of methylation remained more or less the same as the donor plant. However, the alterations in cytosine methylation were introduced by direct as well as indirect embryogenesis pathway at both CG and CHG sequences in *Freesia hybrid* (Gao et al., 2010). Although methylation alteration in regenerated plants was different from donor, the levels and

patterns of cytosine methylation were at similar rates in plants derived from either direct or indirect embryogenesis.

1.3 Rationale of the study

High concentration of antioxidants and other phytonutrients in blueberries help consumers live healthy and prevent several degenerative diseases (Basu et al., 2010; Uysal et al., 2013; Skrovankova et al., 2015). Although lowbush blueberries are reproduced naturally in the forest understory through either seed dispersal or rhizome development or both, they are cultivated in backyard and orchard commercially with planting materials propagated conventionally from stem or rhizome cuttings. Blueberry plants derived from conventional propagation establish and grow slowly due to their poor root development and rhizome production capability. To overcome this problem and produce genetically identical or true-true-type clones of a good cultivar, *in vitro* propagation has been used in several blueberry improvement programs (Nickerson & Hall, 1976; Jamieson & Nickerson, 2003; Debnath, 2017). However, micropropagation may develop somaclonal variation in regenerants being either beneficial or deleterious which should be considered during commercial production of fruit crops especially for perennials. Somaclonal variation among the regenerants and phenotypic variation between regenerant(s) and donor plant may be due to genetic changes or due to epigenetic variation or both (Boxus et al., 2000; Biswas et al., 2009).

Although, micropropagation is successful to regenerate the plants with improved morphological characteristics including high spreading capacity and fruit yield in small berry crops like blueberry, lingonberry and strawberry as discussed in this chapter

(Debnath, 2007b; Biswas et al., 2009; Vyas et al., 2013a), the available data on phenolic content and antioxidant properties of micropropagated blueberries are limited. In addition, the phenolic content of micropropagated blueberry leaf and fruit has not been studied at different maturity stages. The effect of growing season and maturity stage on the phytochemical content of micropropagated blueberry is also incomplete or lacking. Furthermore, the available literature on the epigenetic variation of fully grown micropropagated plants of *Vaccinium* spp. is unavailable. Epigenetic factor especially DNA methylation could be responsible to express variation in morphological and phytochemical changes of *in vitro* derived blueberries. Therefore, the present study was designed to evaluate the morphological and phytochemical characteristics of plants propagated by conventional and *in vitro* methods, and then to assess the possibility of genetic and epigenetic variation among those plants.

1.4 Research aims

The general aim of this series of studies was to determine the changes in plant morphology and antioxidant metabolite content in wild clone and developed cultivar of lowbush blueberry propagated with stem cutting and tissue culture. Especial emphasis was given in genetic and epigenetic fidelity of micropropagated regenerants to assess the possibility of using *in vitro* technique as a sustainable propagation method to increase fruit yield and quality. The objectives of the individual studies were:

1) To determine variation in morphological characteristics, growth and spreading habit, flower bearing trait and fruit yield of wild clone and cultivar of lowbush blueberry propagated *in vitro* and by softwood cutting (Chapter 2).
2) To determine phytochemicals and antioxidant properties of lowbush blueberry leaves and fruits propagated differently (Chapter 3).
3) To determine the content of metabolites and their antioxidant activities in blueberry at different maturity stages (Chapter 3).
4) To evaluate the clonal fidelity of *in vitro* derived lowbush blueberry plants using EST-SSR and EST-PCR molecular markers (Chapter 4).
5) To determine the occurrence of epigenetic variation especially DNA methylation changes in softwood cutting and micropropagated blueberry plants (Chapter 5).

CHAPTER 2

Determination of Propagation Effects on Vegetative Growth, Flower Bearing Traits and Fruit Characteristics of Lowbush Blueberries

This chapter aims to investigate the effects of propagation methods on the morphological characteristics of two lowbush blueberry genotypes 'QB9C' and 'Fundy' obtained from two different propagation methods: by conventional softwood cutting and in vitro shoot proliferation of nodal explants. Parts of this chapter results have been published in the Canadian Journal of Plant Science 93: 1001-1008 and HortScience 50: 888-896 (Goyali et al., 2013, 2015a)[1].

2.1 Introduction

The lowbush blueberry (*Vaccinium angustifolium* Ait.), known as sweet or wild blueberry, is one of the most commercially grown small fruits in North America which belongs to the section *Cyanococcus* Gray in plant family Ericaceae. Lowbush blueberries are rhizomatous, cross-pollinated woody perennial shrubs growing generally in the forest understory. They are native to Newfoundland and Labrador (Vander Kloet, 1988) and produced commercially in Atlantic Canada, Quebec and in Maine in the United States (Kalt et al., 2001b; Strik & Yarborough, 2005) where the bushes are managed with naturally grown native population of plants rather than known genetic stock or cultivar. They form large colonies of genetically identical plants (clones) connecting via underground shoots called rhizomes (Vander Kloet, 1988). The bush of wild blueberries is raised as a visually

[1] The contributions of the author and co-authors to the manuscripts are described in Appendix 1.

distinctive mosaic patchwork (Bell et al., 2010) keeping a lot of bare areas and incomplete coverage in the field. Bare ground may result from unintended kills of blueberry plants from herbicide application or from "scalping" by machinery during weeding, and hence it results lower yields in commercial lowbush blueberry fields (Morrison et al., 2000). Filling in those uncovered areas with high yielding genotypes having rapid establishment and spreading ability is one of the ways to improve productivity in an established farm. Propagation techniques of blueberry may help in this aspect.

Lowbush blueberries are propagated generally by stem cutting using softwood or rhizome or by seeds. Softwood cutting (SC) is not suitable for multiplication of new selections rapidly to evaluate genetic and economic trait or to meet industry planting requirements due to limitation in number of planting materials available from an individual source plant, its poor spread habit, and their extreme precocity of flowering (Jamieson & Nickerson, 2003). The advantages of seed propagation over stem cutting include a lower cost of plants and better establishment in the field with more rhizome formation than SC; but the seed propagated plants yield 50% less than their respective mother clone (Aalders et al., 1979) and increase variability in fruit size and quality (Jamieson & Nickerson, 2003). This limitation may be overcome by micropropagation or *in vitro* propagation which combines benefits of faster spreading growth habits of seedling and the uniform productivity characteristics of stem cutting. Thereby, this method can be potentially more effective over the other two techniques to establish a new blueberry field and to improve an established farm. Micropropagation with selected clones facilitates filling up of incomplete coverage in the fields to recover establishment costs more rapidly and to get higher yields, since

tissue culture (TC) plants spread faster by producing high number of rhizomes and branches (Morrison et al., 2000; Debnath, 2007b; Debnath et al., 2012b) and perform uniform productivity (Frett & Smagula, 1983). Moreover, *in vitro* propagation ensures a rapid and continuous supply of mass production of healthy and pathogen-free planting materials of a desired genotype.

Numerous reports on micropropagation of blueberries (*Vaccinium* spp.) have been published since its first report in mid 70s more than 50 years ago (Barker & Collins, 1963). Shoot proliferation using single or multiple nodal segments is commercially used for lowbush blueberries (Debnath, 2004; Kaldmäe et al., 2006; Debnath, 2007b, 2009b; Georgieva, 2013), while shoot regeneration system in semi-solid media is well-established nowadays. Healthy matured plants are regenerated from excised leaves of greenhouse-grown wild lowbush blueberry in the St. John's Research and Development Centre (SJRDC) by Debnath (2009a). Automated bioreactor system containing liquid medium is used for multiplication of blueberry plantlets derived through shoot proliferation as well as adventitious shoot regeneration systems (Debnath, 2009b, 2011, 2017), which resolves the manual handling of the various stages of micropropagation and reduces unit cost of propagules (Paek et al., 2005). Although morphologically similar and genetically identical clones are regenerated in *in vitro* propagation in *Vaccinium* spp. (Debnath, 2011, 2017), the vegetative growth habit of micropropagated plants is different from stem cutting propagation. Heavier bush resulting from vigorous vegetative growth including higher number of rhizomes, branches and plant height is common in TC blueberry plants (Jamieson & Nickerson, 2003; Debnath, 2007b; Marino et al., 2014). But differential

responses of blueberry species to micropropagation are reported for their fruit production. For instance, *in vitro* propagated half-high 'Northblue' (*V. corymbosum* × *V. angustifolium*) blueberry plants produce higher fruit yield compared to conventional SC counterparts (Read et al., 1989; El-Shiekh et al., 1996), whereas micropropagated wild lowbush and 'Herbert' highbush (*Vaccinium* × *corymbosum* L.) blueberries produce less yield and smaller berries than SC plants (Jamieson & Nickerson, 2003; Litwińczuk et al., 2005; Goyali et al., 2015a, 2015b).

Although variation in the growth habit and yield performance of micropropagated blueberries and other small fruits have been reported, results of TC-derived plants in different growing seasons is limited and contradictory. TC-derived half-high 'Northblue' plants had significantly more lateral branching than SC plants during the three growing seasons (Read et al., 1989), whereas Albert et al. (2009) reported that micropropagated plants of same cultivar produced significantly less and shorter shoots than SC derived plants in the first two of three growing seasons. Propagation method affected plant canopy volume of highbush cultivars 'Emerald' and 'Jewel' during the first season, while no effect was observed by the end of the second growing season (Marino et al., 2014). *In vitro* propagated highbush blueberry plants grew more uniformly and vigorously than SC plants during the first 3 years in the field studies (Litwińczuk et al., 2005). During the 7-years of production trials, TC half-high blueberry plants produced higher berry yield than SC plants only in 2 growing seasons (El-Shiekh et al., 1996). Whereas in red raspberry cv. 'Comet', Deng et al. (1993) reported that micropropagated plants produced same yield as those derived from conventional propagation did in the third growing season of study, although

yield of TC plants was significantly different from conventional propagation in first 2 two years. The objective of the current study is to determine the effect of propagation methods on the growth habit, flower and fruit bearing traits of a wild clone and a cultivar of lowbush blueberry in three consecutive growing seasons under controlled environment in a greenhouse.

2.2 Materials and methods

2.2.1 Plant materials

Two lowbush blueberry genotypes were used for this study. A wild clone 'QB9C' which was grown naturally in the forest understory collected from Longue-Rive in Quebec (48°33′ N and 69°33′ W) in August 2001. Another genotype was the cultivar 'Fundy' developed from open-pollinated seedling of 'Augusta' at the Kentville Research and Development Centre previous name Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada (AAFC), Kentville, NS (Okie, 2002) which had consistently good yield in eastern Canada (Hall et al., 1988). Plants of both genotypes were grown in plastic pots (25 cm in diameter and 18 cm deep, equivalent 6 liter) containing peat:perlite [2:1 (v/v)] soil medium and maintained in a greenhouse at SJRDC previous name Atlantic Cool Climate Crop Research Centre, AAFC, St. John's, NL, under natural light conditions at a maximum photosynthetic photon flux (PPF) of 90 μmol/m^2/s at 20 ± 2 °C temperature and 85% relative humidity since 2001 (Debnath, 2004). Six years of growth in same greenhouse acclimatized the plants of different origins in the similar environmental condition.

For this study, plants were propagated by conventional SC and by shoot proliferation using node explants from the source plants maintained in a greenhouse at the SJRDC, NL (Debnath, 2009b). In 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 (Beaver Plastics, Edmonton, AB) with peat:perlite [2:1 (v/v)] and placed in a humidity chamber equipped with a vaporiser (Controlled Environments Ltd., Winnipeg, MB) at 22 ± 2 °C, 95% relative humidity and 16 h photoperiod provided by fluorescent lights (55 μmol/m^2/s) (Debnath, 2007b). For micropropagation, stem segments (4 - 5 cm) of young, actively growing shoots were surface sterilized in a solution of 0.79% sodium hypochlorite (15% commercial bleach) and 0.1% Tween 20 (polyoxyethylene sorbitan monolaurate) for 20 min, quickly rinsed in 70% ethanol and then washed in sterilized deionized water. Nodal explants (section with three nodes and intact leaves) from the sterile stem segments were cultured in 25 mm × 200 mm glass vial, which contained 12 ml of the modified cranberry medium [detail composition of media in Debnath & McRae (2001a)] containing three-quarter macro-salts and micro-salts of Debnath and McRae's (2001b) shoot proliferation medium D supplemented with sucrose (25 g/L), Sigma A 1296 agar (3.5 g/L) and Gelrite (1.25 g/L) (Sigma Chemical Co., St. Louis, MO, USA) and the growth regulator zeatin (5 μmol/L) (Debnath, 2007b). The medium pH was adjusted to 5.0 before autoclaving at 121 °C for 20 min. After planting explants in culture, tubes were capped with clear permeable polypropylene caps and sealed with parafilm. Tubes were placed upright and maintained at 20 ± 2 °C under a 16 h photoperiod with a PPF density of 30 μmol/m^2/s provided by cool white fluorescent lamps. After 8 weeks, the vegetative shoots were excised and subcultured

by transferring the nodal explants to 175 ml Sigma baby food glass jars with polypropylene clear caps (Sigma Chemical Co., St. Louis), which contained 35 ml of basal medium (Debnath, 2004).

In vitro derived 3 - 5 cm elongated shoots (**Figure 2.1)** were rooted following the same technique used for SC propagation. Rooted stem cuttings and TC-derived plantlets were transplanted into plastic pots (10.5 × 10.5 × 12.5 cm^3) with the same medium used for rooting in 2007. Since then, plants were grown in a greenhouse at SJRDC, NL under natural light conditions (maximum 90 μmol/m^2/s), at 20 ± 2 °C temperature and 85% relative humidity. The plants were pollinated naturally. Fertilization [100 mg/L N from Peters Azalea Neutral Fertilizer 20N–8P–20K (Plant Products Co., Brampton, ON)] and irrigation were applied when 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.

2.2.2 Data collection

Data were collected on the morphological characteristics from five-year-old plants in three consecutive growing seasons of 2011, 2012 and 2013. Four plants were randomly selected for each treatment, and the experiment was replicated four times. Every year when plants were fully covered with green leaves, morphological data of stem and leaf was recorded from same plants for each treatment. Flower data were collected when ≈50% flowers were bloomed, and fruit data were recorded when berries were fully ripe (well-developed blue color). Data were collected on the following characteristics:

i) number of stems per plant

Figure 2.1 Axillary shoots of lowbush blueberry 'QB9C' developed from nodal explants eight weeks after transferring on gelled medium with 5 mM zeatin (bar = 1 cm) (Debnath, 2011)

ii) number of branches per plant

iii) number of branches per stem

iv) diameter of stem (mm)

v) plant height (cm)

vi) length of leaf (mm)

vii) width of leaf (mm)

viii) leaf surface area (mm^2)

ix) plant vigour

x) number of flowers per plant

xi) number of flower clusters per plant

xii) number of flowers per cluster

xiii) fruit setting (%)

xiv) number of fruits per plant

xv) berry diameter (mm)

xvi) individual berry weight (g)

xvii) berry weight per plant (g)

Plant vigour was determined by visual assessment, on a scale of 1 (very poor) to 8 (fully normal and healthy plant with large green leaves and excellent vigour). Leaf characteristics were recorded from ten fully expanded mature leaves selected randomly from each plant. The number of branches per stem, number of flowers per cluster and fruit setting (%) were calculated using following formula:

$$\text{Number of branches per stem} = \frac{\text{Total number of branches per plant}}{\text{Total number of stems per plant}}$$

$$\text{Number of flowers per cluster} = \frac{\text{Total number of flowers per plant}}{\text{Total number of flower clusters per plant}}$$

$$\text{Fruit setting } (\%) = \frac{\text{Total number of fruits per plant}}{\text{Total number of flowers per plant}} \times 100$$

2.2.3 Statistical analysis

Data for each trait were submitted for statistical analysis using the SAS statistical software package version 9.1 (SAS Institute, 2002). Analysis of Variance (ANOVA) was calculated to investigate the effects of genotype, propagation method and growing season and their interaction on all of the morphological characteristics except plant vigour. All data are presented as the means ± SE of four replications. Statistical *F*-tests were evaluated at $P \leq 0.05$ for all the parameters. The treatment means were compared by the least significant difference (LSD) using the *F*-test. The fruit setting (%) was transformed to square root scale due to the values below 20% before ANOVA to stabilize the variance and then back-transformed for presentation (Debnath, 2006). Plant vigour was analysed by using the categorical data modeling procedure (PROC CATMOD in SAS software) and differences between treatment combinations were compared using the contrast statement in the CATMOD procedure. This method is suitable for the analysis of categorical data (Compton, 1994), and it allows assessment of main effects and their interaction terms as ANOVA. The relationships between fruit yield and other morphological characteristics

were analyzed using Pearson's correlation coefficients calculated with the Minitab 17 for Windows software package.

2.3 Results

2.3.1 Stem and leaf morphology

The plants of wild clone 'QB9C' and cultivar 'Fundy' propagated by SC and TC were well established in greenhouse with substantial vegetative growth. TC plants produced denser and larger shoot canopy with the development of higher number of stems and branches than SC plants performed (**Figure 2.2**). Analysis of variance for combined effect of genotype, propagation method and growing season showed that the interactions of genotype × propagation method, propagation method × growing season and genotype × propagation method × growing season were significant ($P \leq 0.05$) for plant vigour in categorical analysis (**Table 2.1** & **2.2**). Propagation method interacted significantly with genotype for the number of stems per plant, number of branches per stem, plant height, whereas the interaction between propagation method and growing season was significant for number of branches per stem. The *F* ratios for genotype and propagation method were much higher in number of stems and branches than that for genotype × propagation method interaction suggesting that the blueberry genotypes reacted similarly to propagation methods for stem and branch development (**Table 2.1**). Whereas, leaf length and leaf area were varied significantly with genotype, propagation method and their interactions (genotypes × propagation method). The *F* ratio for genotype × propagation method interaction was relatively higher in leaf length and area than that for the major factors of

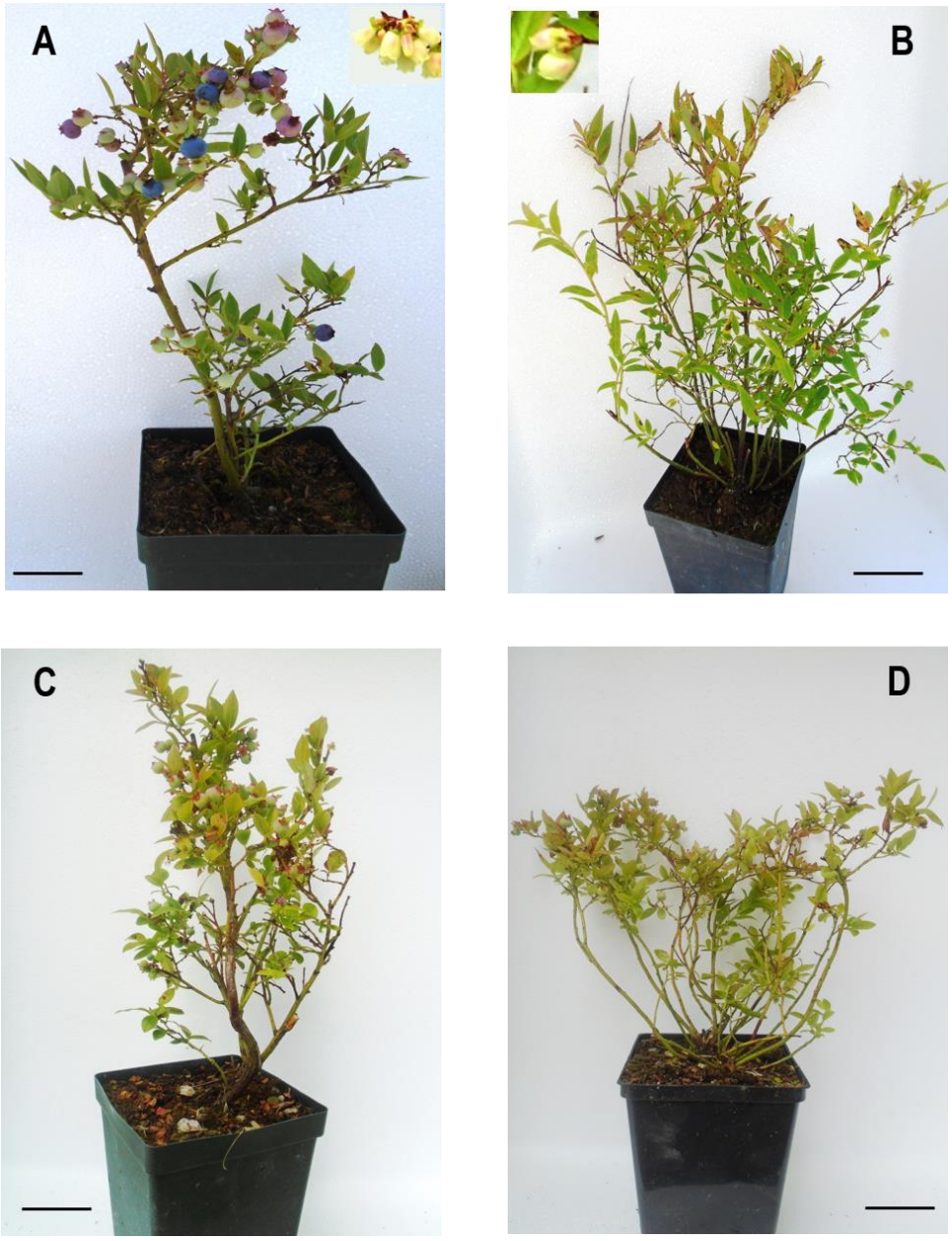

Figure 2.2 Established matured plants of 'QB9C' and 'Fundy' derived from softwood cutting (A and C) and shoot proliferation (B and D) respectively showing growth and development of plant canopy after eight growing seasons (bar =3.5 cm). Inset indicates single flower cluster in 'QB9C' plants.

Table 2.1 Analysis of variance for the effect of genotype, propagation method and growing season and their interactions on the morphological characteristics of two blueberry genotypes assessed in three consecutive growing seasons of 2011, 2012 and 2013

Source of variation	df	Stems (no./plant)		Branches (no./plant)		Branches (no./stem)		Stem diameter (mm)		Plant height (cm)	
		F values	*P* values	*F* values	*P* values	*F* values	*P* values	*F* values	*P* values	*F* values	*P* values
Genotypes (G)	1	37.8	<0.001	12.5	0.001	44.4	<0.001	3.06	0.090	0.33	0.572
Propagation methods (PM)	1	1500	<0.001	195	<0.001	227	<0.001	79.0	<0.001	1.39	0.247
Growing seasons (GS)	2	7.53	0.002	78.5	<0.001	9.55	0.001	8.88	<0.001	5.35	0.010
G×PM	1	6.37	0.017	0.03	0.866	23.5	<0.001	2.60	0.117	14.0	0.001
G×GS	2	0.38	0.685	0.34	0.712	0.79	0.463	0.49	0.616	0.41	0.670
PM×GS	2	0.80	0.456	0.37	0.695	4.55	0.018	1.29	0.288	0.04	0.965
G×PM×GS	2	0.53	0.594	2.72	0.081	1.40	0.260	0.17	0.841	0.08	0.924

Table 2.1 cont'd

Source of variation	df	Leaf length (mm)		Leaf width (mm)		Leaf area (mm^2)		Plant vigour (scale: 1–8)[z]	
		F values	*P* values	*F* values	*P* values	*F* values	*P* values	*F* values	*P* values
Genotypes (G)	1	86.4	<0.001	427	<0.001	56.6	<0.001	6.92	0.013
Propagation methods (PM)	1	27.4	<0.001	1.15	0.292	5.91	0.021	7.38	0.010
Growing seasons (GS)	2	1.24	0.303	2.05	0.145	2.82	0.074	18.9	<0.001
G×PM	1	132	<0.001	40.3	<0.001	72.0	<0.001	4.28	0.047
G×GS	2	18.6	<0.001	38.9	<0.001	31.1	<0.001	0.21	0.810
PM×GS	2	1.09	0.348	0.55	0.581	0.50	0.612	9.17	<0.001
G×PM×GS	2	2.63	0.087	0.03	0.973	0.30	0.744	6.56	0.004

[z]Plant vigour was assessed visually based on plant appearance using a scale of 1 (very poor) to 8 (fully normal healthy shoots with excellent vigour).

Table 2.2 Mean values of the main factors across all the treatments for combined effect of genotype, propagation method and growing season on the morphological characteristics of lowbush blueberry plants assessed in 2011, 2012 and 2013

Parameters	Stems (no./plant)	Branches (no./plant)	Branches (no./stem)	Stem diameter (mm)	Plant height (cm)
Genotypes (G)					
QB9C	4.69b	70.0a	24.1a	4.14a	28.2a
Fundy	5.75a	62.7b	15.2b	4.35a	28.5a
Propagation methods (PM)					
Softwood cutting	1.88b	51.9b	29.7a	4.79a	28.1a
Tissue culture	8.56a	80.8a	9.55b	3.71b	28.6a
Growing seasons (GS)					
2011	4.76b	53.6c	18.8b	3.98b	27.4b
2012	5.35a	61.5b	16.6b	4.16b	28.2ab
2013	5.56a	84.0a	23.6a	4.59a	29.3a
Significant effects	G, PM, GS, G×PM	G, PM, GS	G, PM, GS, G×PM, PM×GS	PM, GS	GS, G×PM

a - c Means within columns and factors followed by different letters indicate significant differences at $P \leq 0.05$.

Table 2.2 cont'd

Parameters	Leaf length (mm)	Leaf width (mm)	Leaf area (mm^2)	Plant vigour (scale: 1–8)[z]
Genotypes (G)				
QB9C	30.8a	10.7b	333b	7.25b
Fundy	27.8b	14.2a	398a	7.46a
Propagation methods (PM)				
Softwood cutting	28.5b	12.3a	355b	7.25b
Tissue culture	30.1a	12.5a	376a	7.46a
Growing seasons (GS)				
2011	29.5a	12.6a	378a	7.67a
2012	29.4a	12.4a	364ab	7.11b
2013	28.9a	12.2a	353b	7.29b
Significant effects	G, PM, G×PM, G×GS	G, G×PM, G×GS	G, PM, G×PM, G×GS	G, PM, GS, G×PM, PM×GS, G×PM×GS

a - c Means within columns and factors followed by different letters indicate significant differences at $P \leq 0.05$. [z]Plant vigour was assessed visually based on plant appearance using a scale of 1 (the poorest plant) to 8 (the best: fully normal healthy shoots with large green leaves and excellent vigour).

genotype and propagation method. It means that blueberry genotypes responded differently to propagation methods for their leaf characteristics.

All the characteristics of stem and leaf except plant height and leaf width were dependent on propagation methods (**Table 2.2**). CATMOD analysis for plant vigour indicated significant differences between propagation methods ($P \leq 0.01$). Across the genotypes and growing seasons, the TC plants produced more stems (4.6 times) and bigger leaves compare to SC plants which produced few or no rhizomes. Although TC plants had higher number of branches per plant (1.6 times of SC plants), branches per stem was less for those plants compared to SC counterparts. However, SC plants had thicker stem than TC plants in both genotypes.

Genotypes in this study differed significantly ($P \leq 0.05$) for their vegetative growth except stem diameter and plant height (**Table 2.2**). Across propagation methods and growing seasons, number of branches per plant, branches per stem and leaf length were higher in 'QB9C' than in 'Fundy'. Whereas 'Fundy' had more vigorous plants with more stems, wider and larger leaves compare to 'QB9C' wild clone.

The detailed growth performance of individual genotypes of 'QB9C' and 'Fundy' propagated by two different methods in three consecutive growing seasons is presented in **Figure 2.3, 2.4** & **2.5.** Number of stems and branches per plant were higher in TC plants compared to SC counterparts of both genotypes in all three growing seasons, whereas the number of branches per stem was higher in SC plants than in TC counterparts (**Figure 2.3**). Every year TC plants showed a tendency to produce more stems than in the previous year,

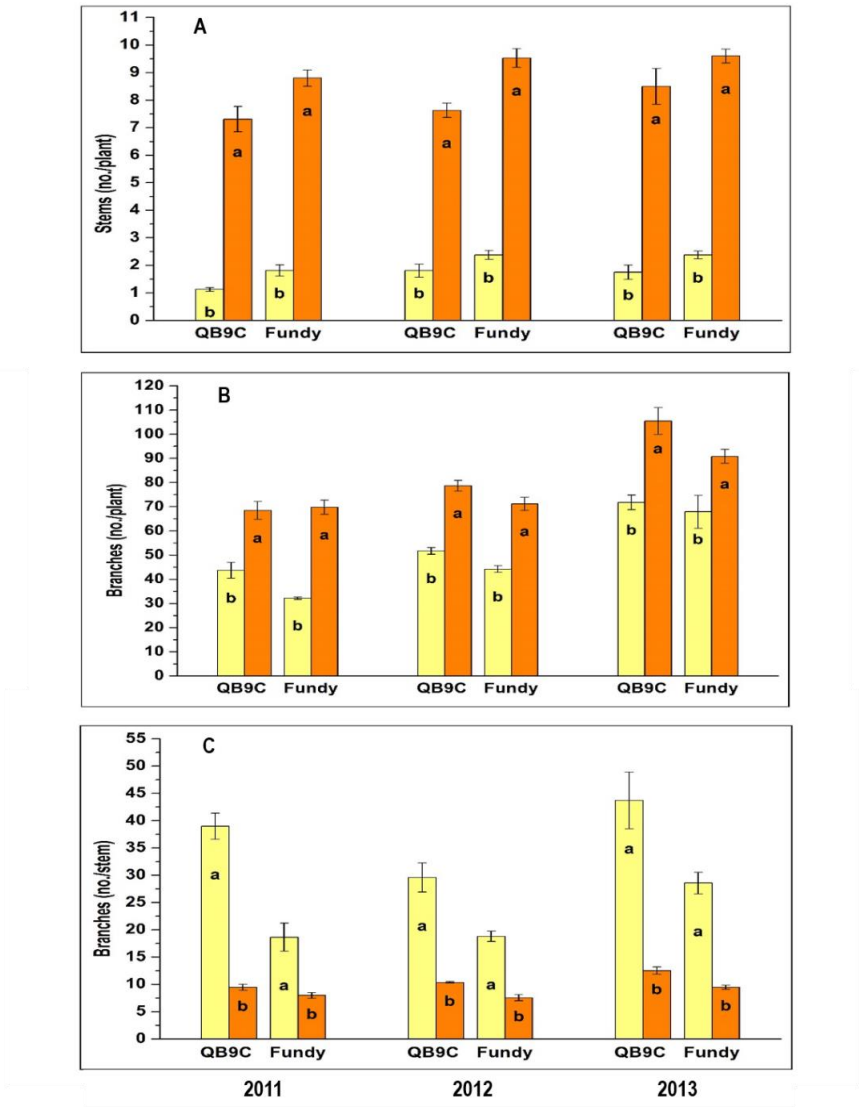

Figure 2.3 Effect of propagation method on number of stems per plant (A), number of branches per plant (B), number of branches per stem (C) of blueberry genotypes obtained by softwood cutting (light yellow bars) and tissue culture (orange bars) measured in 2011, 2012 and 2013. Different letters (a, b) indicate significant differences between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 4).

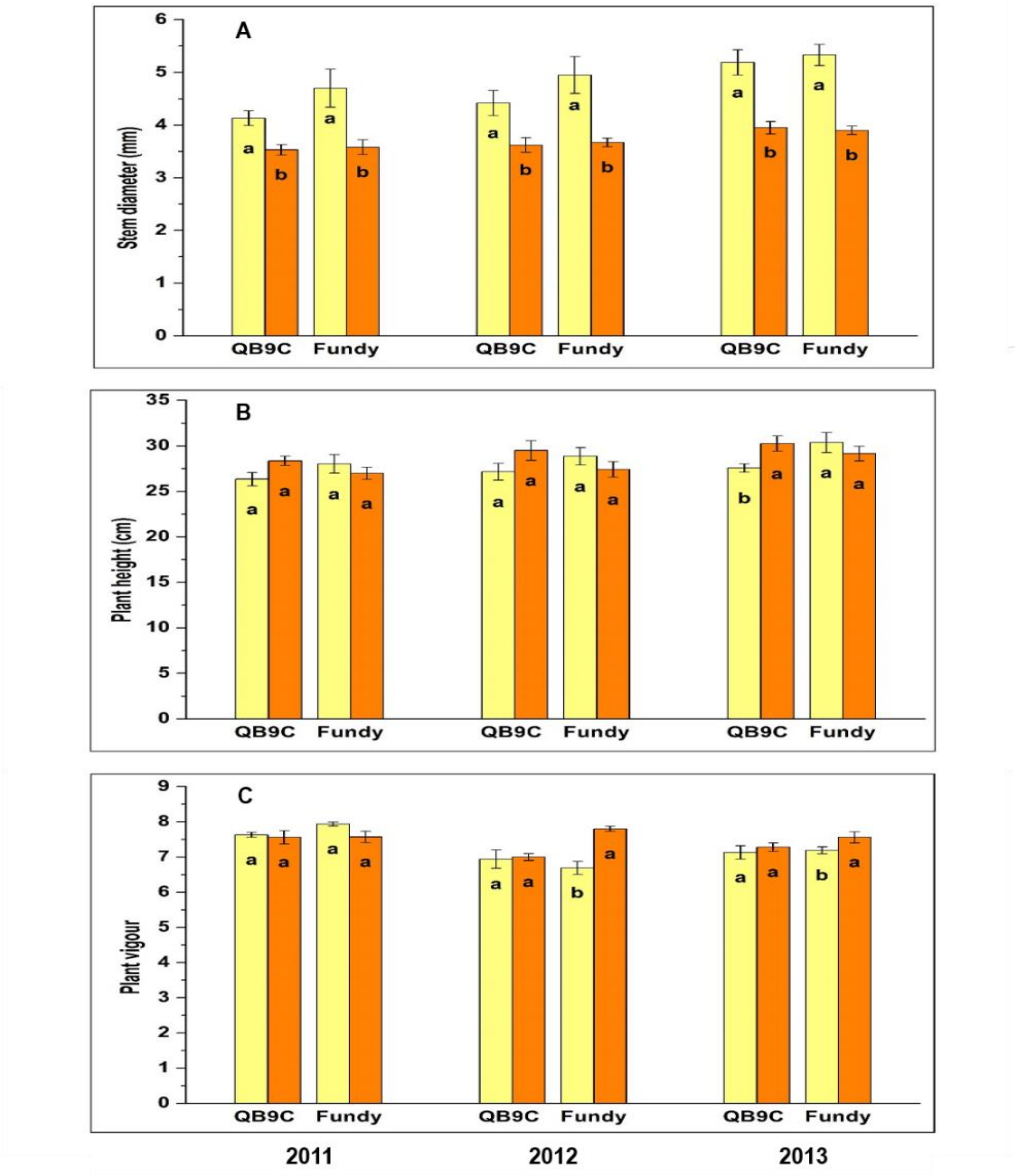

Figure 2.4 Effect of propagation method on stem diameter (A), plant height (B) and plant vigour (C) of blueberry genotypes obtained by softwood cutting (light yellow bars) and tissue culture (orange bars) measured in 2011, 2012 and 2013. Different letters (a, b) indicate significant differences between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 4).

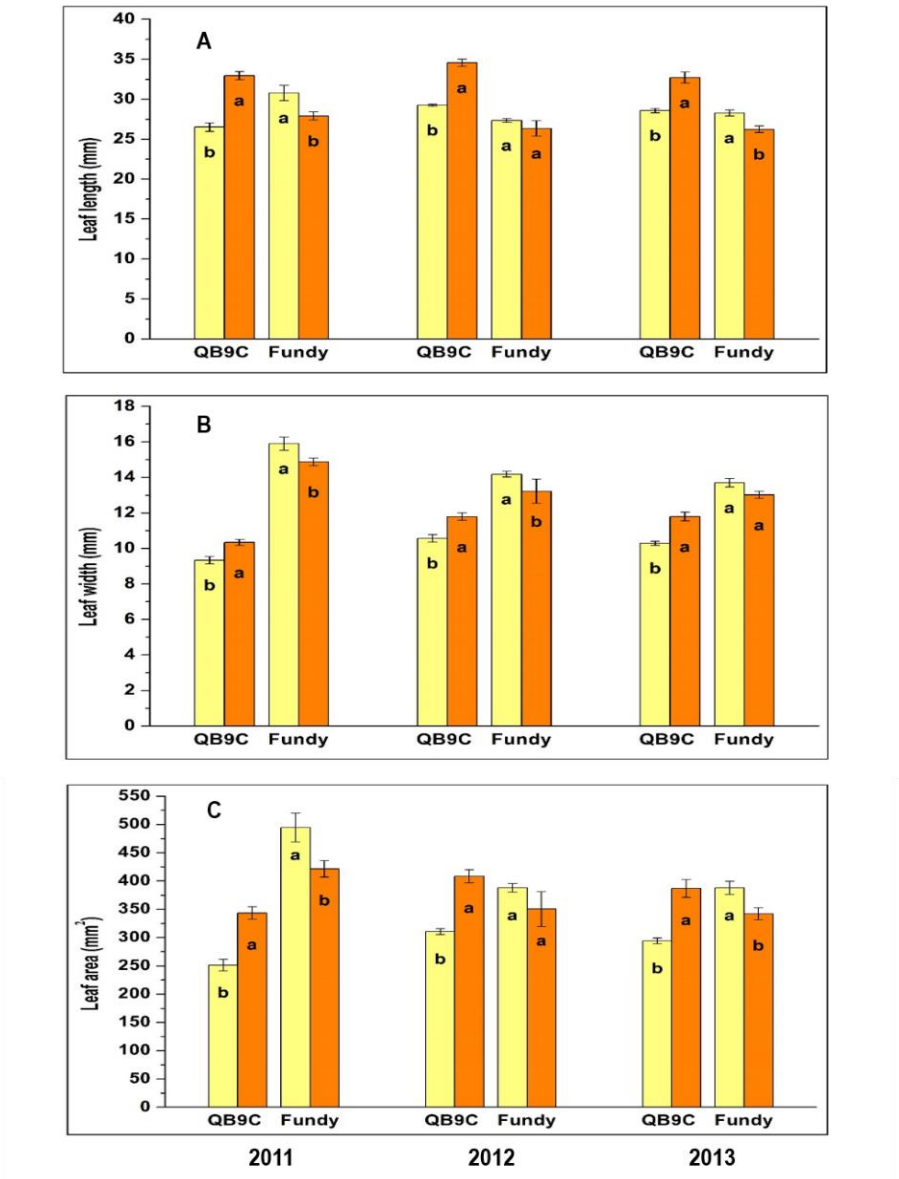

Figure 2.5 Effect of propagation method on leaf length (A), leaf width (B) and leaf area (C) of blueberry genotypes obtained by softwood cutting (light yellow bars) and tissue culture (orange bars) measured in 2011, 2012 and 2013. Different letters (a, b) indicate significant differences between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 4).

whereas shoot proliferation of SC plants remained stable in 2012 and 2013 in 'Fundy' (**Figure 2.3A**). SC plants of both genotypes produced thicker stems compared to TC plants in all three growing seasons (**Figure 2.4A**). Although the 'QB9C' plants propagated by SC and TC were vigorous in similar level, for TC 'Fundy' plants were more vigorous than SC plants in 2012 and 2013 (**Figure 2.4F**). Plant height of either genotypes was not changed significantly due to different propagation methods except for 'QB9C' in 2013 (**Figure 2.4B**).

The effect of propagation methods on leaf characteristics was different in the wild clone 'QB9C' from 'Fundy' (**Figure 2.5**). The length, width and area of individual leaves were higher in micropropagated 'QB9C', plants than in SC counterparts in all three production years while the leaves of TC 'Fundy' plants were significantly smaller in size than of SC plants in 2011 and 2013.

2.3.2 Flower and fruit characteristics

In the present study, all the morphological characteristics of flowers and fruits were significantly affected by propagation method. Interactions among genotype, propagation method and growing season were significant for the number of flowers, flower clusters and fruits per plant, and berry weight per plant (**Table 2.3 & 2.4)**. Number of flowers per cluster, fruit setting, number of fruits and berry weight per plant were varied significantly with genotype, propagation method and their interactions (genotype × propagation) with propagation being the major influence (**Table 2.3**). TC plants produced less number of flowers, and fruits, and had low fruit setting and berry yield compared to SC plants

Table 2.3 Analysis of variance for the effect of genotype, propagation method and growing season on flower and fruit characteristics of two lowbush blueberry genotypes measured in three consecutive growing seasons of 2011, 2012 and 2013

Source of variation	df	Flowers (no./plant)		Flower clusters (no./plant)		Flowers (no./cluster)		Fruit setting (%)[z]	
		F values	*P* values	*F* values	*P* values	*F* values	*P* values	*F* values	*P* values
Genotypes (G)	1	540	<0.001	207	<0.001	4.34	0.045	39.7	<0.001
Propagation methods (PM)	1	924	<0.001	172	<0.001	263	<0.001	121	<0.001
Growing seasons (GS)	2	106	<0.001	56.8	<0.001	0.97	0.391	112	<0.001
G×PM	1	1219	<0.001	280	<0.001	231	<0.001	39.7	<0.001
G×GS	2	40.2	<0.001	18.5	<0.001	4.87	0.014	16.7	<0.001
PM×GS	2	26.2	<0.001	7.37	0.002	6.45	0.004	7.69	0.002
G×PM×GS	2	52.0	<0.001	11.5	<0.001	1.19	0.316	2.83	0.074

[z]Square root transformation.

Table 2.3 cont'd

Source of variation	df	Fruits (no./plant)		Berry diameter (mm)		Individual berry weight (g)		Berry weight (g/plant)	
		F values	*P* values	*F* values	*P* values	*F* values	*P* values	*F* values	*P* values
Genotypes (G)	1	162	<0.001	245	<0.001	336	<0.001	6.45	0.016
Propagation methods (PM)	1	318	<0.001	5.52	0.024	5.99	0.043	264	<0.001
Growing seasons (GS)	2	87.8	<0.001	2.39	0.050	1.48	0.513	81.6	<0.001
G×PM	1	183	<0.001	3.07	0.088	10.8	0.227	80.7	<0.001
G×GS	2	11.7	<0.001	0.01	0.994	3.27	0.337	0.11	0.896
PM×GS	2	28.4	<0.001	0.69	0.509	0.72	0.751	13.8	<0.001
G×PM×GS	2	31.1	<0.001	0.69	0.509	4.08	0.762	16.7	<0.001

Table 2.4 Mean values of the main factors across all the treatments for combined effect of genotype, propagation method and growing season on flower and fruit characteristics of two lowbush blueberry genotypes assessed in 2011, 2012 and 2013

Parameters	Flowers (no./plant)	Flower clusters (no./plant)	Flowers (no./cluster)	Fruit setting (%)
Genotypes (G)				
QB9C	104a	26.0a	3.31b	12.9b
Fundy	47.3b	13.8b	3.50a	16.6a
Propagation methods (PM)				
Softwood cutting	113a	25.5a	4.16a	18.2a
Tissue culture	38.6b	14.4b	2.65b	11.4b
Growing season (GS)				
2011	55.1c	14.4c	3.49a	17.9a
2012	98.7a	25.5a	3.45a	18.5a
2013	73.8b	19.9b	3.33a	7.87b
Significant effects	G, PM, GS, G×PM, G×GS, PM×GS, G×PM×GS	G, PM, GS, G×PM, G×GS, PM×GS, G×PM×GS	G, PM, G×PM, G×GS, PM×GS	G, PM, GS, G×PM, G×GS, PM×GS

a - c Means within columns and factors followed by different letters indicate significant differences at $P \leq 0.05$.

Table 2.4 cont'd

Parameters	Fruits (no./plant)	Berry diameter (mm)	Individual berry weight (g)	Berry weight (g/plant)
Genotypes (G)				
QB9C	15.0a	7.42b	0.17b	2.81b
Fundy	7.09b	11.3a	0.47a	3.16a
Propagation methods (PM)				
Softwood cutting	17.8a	9.67a	0.34a	4.56a
Tissue culture	4.32b	9.08b	0.30b	1.40b
Growing season (GS)				
2011	9.67b	9.29b	0.32a	2.59b
2012	17.3a	9.10b	0.31a	4.72a
2013	6.16c	9.80a	0.33a	1.64c
Significant effects	G, PM, GS, G×PM, G×GS, PM×GS, G×PM×GS	G, PM, GS	G, PM	G, PM, GS, G×PM, PM×GS, G×PM×GS

a - c Means within columns and factors followed by different letters indicate significant differences at $P \leq 0.05$.

(**Table 2.4**). The berry diameter and individual berry weight showed significant variation with genotype and propagation methods. For berry diameter, *F* ratios for genotypes and propagation methods were much higher than *F* ratios for their interactions (genotype × propagation method) suggesting that the blueberry genotypes countered similarly to propagation techniques for berry size (**Table 2.3**).

The micropropagated plants of both genotypes produced smaller and lighter berries than SC plants did (**Table 2.4**). Across propagation methods and growing seasons, 'QB9C' produced more flowers, flower clusters and fruits per plant than 'Fundy' did, whereas, number of flowers per cluster, fruit setting, berry diameter, individual berry weight and berry weight per plant were higher in 'Fundy' plants compare to 'QB9C' counterparts. Overall, flower and fruit bearing capability of blueberry plants were better in the growing season of 2012 compare to in 2011 and 2013.

Three-year flower and fruit bearing performance of both genotypes 'QB9C' and 'Fundy' are presented in **Figure 2.6** & **2.7**. The differences between SC and TC plants for all the characteristics studied except fruit setting in percentage were higher in 'QB9C' than those in 'Fundy' in all three growing seasons. Number of flowers and flower clusters per plant, number of flowers per cluster, number and weight of berries per plant and berry diameter were higher in SC 'QB9C' plants compared to TC plants. Whereas for 'Fundy', none of the above characteristics except fruit setting and berry weight per plant was changed significantly in all three growing seasons. Fruit setting percentage (**Figure 2.6D)** and berry weight per plant (**Figure 2.7D)** were less in TC plants of 'Fundy' than in SC counterparts

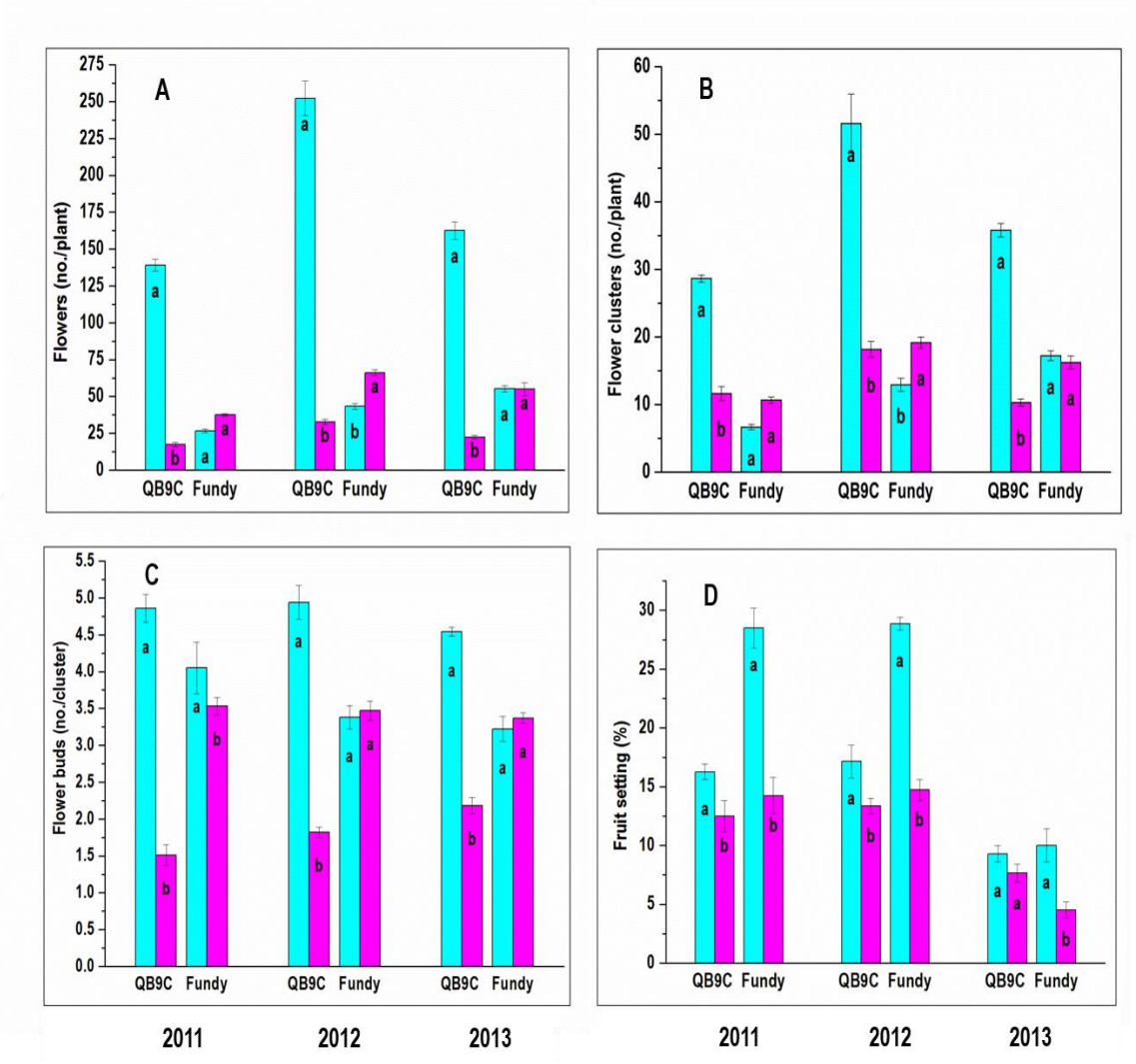

Figure 2.6 Effect of propagation method on number of flowers per plant (A), number of flower clusters per plant (B), number of flowers per cluster (C) and fruit setting in percentage (D) of blueberry genotypes obtained by softwood cutting (cyan bars) and tissue culture (magenta bars) measured in 2011, 2012 and 2013. Different letters (a, b) indicate significant differences between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 4).

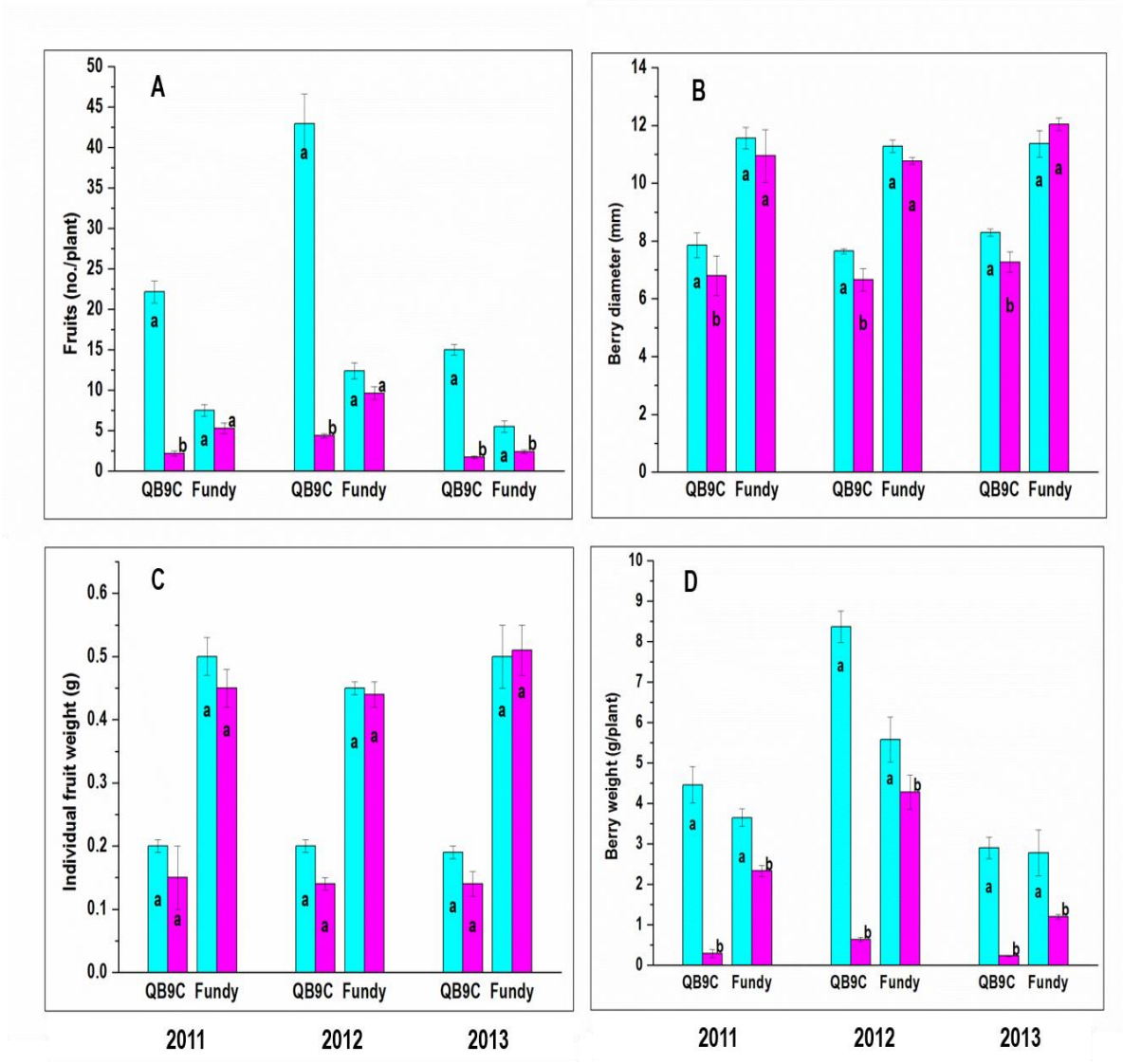

Figure 2.7 Effect of propagation methods on number of fruits per plant (A), berry diameter (B), individual berry weight (C) and berry weight per plant (D) of blueberry genotypes obtained by softwood cutting (cyan bars) and tissue culture (magenta bars) measured in 2011, 2012 and 2013. Different letters (a, b) indicate significant differences between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 4).

of same genotype**,** while TC 'Fundy' plants bore higher number of floral buds in the season of 2011 and 2012 compared to SC counterparts (**Figure 2.6A**). *In vitro* derived plants of 'QB9C' wild clone, had mostly single flowers rather than a standard size cluster having 4 - 5 flower buds (**Figure 2.2A** & **B**).

2.3.3 Correlation between berry yield and other morphological characteristics

In the correlation study among the berry weight per plant and other agro-morphological characteristics revealed that correlations were different in case of two different propagation methods. The berry weight of micropropagated blueberry plants exhibited significant positive association with number of stems per plant, leaf width, plant vigour, number of flowers per cluster, fruit setting, berry diameter and individual berry weight, however berry yield was negatively correlated with number of branches per stem, plant height and leaf length (**Table 2.5**). None of the above correlations was significant in SC propagated blueberry plants (**Table 2.6**). Whereas, berry yield per plant was correlated positively with number of flowers, flower clusters and berries per plant in both propagation methods in all three growing seasons (**Table A.1, A.2** & **A.3 in Appendix 2**) and in combined analysis (**Table 2.7**).

2.4 Discussion

The methods of propagation exhibited a remarkable influence on growth habit of lowbush blueberries. The faster vegetative growth with dense and large canopy of micropropagated blueberry plants in this study is in agreement with previous reports in lowbush blueberry

Table 2.5 Pearson's correlation coefficients between pairs of morphological characteristics in blueberries propagated by tissue culture: number of stems per plant (NSP), number of branches per plant (NBrP); number of branches per stem (NBrS), plant height (PH; cm), stem diameter (SD; mm), leaf length (LL; mm), leaf width (LW; mm), leaf area (LA; mm^2), plant vigour (PV), number of flowers per plant (NFP), number of flower clusters per plant (NCP), number of flowers per cluster (NFC), fruit setting percentage (FSP), number of berries per plant (NBP), berry diameter (BD; mm), individual berry weight (IBW; g) and berry weight per plant (BWP; g). Data was combined from two genotypes 'QB9C' and 'Fundy' grown in three growing seasons of 2011, 2012 and 2013.

Characters	NBrP	NBrS	PH	SD	LL	LW	LA	PV	NFP	NCP	NFC	FSP	NBP	BD	IBW	BWP
NSP	0.2	-0.5*	-0.2	0.2	-0.7**	0.5*	-0.2	0.4*	0.6**	0.2	0.7**	-0.2	0.3	0.6**	0.6**	0.5*
NBrP		0.7**	0.6**	0.6**	0.1	-0.1	-0.1	-0.2	-0.1	-0.1	-0.1	-0.6**	-0.5*	-0.1	-0.2	-0.4*
NBrS			0.6**	0.4*	0.6**	-0.4*	0.1	-0.6**	-0.6**	-0.3	-0.6**	-0.4	-0.6**	-0.5*	-0.6**	-0.7**
PH				0.4*	0.3	-0.4*	-0.1	-0.2	-0.2	0.0	-0.3	-0.3	-0.4*	-0.4	-0.4	-0.4*
SD					0.1	0.1	0.1	-0.1	0.1	0.0	0.1	-0.5*	-0.3	0.1	0.0	-0.2

* and ** Correlarion is significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table 2.5 cont'd

Characters	NBrP	NBrS	PH	SD	LL	LW	LA	PV	NFP	NCP	NFC	FSP	NBP	BD	IBW	BWP
LL						-0.6**	0.4*	-0.6**	-0.8**	-0.2	-0.9**	0.1	-0.5*	-0.8**	-0.9**	-0.7**
LW							0.6**	0.2	0.6**	0.1	0.8**	0.1	0.3	0.7**	0.7**	0.5*
LA								-0.5*	-0.2	-0.2	0.1	0.2	-0.1	-0.1	-0.2	-0.2
PV									0.4	0.1	0.4*	0.1	0.3	0.5*	0.5*	0.5*
NFP										0.7**	0.8**	-0.1	0.7**	0.8**	0.8**	0.7**
NCP											0.2	0.1	0.5*	0.2	0.2	0.4
NFC												0.1	0.6**	0.9**	0.9**	0.7**
FSP													0.7**	-0.3	0.0	0.5*
NBP														0.4	0.5*	0.9**
BD															0.9**	0.6**
IBW																0.6**

* and ** Correlarion is significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table 2.6 Pearson's correlation coefficients between pairs of morphological characteristics in blueberries propagated by softwood cutting: number of stems per plant (NSP), number of branches per plant (NBrP); number of branches per stem (NBrS), plant height (PH; cm), stem diameter (SD; mm), leaf length (LL; mm), leaf width (LW; mm), leaf area (LA; mm^2), plant vigour (PV), number of flowers per plant (NFP), number of flower clusters per plant (NCP), number of flowers per cluster (NFC), fruit setting percentage (FSP), number of berries per plant (NBP), berry diameter (BD; mm), individual berry weight (IBW; g) and berry weight per plant (BWP; g). Data was combined from two genotypes 'QB9C' and 'Fundy' grown in three growing seasons of 2011, 2012 and 2013.

Characters	NBrP	NBrS	PH	SD	LL	LW	LA	PV	NFP	NCP	NFC	FSP	NBP	BD	IBW	BWP
NSP	0.2	-0.6**	0.2	0.3	0.2	0.6**	0.5*	0.6**	-0.4	-0.3	-0.6**	0.1	-0.4	0.5*	0.6**	-0.1
NBrP		0.6**	0.1	0.4	-0.1	-0.3	-0.4	-0.3	0.3	0.4	-0.1	-0.8**	-0.1	-0.2	-0.2	-0.3
NBrS			-0.1	0.0	-0.3	-0.8**	-0.7**	0.2	0.5*	0.5*	0.5*	-0.6**	0.3	-0.6**	-0.7**	-0.1
PH				0.3	0.2	0.4*	0.4	0.0	-0.4	-0.4	-0.4*	0.0	-0.4	0.6**	0.6**	-0.2
SD					0.0	0.2	0.2	-0.1	-0.3	-0.2	-0.6**	-0.1	-0.4	0.4	0.4	-0.3

* and ** Correlarion is significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table 2.6 cont'd

Characters	NBrP	NBrS	PH	SD	LL	LW	LA	PV	NFP	NCP	NFC	FSP	NBP	BD	IBW	BWP
LL						0.5*	0.7**	0.2	-0.1	-0.1	0.1	0.2	-0.1	0.3	0.3	0.1
LW							0.9**	0.1	-0.8**	-0.8**	-0.6**	0.6**	-0.7**	0.9**	0.9**	-0.3
LA								0.2	-0.7**	-0.7**	-0.5*	0.6**	-0.6**	0.8**	0.8**	-0.2
PV									-0.2	-0.3	0.2	0.1	-0.2	0.1	0.1	-0.3
NFP										0.9**	0.7**	-0.5*	0.9**	-0.9**	-0.9**	.0.6**
NCP											0.6**	-0.5*	0.8**	-0.8**	-0.8**	0.6**
NFC												-0.2	0.7**	-0.8**	-0.8**	0.4
FSP													-0.1	0.4*	0.5*	0.4
NBP														-0.8**	-0.7**	0.8**
BD															0.9**	-0.4
IBW																-0.3

* and ** Correlarion is significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table 2.7 Pearson's correlation coefficients between pairs of morphological characteristics in blueberries: number of stems per plant (NSP), number of branches per plant (NBrP), number of branches per stem (NBrS), plant height (PH; cm), stem diameter (SD; mm), leaf length (LL; mm), leaf width (LW; mm), leaf area (LA; mm^2), plant vigour (PV), number of flowers per plant (NFP), number of flower clusters per plant (NCP), number of flowers per cluster (NFC), fruit setting percentage (FSP), number of berries per plant (NBP), berry diameter (BD; mm), individual berry weight (IBW; g) and berry weight per plant (BWP; g). Data was combined from two genotypes 'QB9C' and 'Fundy' propagated by softwood cutting and tissue culture, and grown in three growing seasons of 2011, 2012 and 2013.

Characters	NBrP	NBrS	PH	SD	LL	LW	LA	PV	NFP	NCP	NFC	FSP	NBP	BD	IBW	BWP
NSP	0.7**	-0.8**	0.1	-0.7**	0.1	0.2	0.2	0.2	-0.6**	-0.4*	-0.6**	-0.6**	-0.6**	0.1	0.1	-0.6**
NBrP		-0.4*	0.4*	-0.4*	0.2	-0.2	-0.1	-0.1	-0.2	-0.2	-0.5**	-0.8**	-0.5**	-0.2	-0.2	-0.6**
NBrS			-0.1	0.6**	-0.2	-0.5**	-0.5**	-0.1	0.7**	0.6**	0.6**	0.1	0.6**	-0.1	-0.2	0.4*
PH				0.1	0.3	0.1	0.2	-0.1	-0.4*	-0.4*	-0.4*	-0.2	-0.4*	0.1	0.1	-0.4*
SD					-0.2	0.1	0.0	-0.3	0.3	0.2	0.4*	0.2	0.3	0.3	0.2	0.4*

* and ** Correlarion is significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table 2.7 cont'd

Characters	NBrP	NBrS	PH	SD	LL	LW	LA	PV	NFP	NCP	NFC	FSP	NBP	BD	IBW	BWP
LL						-0.1	0.5**	-0.2	-0.4*	-0.2	-0.6**	0.1	-0.3	-0.6**	-0.6**	-0.6**
LW							.0.8**	0.1	-0.5**	-0.6**	-0.1	0.4*	-0.4*	0.7**	0.8**	-0.1
LA								0.1	-0.6**	-0.6**	-0.4*	0.4*	-0.5**	0.4*	0.4*	-0.3
PV									-0.2	-0.2	0.0	-0.1	-0.2	0.2	0.2	-0.2
NFP										0.9**	0.7**	0.1	0.9**	-0.3	-0.4*	0.7**
NCP											0.6**	-0.1	0.8*	0.4*	-0.4*	0.6**
NFC												0.3	0.7**	0.2	0.2	0.7**
FSP													0.4*	0.2	0.3	0.6**
NBP														-0.2	-0.2	0.9**
BD															0.9**	0.1
IBW																0.1

* and ** Correlarion is significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

cultivar ‘Dwarf Tophat’ (Georgieva, 2013) and wild clone ‘NB 284’ (Debnath, 2007b). They reported that TC plants had vigorous growth, longer and more stems with more leaves per stem, and produced larger canopy than the conventional cuttings. *In vitro* derived Southern highbush blueberry (*V. corymbosum* interspecific hybrids) cultivars ‘Emerald’ and ‘Jewel’ had larger canopy volume than SC plants (Marino et al., 2014). Generally, lowbush blueberry plants are established and bloomed in 3 - 5 years after planting or seed germination. In the present study, plants were grown in greenhouse for more than 5 years in greenhouse before data collection was started. The propagation effect on morphological characteristics for a period of 3 consecutive growing seasons revealed that TC-derived plants grew more vigorously and produced significantly more stems, branches and bigger leaves than SC plants. Higher number of shoots and rhizomes in micropropagated plants were reported in lingonberry (Vyas et al., 2013a), highbush (Marino et al., 2014), half-high (El-Shiekh et al., 1996) and lowbush (Debnath, 2007b) blueberries. Branches per plant were more in *in vitro* derived half-high blueberry plants (El-Shiekh et al., 1996), but not in lowbush blueberries (Morrison et al., 2000). Plant height in this study was not affected by propagation methods. Taller rhizomes were reported in TC highbush (Litwińczuk et al., 2005) and half-high blueberries (El-Shiekh et al., 1996), whereas shorter and less-vigorous shoots were produced in TC plants of lingonberry (Debnath, 2006). Although TC plants produced higher number of rhizomes than other propagation methods in *Vaccinium* spp., the micropropagation effects on plant height is species specific.

Leaf size in blueberry plants was significantly influenced by propagation methods. Litwińczuk et al. (2005) reported that micropropaged plants of highbush blueberry cultivar

'Herbert' produced wider leaves compared to SC platns. Conversely, Brissette et al. (1990) found that reversion of field-grown matured lowbush blueberry plants to juvenile state produced smaller and rounder leaves in micropropagation using bud cultures. Micropropagated lingonberry plants also bore smaller leaves than SC counterparts.

The increasing number of shoots and branches is the result of the escalation in vegetative growth of micropropagated plants. The differences in growth habit observed can be attributed to different genotypes, growing seasons and/or the culture conditions used for propagation. A direct result of residual action of growth hormones used during *in vitro* propagation to promote the multiplication and elongation of shoots might have influenced the vegetative growth of TC plants (Debnath et al., 2012b). It is proposed that the left-over cytokinin from culture media within young TC plants of blueberry (Morrison et al., 2000) and of other *Vaccinium* species (Debnath, 2005a; Debnath & McRae, 2005) apparently induces the juvenile branching characteristics that enhance rhizome production ultimately influencing vegetative growth. In general, the shoot apex grows predominantly in intact plants and inhibits outgrowth of axillary buds. This phenomenon, known as apical dominance, is controlled by endogenous growth hormones auxin and cytokinin (Cline et al., 1997). Auxin, derived from shoot apex represses outgrowth of axillary buds, while cytokinin promotes outgrowth of axillary buds. In micropropagation with nodal explants, enhanced axillary branching involves the abolition of auxin derived at shoot apex and thus inhibits apical dominance resulting in the de-repression and multiplication of axillary buds. It has been demonstrated in *Pisum sativum* that auxin negatively regulates local cytokinin biosynthesis in the nodal stem by controlling the expression level of adenosine phosphate-

isopentenyltransferase (*PsIPT*) gene which encodes a key enzyme in cytokinin biosynthesis (Tanaka et al., 2006). Due to the simplicity of approach and faster propagation rate, micropropagation with nodal explants is extensively utilized in commercial micropropagation.

Possibly supernumerary rhizome or stem and branch formation in this study resulting from *in vitro* culture could have been a symptom for the rejuvenation characteristics in lowbush blueberry plants (Debnath, 2010) which has been continued for more than six years. Micropropagated thornless blackberry plants had greater shoot growth and more canes and branches per plant than plants from SCs at first year after propagation, but had similar lateral bud activity after 2 years of growth (Swartz et al., 1983). El-Shiekh et al. (1996) reported that higher branching and greater spreading characteristics of TC blueberry plants remained for long time even after 10 years of field trial in cold areas with short growing seasons. Cold climate and short growing seasons of Newfoundland are suitable for continuing rhizome production in lowbush when it is propagated *in vitro*.

In the present study, the characteristics of flower and fruit especially number of flowers, berry yield and yield components were significantly affected by propagation methods. Although SC plants flowered more abundantly, bore significantly higher number of berries, thus apparently yielded better than TC plants, the significant interaction between genotype and propagation method reflected that the effect of propagation methods was genotype specific for those characteristics. Jamieson and Nickerson (2003) reported significant genotype × propagation method interaction for berry weight and yield in the field

performance study of three lowbush blueberry clones propagated by SC, micropropagation and grown from open-pollinated seeds. They also reported less flower and fruit number and berry yield in micropropagated blueberry plants than in SC counterparts. Litwińczuk et al. (2005) and Vyas et al. (2013a) reported similar results for highbush blueberry and lingonberry, respectively. On the contrary, better yielding of TC plants without deteriorating fruit quality was reported for half-high 'Northblue' cultivar (El-Shiekh et al., 1996) and lingonberry (Gustavsson & Stanys, 2000). Whereas, no difference was found between established field-grown SC and micropropagated plants of half-high and lowbush blueberries for the number of flower buds per branch and berry weight per plant (Morrison et al., 2000). Although TC-derived plants produced less fruits than the plants propagated by SC, their even canopy structure with fewer branches per stem would be more amenable to mechanical harvesting.

The berry yield per plant was higher in SC plants in all three years of study, even after the plants had been growing for five years in greenhouse. The interactions between propagation method and growing season for all the flower and fruit characters except individual berry weight and berry diameter were similar as in El-Shiekh et al. (1996) study. They reported higher yield in TC-derived blueberry plants compare to SC counterparts in field trials. Read et al. (1989) also reported similar result in the first 3 years after planting. Whereas, in other genus of small fruits, raspberry for example, Deng et al. (1993) reported micropropagation produced same berry yield as those of conventionally propagated plants did in the third growing season.

Fruit development requires substantial metabolic inputs in the form of nutrients and energy. Plants derived from *in vitro* propagation directed significant amounts of energy into the production of new axillary shoots and rhizomes, and were therefore potentially limited by a commitment to vegetative growth that might have restricted the size and weight of fruit (Foley & Debnath, 2007). In contrast, SC plants showed energy conservation by producing fewer, if any, rhizomes and only one primary shoot (Debnath et al., 2012b) thereby allowing bigger size fruit ultimately increased berry weight.

The higher berry yield per plant in SC plants is mostly the result from developing more flowers per plant and larger size flower cluster. The large flower cluster with brilliant color and aromatic scent is generally attractive to insect pollinators like honey bees and other native bees (Hicks, 2011). Thus, single flowers or small size clusters of 2 - 3 flowers produced in *in vitro* derived 'QB9C' plants might affect pollination which is essential for fruit development. Since lowbush blueberries are genetically heterozygous and self-incompatible in nature, natural pollinators play a significant role in successful and adequate pollination and in fruit setting ultimately berry yield.

Differential response of blueberry genotypes to propagation methods for their flower and fruit characteristics is common. The number of flower buds per plant was more than double in 'QB9C' to 'Fundy' which might be due to more number of branches in 'QB9C' than that of in 'Fundy' cultivar. While, 'QB9C' is a wild selection from Quebec, 'Fundy' was selected from open-pollinated seedlings of cultivar 'Augusta', the first wild clone released as a cultivar (Aalders et al., 1975). The genotypes 'Fundy' and 'QB9C' belong to a complex

tetraploid *V. angustifolium* (2n = 4x = 48) species and the proposed origin of this species is as an autotetraploid of *V. boreale* (Camp, 1945) or an allotetraploid of two diploid species either *V. boreale* × *V. palladium* or *V. boreale* × *V. myrtilloides* (Vander Kloet, 1977), but with tetrasomic inheritance (Hokanson & Hancock, 1998). Hence, both genotypes are tetraploid and morphologically and genetically polymorphic as proved in DNA based molecular system analysis using expressed sequence tag-simple sequence repeat (EST-SSR), expressed sequence tag-polymerase chain reaction (EST-PCR) and inter simple sequence repeat (ISSR) markers (Debnath, 2009d, 2014b; Goyali et al., 2015a; Tailor et al., 2017). Since both clones originated from open pollinated genotypes and are different at the genetic level, their responses to the propagation methods are different for their morphological and reproductive characteristics. However, better performance of 'QB9C' with respect to the number of fruits, flowers and branches need to be confirmed in replicated field trials over years.

Berry weight per plant, the ultimate results of correlation among other agro-morphological characteristics, was highly affected by propagation methods in both genotypes. The significant positive correlations between berry yield and number of stems per plant, leaf width, number of flowers per cluster, berry diameter and individual berry weight in micropropagated plants (**Table 2.5**) revealed that those traits had certain inherent potential to increase berry yield when the plants are propagated *in vitro*. Micropropagated plants produce higher number of stems which may provide good fruit performance as the correlation showed in this study.

Propagation methods and growing seasons appeared to have pronounced effect on vegetative growth habit showing changes in morphological characteristics, as well as fruit number and yield in lowbush blueberry. While conventional SC stimulates various agro-morphological characteristics: flower number, inflorescence/cluster size, fruit number, berry size and yield, micropropagation influences vegetative growth of blueberry plants having faster spreading capacity with vigorous and large canopy which could help a producer recover the costs of establishment of new field more rapidly and help to cover the bare area of established field to get large scale production.

CHAPTER 3

Phytochemical Content and Antioxidant Activity in Lowbush Blueberries under Different Propagation Methods and Maturity Stages

This chapter is on determination of the effects of propagation methods and maturity stages on the phytochemical content and antioxidant property of leaves and fruits of two lowbush blueberry genotypes 'QB9C' and 'Fundy' propagated by softwood cutting and micropropagation. Parts of the results of this chapter have been published in the Canadian Journal of Plant Science 93: 1001-1008, Acta Horticulturae 1098: 137-142 and HortScience 50: 888-896 (Goyali et al., 2013, 2015a, 2015b[2]).

3.1 Introduction

Blueberry is one of the important sources of food and nutraceutical ingredients, and is distinguished for high antioxidant potential (Sellappan et al., 2002; Yi et al., 2005; Wolfe & Liu, 2007; Huang et al., 2012b). The major sources of the antioxidant properties of blueberries have been directly attributed to its intense phenolic, flavonoid and anthocyanin content (Howell et al., 2001; Krupa & Tomala, 2007). These phenolic-linked bioactive phytochemicals are present in higher level in lowbush blueberries (*Vaccinium angustifolium* Ait.) compared to other fruits and vegetables (Prior et al., 1998; Smith et al., 2000; Kalt et al., 2001b; Koca & Karadeniz, 2009; Piljac-Zegarac et al., 2009). It is interesting that the leaves of the wild blueberry, a byproduct of blueberry harvesting and processing, have higher polyphenol and proanthocyanidin content than fruits (Percival & MacKenzie, 2007; Riihinen et al., 2008). Although plants synthesize antioxidant

[2] The contributions of the author and co-authors to the manuscript are described in Appendix 1.

compounds for their own defense against an attack on the plant tissue or oxidative stress (Dias et al., 2016), certain types of dietary phytochemicals are present in sufficient level to contribute significantly to the antioxidant complement found in diet. In addition to the protective properties, these phenolic metabolites are free radical and metal scavengers (Wang et al., 1996) and help in mitigating oxidative damage to lipids, proteins, and nucleic acids which caused carcinogenesis in the human body (Neto, 2007). There are numerous products prepared from blueberry fruit and leaf extract utilized as dietary supplements in the world market (Yuan et al., 2011). *In vitro* and *in vivo* research demonstrates that the phytochemicals of those products and fruits itself have ability to reduce risk of development, and to treat of cancers (Matchett et al., 2005; Adams et al., 2010), stroke (Basu et al., 2010), cardiovascular disorders (Shaughnessy et al., 2009), diabetes (Cheplick et al., 2015; Kang et al., 2016) and aging related diseases (Shukitt-Hale, 2012). Blueberry extract has urinary tract protective anti-adhesion and colonic health protective anti-inflammatory and anti-microbial properties (Kalt et al., 2007). Due to the contribution of those 'life span essentials' in maintaining body function and health throughout the adult stages of life, blueberry is called health promoting 'Superfruit', and the consumer and nutraceutical market demand for polyphenolic-rich wild blueberry products has been increased.

The profile and quantitative composition of phenolics and flavonoids of blueberry vary with the internal physiological development of fruit as well as external stimuli. Genera, species, types and selections of blueberry vary with respect to content of these phytochemicals and their antioxidant properties (Howard et al., 2003). Phenolic and

anthocyanin content in berries are affected by the degree of maturity at harvest (Zadernowski et al., 2005). Blueberries at green stage have highest total phenolic and lowest anthocyanin content compared to the berries at advance maturity stages (Allan-Wojtas et al., 2001; Kalt et al., 2003; Forney et al., 2012). The genotype specific changes in the levels of those metabolites are predominant between the transition from semi-ripe (purple) to ripe stages. Total soluble phenolic content of semi-ripe (green/pink) and ripe blueberry fruits of highbush blueberry cultivar 'Bluecrop' were similar and remained unchanged in these stages of fruit maturity (Cheplick et al., 2015), while phenolic level was greater in ripe fruits of 'Bluecrop', 'Puru' and 'Berkeley' than in berries of turning maturity stage (Castrejón et al., 2008; Forney et al., 2012). Whereas the decrease level of phenolic content in ripe blueberries from the purple stages was reported in cultivar 'Reka' (Castrejón et al., 2008). In lowbush blueberry, chlorogenic and coumaric acid content decreased when fruits are transformed from green to blue, but caffeic acid increased gradually in fruits of more advanced stages of ripeness (Kalt & McDonald, 1996). Two common classes of flavonoids in *Vaccinium* spp., proanthocyanidins and anthocyanins, exhibited the opposite trend in their content with the fruit developmental stages. Concentration of proanthocyanidins is highest in flower ovaries and continued to decline gradually to a minimum at fully ripe cranberry and blueberry fruits (Vvedenskaya & Vorsa, 2004; Zifkin et al., 2012). But anthocyanin synthesis in those species is initiated after fruit growth ceased and is increased from unripe green to ripe blue stage of maturity (Vvedenskaya & Vorsa, 2004; Castrejón et al., 2008; Forney et al., 2012). It is higher in red leaves than in green ones (Percival & MacKenzie, 2007). Although total phenolic

content is in elevated levels in blueberries at green stage, anthocyanin level is dominant at mature stage.

Antioxidant activity is altered with the maturity stages of fruits as phenolic or flavonoid content does. Antioxidant activity in blueberry fruits of highbush cultivars 'Bergitta', Bluegold', Nelson' and 'Bluecrop' is higher at early developmental stages and decreased as fruit maturity is progressed (Kalt et al., 2003; Castrejón et al., 2008), while it remains unchanged in fruits of green, pink and blue (ripe) stages in 'Bluecrop' cultivar when phenolic content is adjusted to 100 μg/ml fruit extract (Cheplick et al., 2015). Ribera et al. (2010) reported that in blueberry cultivars 'Legacy', 'Brigitta', and 'Bluegold', total antioxidant activity of whole fruits decreased 68–85% at increasing maturity from unripe green to red stage (75% red color) whereas, fruits at ripe blue stage gained 68–83% higher antioxidant activity than in fruits at red stage. Increased maturity at harvest increases the antioxidant properties in 'Brightwell' and 'Tifblue' cultivars of rabbiteye (*V. ashei* Reade) blueberry (Prior et al., 1998). Water-soluble total antioxidant activity is increased in strawberry fruits from green to ripe stage (Olsson et al., 2004). It is vice-versa in case of water-insoluble antioxidant activity.

The phytochemical content and antioxidant properties in berries are affected by external factors such as growing environment, foliar application of growth regulators, pre- or post-harvest environmental conditions, year of production (Connor et al., 2002a; Howard et al., 2003; Kalt et al., 2003; Krupa & Tomala, 2007; Percival & MacKenzie, 2007). Although anthocyanin pigment formation and antioxidant properties of blueberry have been previously analyzed in the fruits of different developmental stages which have been

affected by harvest year and postharvest storages (Kalt et al., 2003), the role of propagation methods on the levels of phenolics, flavonoid, anthocyanin content and antioxidant properties in lowbush blueberry at different maturity stages have not been investigated.

Although the lowbush blueberry industries are depended generally on managing wild native stands, few improved cultivars such as 'Blomidon', 'Fundy' are cultivated commercially in small scale and in backyard gardens in North America and China (Li & Hong, 2009). It is proven that micropropagation is a more demanding and potentially more effective method for improving lowbush blueberry fields, comparable in its requirements with growing and setting out seedlings (Morrison et al., 2000). Micropropagated blueberry performed better for quick establishment in field due to their higher number of rhizomes and branches. Although micropropagation for lowbush blueberry started in the mid-1980s with single-bud explants of mature tissue to obtain multiple shoots (Frett & Smagula, 1983), most of those studies are on morphological characteristics including fruit yield. The synthesis of antioxidant phenolics is triggered often within plants as a response to tissue culture. For example, micropropagated strawberry and lingonberry fruits have higher level of polyphenols, flavonoids and anthocyanins as well as antioxidant activities than in fruits of stem cuttings (Foley & Debnath, 2007; Debnath, 2009c; Vyas et al., 2013a). However, lower concentration of phenolic compounds has been found in leaves of established tissue culture derived *Fragaria vesca* L. species (Yildirim & Turker, 2014). Little is known about those phytochemicals or their antioxidant activities in blueberry fruits and leaves originated from micropropagation. The present study was carried out to investigate the effect of propagation methods on the total phenolic, flavonoid, anthocyanin and proanthocyanidin

content and their antioxidant capacity in fruits and leaves in different growing seasons. The content of those phytochemicals and their antioxidant activities were evaluated in fruits and leaves at different maturity stages. The potential of micropropagation on the developmental stages for those antioxidant metabolites was also investigated. The main goal was to assess the possibility of using *in vitro* technique as a sustainable propagation method to increase fruit quality.

3.2 Materials and methods

3.2.1 Plant materials

Same plant materials, a wild lowbush blueberry clone 'QB9C' and a cultivar 'Fundy' (described in Chapter 2), derived from softwood cutting (SC) propagation and by tissue culture using node explant (TC) were used to assess the effect of propagation methods on the antioxidant metabolites and activities. Fresh fully expanded green leaves (**Figure 3.1**) with approximately equivalent physiological stages were collected separately from four plants per treatment in 3rd week of May in 2011 and 2012. Each treatment was replicated four times. Leaves were shock-frozen in liquid nitrogen immediately after collection and stored at -80 °C until the antioxidant phytochemicals were extracted. Fully ripe (well-developed blue color) fruits were picked from those four plants in each treatment in 2011, 2012 and 2013, weighed them and stored at -80 °C. A separate batch of fruits and leaves of different growth stages were collected in the growing season of 2014 to determine the effect of maturity stages on the phytochemical properties. Berries based on skin and pulp color (Kalt et al., 2003) were collected from five randomly selected plants of both

genotypes propagated by SC and TC: a) unripe or green fruit (green skin, pulp and whitish seed); b) half-ripe or red fruit (red skin and reddish or colorless pulp); and c) ripe fruit (blue skin, bluish pulp and brown seeds) (**Figure 3.2**). Green leaves were collected in 3rd week of May and red leaves in 3rd week of September from same plants. They were sock-frozen after collection and stored at -80 °C (**Figure 3.1**).

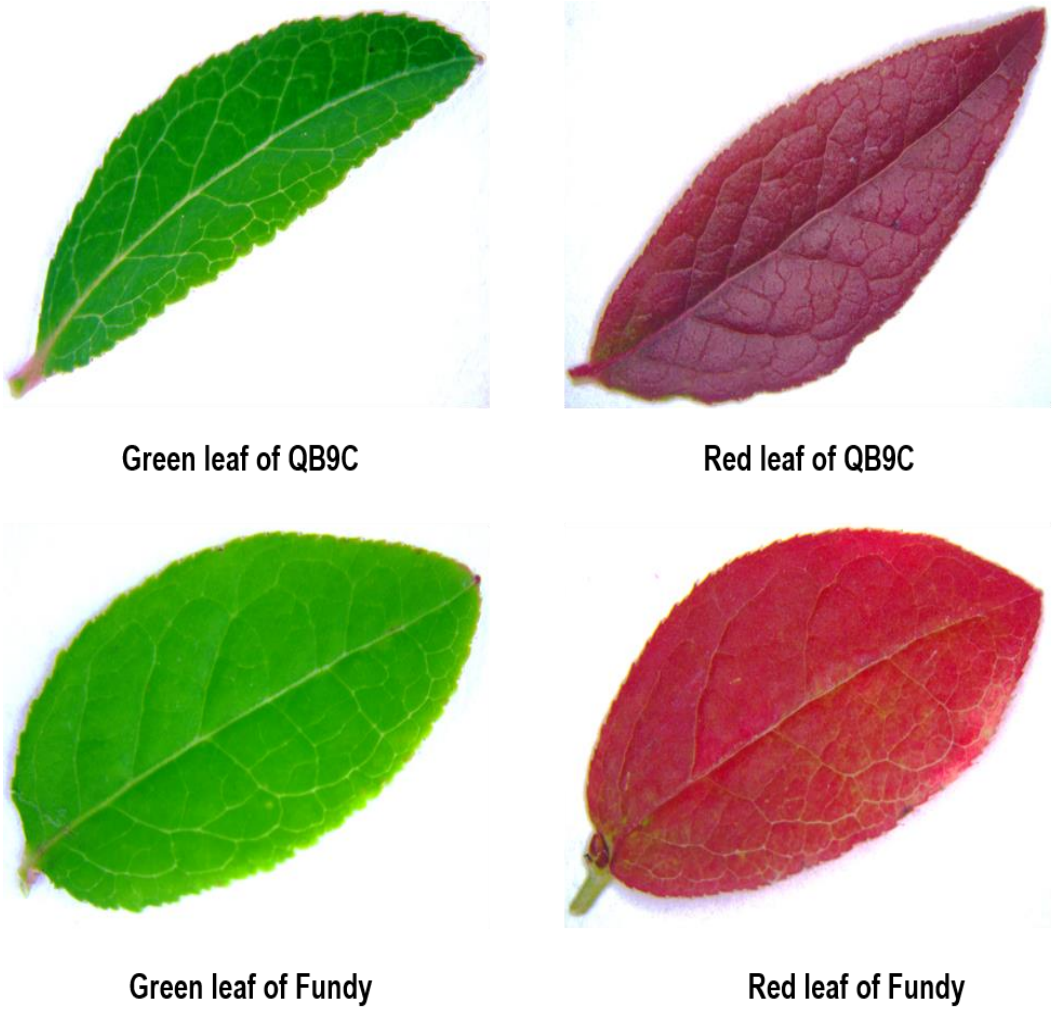

Figure 3.1 Healthy fully expanded blueberry leaves at different maturity stages

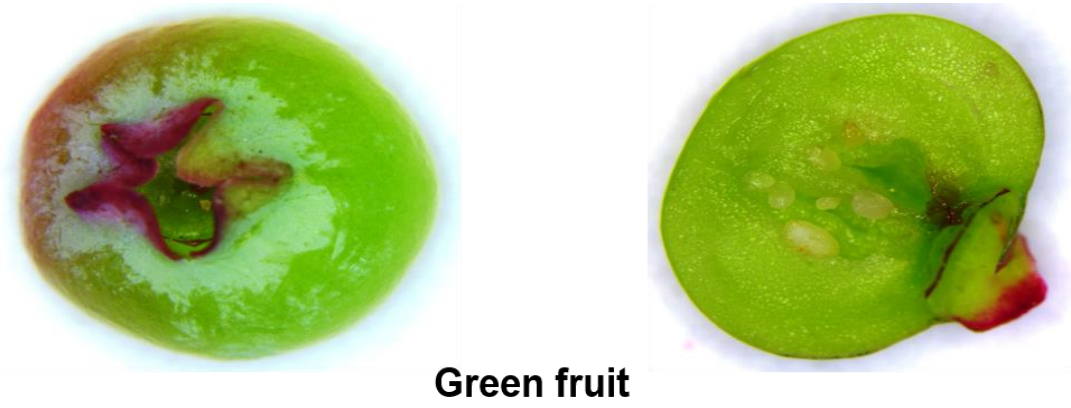

Figure 3.2 Whole fruits (left side) and halves of fruits (right side) of lowbush blueberry cultivar 'Fundy' at three stages of maturity based on the skin and pulp color of fruits

3.2.2 Chemicals

ACS grade acetone, Folin–Ciocalteau reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, catechin, vanillin, aluminium chloride, sodium nitrite, monobasic and dibasic potassium phosphates, sodium chloride, ascorbic acid, potassium ferricyanide, trichloroacetic acid, ferric chloride, cyanidin-3-glucoside, potassium chloride and sodium acetate were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON, Canada. Absolute methanol, sodium carbonate, sudium hydroxide, formic acid and hydrochloric acid (HCl) were purchased from Fisher Scientific Ltd., Ottawa, ON, Canada.

3.2.3 Extraction of polyphenolics from leaves and fruits

Frozen leaf tissues (green and red) were ground to a fine powder using a mortar and pestle in liquid nitrogen. A 200 - 500 mg ground leaf tissues was added with extraction solvent [80% (v/v) aqueous acetone containing 0.2% (v/v) formic acid] into a 2 ml safe-lock centrifuge tube at a ratio of 1:4 (g/ml) and vortex in high speed to homogenize. The ground green and red leaf tissues collected in 2014 were homogenized with same extraction solvent using FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Irvine, CA) at 5.5 m/s speed for 2 times at 45 s each with 5 min rest periods. The homogenate was shaken at 4 °C for 30 min and then centrifuged at 15000 *g* using a Benchtop Centrifuge, Model: Allegra 64R (Beckman Coulter Inc., Palo Alto, CA, USA) at 4 °C for 15 min. The supernatant, called extract, was separated and the residual tissue pellets were re-extracted following the same steps and conditions. The two extracts were combined (designated as concentrated extract) and stored at -80 °C until biochemical assays were carried out. The fruits from

each plant were cut into pieces in 15 ml safe-lock centrifuge tubes and homogenized with 80% aqueous acetone using FastPrep-24 Tissue and Cell Homogenizer. The remainder of the extraction steps was same as outlined for leaves.

3.2.4 Determination of total phenolic content

Total phenolic content in leaf and fruit extract was determined by the photometric method using the Folin-Ciocalteu reagent, an acidic phosphomolybdo-tungstate solution which oxidizes phenolate anions and develops blue colored chromogen, following Singleton and Rossi (1965) with few modifications. Folin-Ciocalteu reagent (100 µl) was added to 100 µl of appropriately diluted sample extract and standard solution (gallic acid) and mixed well. Within 30 s to 2 min, saturated sodium carbonate solution (200 µl) was added to develop basic condition. The final volume was adjusted to 1.8 ml by adding distilled water and mixed thoroughly. As a negative control or blank, 100 µl 80% aqueous acetone was used with all other components instead of extract (Xu & Chang, 2007). The mixture was placed in the dark for 35 min at ambient temperature followed by centrifuged at 4000 *g* for 10 min. The absorbance was read at 725 nm with a Libra S32 PC UV/Visible Spectrophotometer (Biochrom Ltd. Cambridge, UK) against blank. To select the appropriate concentration of leaf extract for phenolic assay, a series of dilutions were prepared from concentrated extracts of three SC ‘QB9C’ plants and measured the absorbance of those dilutions at 725 nm following the same steps above. The diluted leaf extracts which showed the absorbance ranged from 0.4 to 0.8, the concentration of that dilution was selected for each sample of SC ‘QB9C’ plants for all the biochemical assays.

Similar way the appropriate concentrations of leaf and fruit extracts were selected from all other treatment. Total phenolic content of each sample was measured as milligram of gallic acid equivalents (GAE) per gram fresh leaves and fruits. The test was performed three times on each sample and the mean was calculated.

3.2.5 Determination of total flavonoid content

Total flavonoid content was assessed using aluminium chloride colorimetric assay developed by Zhishen et al. (1999) with few modifications. A 500 µl aliquot of extract and standard solution of catechin was added with 2 ml of distilled water (dH_2O) and 150 µl of 5% (w/v) sodium nitrite. A 150 µl of 10% (w/v) aluminium chloride was mixed after 5 min, followed by 1 ml of 1 M sodium hydroxide solution was added after 6 min of adding aluminum chloride. The volume was adjusted to 5 ml with distilled water and absorbance of the mixture (pink in color) was read at 510 nm against the appropriate blank. Total flavonoid content in leaves and fruits was expressed in milligram of catechin equivalents (CE) per gram of leaves and fruits.

3.2.6 Determination of anthocyanin content

Quantification of monomeric anthocyanin content of the blueberry leaf and fruit extracts was carried out using the pH-differential method following Chen et al. (2012). This method estimates monomeric anthocyanin content based on the reversible change in color with a change in pH; the colored oxonium form exists at pH 1.0, and the colorless hemiketal form predominates at pH 4.5 (Lee et al., 2005). 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 a UV spectrophotometer after incubating in the dark at room temperature for 20 min. Total anthocyanin content was calculated using the following formula:

$$\text{Anthocyanin content (mg/L)} = \frac{\text{A} \times \text{MW} \times \text{DF} \times 1000}{\varepsilon \times 1}$$

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 g mol^{-1} for cyanidin-3-glucoside; DF = dilution factor; ε = 26900 molar extinction coefficient in L × mol^{-1} × cm^{-1} for cyanidin-3-glucoside; and l = path length (in cm) of the spectrophotometer. The total anthocyanin pigment concentration was expressed in milligram of cyanidin-3-glucoside equivalents (C3GE) per gram of leaves and fruits.

3.2.7 Determination of proanthocyanidin content

Proanthocyanidin content of leaf and fruit extracts was determined spectrophotometrically using modified vanillin methods developed by Price et al. (1978) with few modifications. A 0.5% (w/v) vanillin-HCl reagent was prepared by adding 0.5 g vanillin and 4 ml HCl in 96 ml absolute methanol. 2.5 ml vanillin-HCl reagent was added with 0.5 ml of diluted extract and standard (catechin) solution, mixed thoroughly and incubated at 30 °C in the dark for 20 min. The absorbance was recorded at 500 nm against the corresponding blank (80% aqueous acetone). Proanthocyanidin content of leaves and fruits was expressed in milligram of CE per gram of leaves and fruits.

3.2.8 Measurement of chlorophyll content

Chlorophyll concentration of leaf was determined non-destructively using an SPAD-502 portable chlorophyll meter (Minolta Camera Co., Osaka, Japan) in relative SPAD (Soil Plant Analysis Development) units. The average of readings was recorded for 10 fully matured green leaves of the third upper canopy of each plants of four in each treatment.

3.2.9 Determination of total antioxidant activity

The radical scavenging activity of leaf and fruit extracts of lowbush blueberry was carried out using a stabilized artificial free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH). In the radical form, DPPH molecule has an absorbance at 517 nm that disappears with acceptance of an electron from an antioxidant compound to become a stable diamagnetic molecule. The method was published by Hatano et al. (1988). A freshly prepared 60 µM DPPH solution (1.7 ml) in absolute methanol was added with an aliquot (100 µl) of leaf and fruit extracts or standard solution (gallic acid), mixed thoroughly, and left to stand in the dark for 45 min. The absorbance of the resulting solution was recorded at 517 nm. The DPPH scavenging activity of leaf and fruit extracts 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). Percent of inhibition was calculated by using the following formula (Khalaf et al., 2008):

$$\text{DPPH quenching (\%)} = \frac{\text{A} - \text{B}}{\text{A}} \times 100$$

Where, A is optical density of the blank and B is optical density of the leaf and fruit extract. Measurements were performed in triplicate. The gallic acid standard curve was used to express the total antioxidant activity in milligram of GAE per gram leaves and fruits.

3.2.10 Determination of reducing power

The reducing power of leaf extract and fruit extract was assessed using reducing power of iron (III) in ferricyanide complex according to the method explained by Chandrasekara and Shahidi (2010) with modifications. The extract (0.5 ml) was mixed with 1.25 ml of 0.2M phosphate buffer solution (pH 6.6) and 1.25 ml of 1% (w/v) potassium ferricyanide in a centrifuge tube. The mixture was incubated at 50 °C for 20 min, and then 1.25 ml of 10% (w/v) trichloroacetic acid was added, followed by centrifugation at 1800 *g* for 10 min at room temperature. The supernatant (1.25 ml) was transferred into a tube containing 1.25 ml of deionized water. A 0.25 ml of 0.1% (w/v) ferric chloride was added and mixed thoroughly. The absorbance was read at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Reducing power was expressed in milligram of ascorbic acid equivalent (AAE) per gram fresh leaves and fruits.

3.2.11 Statistical analysis

Data for all characterisctics were subjected to analysis of variance (ANOVA) using the General Linear Model of SAS statistical software package (SAS Institute, 2002). All data are presented as the mean ± SE of four replications. Statistical *F*-tests were evaluated at $P \leq 0.05$. The treatment means were compared by the least significant difference (LSD) using

the *F*-test. The relationships among antioxidant activities and other biochemical and morphological characteristics of fruits and leaves were determined using Pearson's correlation coefficients calculated with the Minitab 1.2 for Windows software package.

3.3 Results

3.3.1 Biochemical properties of leaves in SC and TC blueberry plants

3.3.1.1 Phytochemical content in leaves in different growing seasons

The phenolic content showed significant variation with genotype, propagation method and growing season and with the interactions of genotype × propagation method, genotype × growing season (**Table 3.1** & **3.2**). Significant interactions of genotype × propagation method × growing season were observed for flavonoid and proanthocyanidin content in leaf extracts. The *F* values both for genotype and propagation method were much higher than those for their interactions, suggested that the propagation method affected blueberry genotypes similar way for the phytochemical content in leaves (**Table 3.1**). Propagation method interacted significantly with genotype for the total phenolic, proanthocyanidin and chlorophyll content, and with growing season for total flavonoid and proanthocyanidin content in leaves. Genotypic performance for the total phenolic, flavonoid and proanthocyanidin content was depended on the growing season as found in significant interactions between genotype and growing season for those characteristics. Across the genotypes and growing seasons, all the phytochemicals studied in blueberry leaves except chlorophyll content were higher in leaves of SC plants than in leaves of TC ones. The

Table 3.1 Analysis of variance for combined effect of genotype, propagation method and growing season on total phenolic, flavonoid, proanthocyanidin and chlorophyll content, and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DRSA) in green leaves of lowbush blueberry genotypes 'QB9C' and 'Fundy' measured in 2011 and 2012

Source of variation	df	Total phenolic content (mg GAE/g F.L.)		Total flavonoid content (mg CE/g F.L.)		Proanthocyanidin content (mg CE/g F.L.)		Chlorophyll content (SPAD[z] unit)		DRSA (mg GAE/g F.L.)	
		F values	*P* values	*F* values	*P* values	*F* values	*P* values	*F* values	*P* values	*F* values	*P* values
Genotypes (G)	1	41.3	<0.001	303	<0.001	42.8	<0.001	169	<0.001	0.01	0.909
Propagation methods (PM)	1	78.1	<0.001	63.5	<0.001	37.1	<0.001	0.01	0.928	62.8	<0.001
Growing seasons (GS)	1	95.5	<0.001	1.90	0.182	2.44	0.133	10.8	0.004	19.5	<0.001
G×PM	1	4.78	0.040	0.03	0.866	6.31	0.020	10.9	0.003	46.1	<0.001
G×GS	1	20.1	<0.001	22.2	<0.001	24.1	<0.001	1.26	0.274	19.5	<0.001
PM×GS	1	2.40	0.136	33.1	<0.001	4.06	0.050	0.43	0.518	3.21	0.088
G×PM×GS	1	0.02	0.892	29.6	<0.001	5.65	0.027	1.26	0.274	6.70	0.017

GAE = gallic acid equivalents; CE = catechin equivalents; F.L. = fresh leaf; [z]SPAD unit = soil plant analysis development unit.

Table 3.2. Mean values of the main factors across all the treatments for combined effect of genotype, propagation method and growing season on total phenolic, flavonoid, proanthocyanidin and chlorophyll content, and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DRSA) in green leaves of lowbush blueberries measured in 2011 and 2012

Parameters	Total phenolic content (mg GAE/g F.L.)	Total flavonoid content (mg CE/g F.L.)	Proanthocyanidin content (mg CE/g F.L.)
Genotypes (G)			
QB9C	41.3a	22.3a	6.51a
Fundy	35.7b	12.8b	4.70b
Propagation methods (PM)			
Softwood cutting	42.4a	19.7a	6.44a
Tissue culture	34.7b	15.3b	4.76b
Growing seasons (GS)			
2011	42.7a	17.9a	5.82a
2012	34.3b	17.5a	5.39a
Significant effects	G, PM, GS, G×PM, G×GS	G, PM, G×GS, PM×GS, G×PM×GS	G, PM, G×PM, G×GS, PM×GS, G×PM×GS

a, b Means within columns and factors followed by different letters indicate significant differences at $P \leq 0.05$; GAE = gallic acid equivalents; CE = catechin equivalents; F.L. = fresh leaf.

Table 3.2. cont'd

Parameters	Chlorophyll content (SPAD[z] unit)	DRSA (mg GAE/g F.L.)
Genotypes (G)		
QB9C	31.1a	31.7a
Fundy	23.0b	31.6a
Propagation methods (PM)		
Softwood cutting	27.1a	33.6a
Tissue culture	27.0a	29.8b
Growing seasons (GS)		
2011	26.0b	32.7a
2012	28.1a	30.6b
Significant effects	G, GS, G×PM	PM, GS, G×PM, PM×GS, G×PM×GS

a, b Means within columns and factors followed by different letters indicate significant differences at $P \leq 0.05$; F.L. = fresh leaf; [z]SPAD unit = soil plant analysis development unit.

leaves of wild clone ‘QB9C’ had higher content of all the phytochemicals studied compared with the leaves of ‘Fundy’.

The detailed performance of individual blueberry genotypes for antioxidant metabolite content in leaves in two growing seasons is shown in **Figure 3.3 A - D**. Although the leaf extract from SC plants of ‘QB9C’ had higher phenolic content than of TC plants in both growing seasons, no significant difference between SC and TC ‘QB9C’ plants was found in 2012 for total flavonoid and proanthocyanidin content. Whereas, SC ‘Fundy’ plants performed better for total phenolic, flavonoid and proanthocyanidin content than TC plants in both growing seasons.

3.3.1.2 Antioxidant activity in leaves in different growing seasons

The total antioxidant activity in the leaf extract of both blueberry genotypes measured as DPPH radical scavenging activity was influenced by propagation method and growing season. DPPH radical scavenging activity varied significantly with propagation method, growing season and the propagation method × growing season interaction with propagation method being major influence (**Table 3.1** & **3.2**). Across the genotypes and growing seasons, leaves from SC plants were superior to TC leaves for antioxidant capacity. DPPH radical scavenging activity in leaves was higher in 2011 compared to in 2012.

The detailed performance of individual blueberry genotypes for antioxidant activity in leaves in two growing seasons is shown in **Figure 3.4**. Although the leaf extract of SC ‘QB9C’ plants exerted significantly higher DPPH radical scavenging capacity in 2012, no

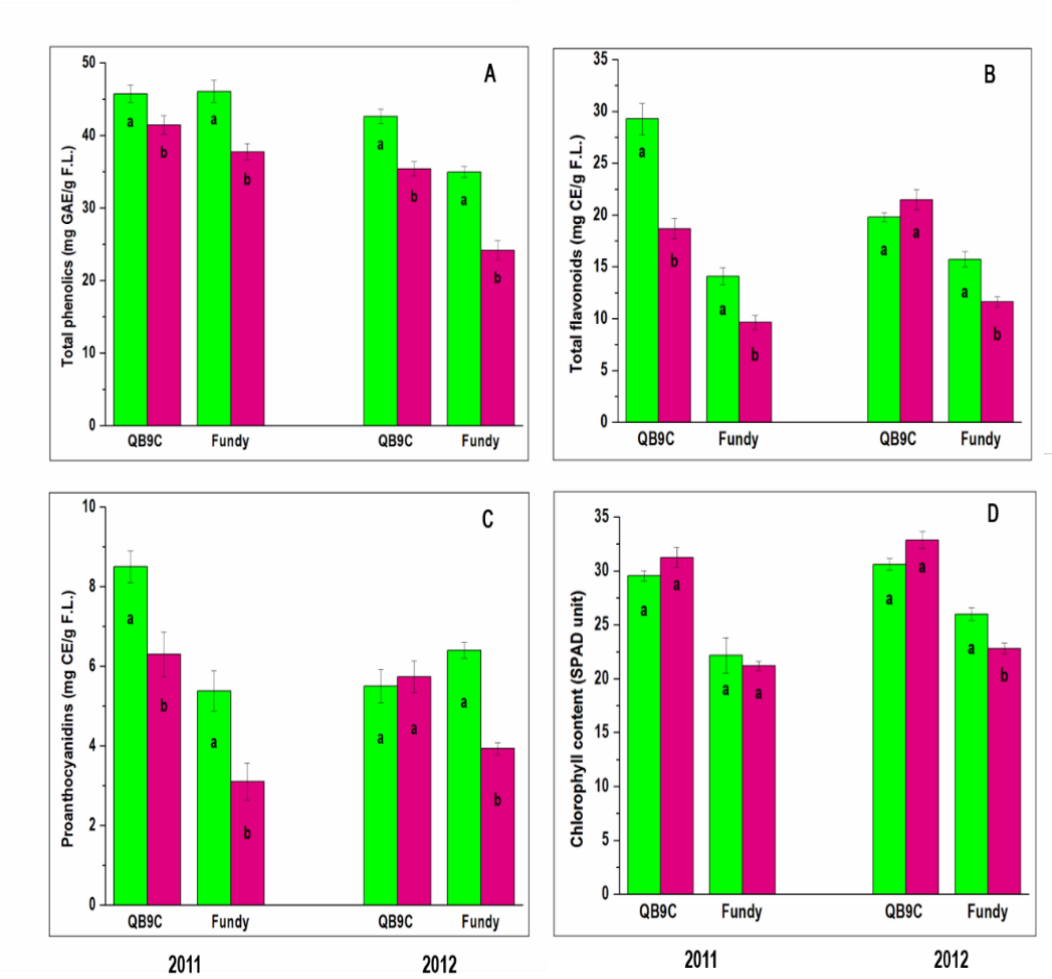

Figure 3.3 Effect of propagation method on the content of phenolics (A), flavonoids (B), proanthocyanidins (C) and chlorophyll (D) in leaves of blueberry wild clone 'QB9C' and cultivar 'Fundy' propagated by softwood cutting (green bars) and tissue culture (red bars) measured in two growing seasons of 2011 and 2012. GAE = gallic acid equivalents; CE = catechin equivalents; SPAD = soil plant analysis development; F.L. = fresh leaf. Different letters (a, b) indicate significant differences between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 4).

difference was found in antioxidant activity in 2011. Whereas, the leaf extract from SC 'Fundy' plants performed better in antioxidant activity than the leaf extract from TC plants in both growing seasons.

3.3.1.3 Phytochemical content in leaves at different maturity stages

The phenolic content in leaves varied significantly with the genotype, maturity stage, and the two-way (maturity stage × propagation method) and three-way (genotype × maturity stage × propagation method) interactions (**Table 3.3** & **3.4)**. Flavonoid content varied with the genotype, maturity stage and their interaction. The *F* ratios both for genotype and maturity stage were much higher than that for the interactions, suggesting that the genotypes reacted similarly to maturity stage for those phytochemicals (**Table 3.3)**. The anthocyanin content showed significant variation with genotype, maturity stage, propagation method and with genotype × propagation method, maturity stage × propagation method and genotype × maturity stage × propagation method interactions, with maturity stage being the major influence. The proanthocyanidin content varied significantly with maturity stage and the genotype × maturity stage × propagation method interaction. The *F* ratio for the three-way interaction was much lower than the *F* ratio for maturity stage, meaning that although there were some variations in the pattern of proanthocyanidin content in leaves of SC plants from TC leaves, they were smaller than the trends for maturity stage. Across the genotypes and propagation methods, the extract from red leaves had higher content of phenolics, flavonoids, anthocyanins and proanthocyanidins than those in the extract from green leaves (**Table 3.4**). Although the

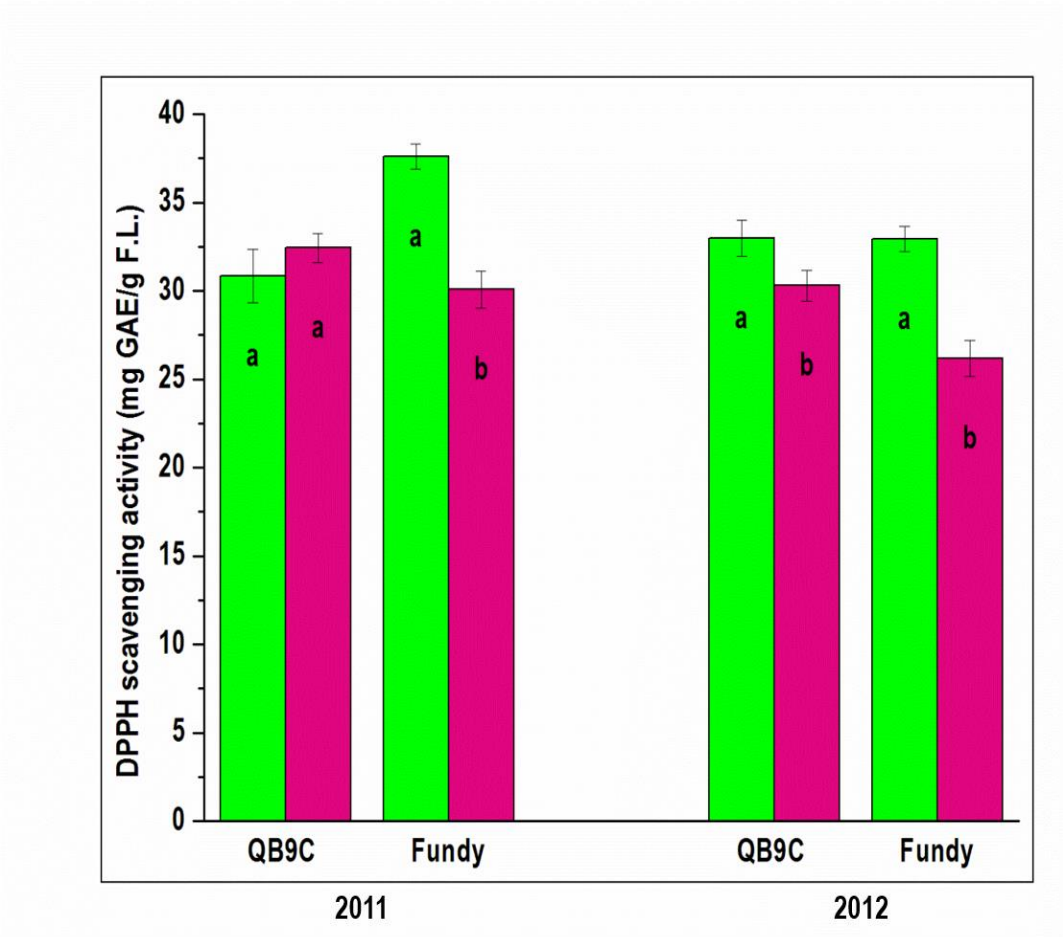

Figure 3.4 Effect of propagation method on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity in green leaves of blueberry wild clone 'QB9C' and cultivar 'Fundy' propagated by softwood cutting (green bars) and tissue culture (red bars) measured in two growing seasons of 2011 and 2012. GAE = gallic acid equivalents; F.L. = fresh leaf. Different letters (a, b) indicate significant differences between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 4).

Table 3.3 Analysis of variance for combined effect of genotype, maturity stage and propagation method on total phenolic, flavonoid, anthocyanin and proanthocyanidin content, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DRSA) and reducing power in green and red leaves of two lowbush blueberry genotypes measured in 2014

Source of variation	df	Phenolic content (mg GAE/g F.L.)		Flavonoid content (mg CE/g F.L.)		Anthocyanin content (mg C3GE/g F.L.)		Proanthocyani-din content (mg CE/g F.L.)		DRSA (mg GAE/g F.L.)		Reducing power (mg AAE/g F.L.)	
		F values	*P* values	*F* values	*P* values	*F* values	*P* values	*F* values	*P* values	*F* values	*P* values	*F* values	*P* values
Genotypes (G)	1	118	<0.001	133	<0.001	10.9	0.003	0.14	0.712	136	<0.001	0.28	0.603
Maturity stages (MS)	1	71.3	<0.001	92.1	<0.001	403	<0.001	80.9	<0.001	98.6	<0.001	147	<0.001
Propagation methods (PM)	1	0.52	0.474	2.25	0.145	5.37	0.028	1.4	0.246	3.28	0.081	8.00	0.009
G×MS	1	2.90	0.098	13.3	0.001	0.08	0.777	3.94	0.057	30.9	<0.001	4.10	0.053
G×PM	1	1.26	0.270	1.49	0.233	17.1	<0.001	3.89	0.058	3.25	0.082	2.00	0.169
MS×PM	1	5.21	0.029	3.29	0.080	16.7	<0.001	1.18	0.287	0.17	0.681	5.62	0.025
G×MS×PM	1	21.6	<0.001	3.03	0.093	24.7	<0.001	7.45	0.011	0.40	0.532	11.3	0.002

GAE = gallic acid equivalents; CE = catechin equivalents; C3GE = cyanidine-3-glucoside equivalents; AAE = ascorbic acid equivalents; F.L. = fresh leaf.

Table 3.4 Mean values of the main factors across all the treatments for combined effect of genotype, maturity stage and propagation method on total phenolic, flavonoid, anthocyanin and proanthocyanidin content, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DRSA) and reducing power in green and red leaves of lowbush blueberries determined in 2014

Parameters	Phenolic content (mg GAE/g F.L.)	Flavonoid content (mg CE/g	Anthocyanin content (mg C3GE/g F.L.)	Proanthocyani-din content (mg CE/g F.L.)
Genotypes (G)				
QB9C	59.3a	30.4a	0.47b	10.8a
Fundy	48.0b	20.2b	0.53a	10.9a
Maturity stages (MS)				
Green leaf	49.3b	21.1b	0.30b	9.40b
Red leaf	58.0a	29.5a	0.69a	12.2a
Propagation methods				
Softwood cutting	54.0a	26.0a	0.52a	11.0a
Tissue culture	53.3a	24.6a	0.48b	10.6a
Significant effects	G, MS, MS×PM G×MS×PM	G, MS, G×MS	G, MS, PM, G×PM, MS×PM, G×MS×PM	MS, G×MS×PM

a, b Means within columns and factors followed by different letters indicate significant differences at $P \leq 0.05$; GAE = gallic acid equivalents; CE = catechin equivalents; C3GE = cyanidine-3-glucoside equivalents; F.L. = fresh leaf.

Table 3.4 cont'd

Parameters	DRSA (mg GAE/g F.L.)	Reducing power (mg AAE/g F.L.)
Genotypes (G)		
QB9C	42.4b	87.9a
Fundy	49.7a	89.1a
Maturity stages (MS)		
Green leaf	42.9b	74.9b
Red leaf	49.1a	102a
Propagation methods		
Softwood cutting	45.5a	91.7a
Tissue culture	46.6a	85.3b
Significant effects	G, MS, G×MS	MS, PM, MS×PM, G×MS×PM

a, b Means within columns and factors followed by different letters indicate significant differences at $P \leq 0.05$; GAE = gallic acid equivalents; AAE = ascorbic acid equivalents; F.L. = fresh leaf.

total phenolic, flavonoid and proanthocyanidin content did not varied significantly with propagation method, the leaf extract from SC plants had higher anthocyanin content than TC counterparts.

The phytochemical content in leaf extract of individual blueberry genotypes studied in two maturity stages is shown in **Figure 3.5A-D**. The extract from green leaves of 'QB9C' plants propagated by TC had higher content of phenolics and proanthocyanidins compared to those in the leaf extract of SC 'QB9C' plants. Whereas red leaves from SC 'QB9C' plants contained higher phenolic, flavonoid and anthocyanin content than in red leaves of TC plants. For 'Fundy', green leaves of SC plants had higher proanthocyanidin content than the green TC counterparts, and SC red leaves contained less phenolics than that in red leaves of TC plants.

3.3.1.4 Antioxidant activities in leaves at different maturity stages

DPPH radical scavenging activity showed significant variation with genotype, maturity stage and the interaction between those two factors (**Table 3.3** & **3.4)**. The *F* ratios both for genotype and maturity stage were much higher than the *F* ratio for the interaction of genotype × maturity stage, suggesting that the both genotypes studied responded similarly to the maturity stage for antioxidant activity in leaves (**Table 3.3)**. Reducing power in leaves varied significantly with the maturity stage, propagation method and the two-way (maturity stage × propagation method) and three-way (genotype × maturity stage × propagation method) interactions. The *F* values for both maturity stage and propagation method are much higher than that for the interaction of maturity stage × propagation

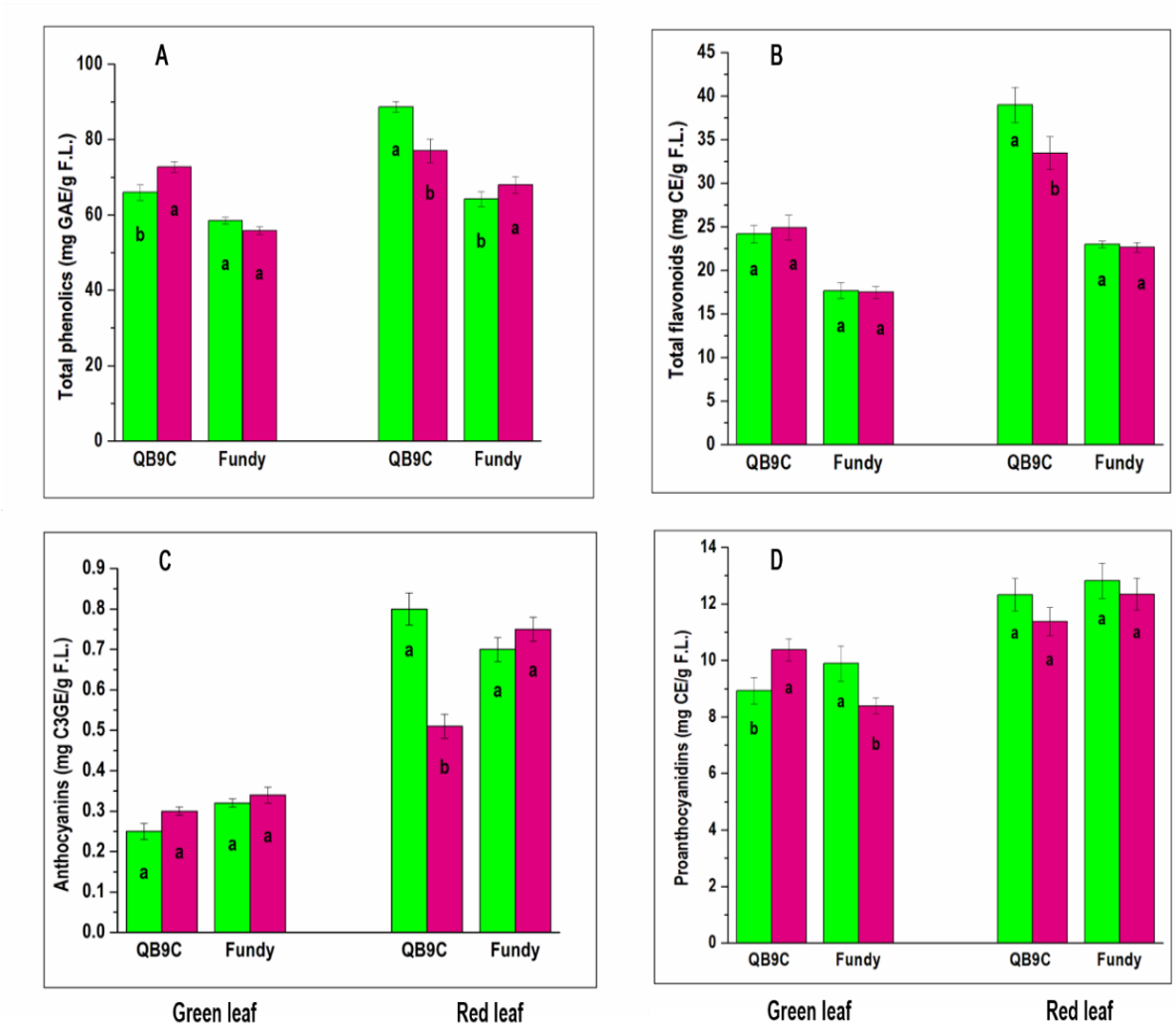

Figure 3.5 The levels of phenolic (A), flavonoid (B), anthocyanin (C) and proanthocyanidin (D) content in green and red leaves of blueberry genotypes propagated by softwood cutting (green bars) and tissue culture (red bars). GAE = gallic acid equivalents; CE = catechin equivalents; C3GE = cyanidine-3-glucoside equivalents; F.L. = fresh leaf. Different letters (a, b) indicate significant differences between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 5).

method. It was also observed that variation in maturity stages were greater than variations between propagation methods. Across the genotypes and propagation methods, the extract from red leaves exhibited higher antioxidant activities measured as scavenging capacity of DPPH radicals and reducing power of ferric ions than those in green leaf extract (**Table 3.4)**. Across the genotypes and maturity stages, the leaves from SC plants exerted higher reducing power than the leaves from TC plants. The cultivar 'Fundy' performed better DPPH radical scavenging capacity than the wild clone 'QB9C'.

The antioxidant activities in leaf extract at two maturity stages of 'QB9C' and 'Fundy' are shown detailed in **Figure 3.6A** & **B**. The extract from red leaves of SC 'QB9C' plants performed better in reducing power than that from red leaf of TC plants of same genotype. Whereas, DPPH radical scavenging capacity and reducing power were not varied significantly with propagation methods in 'Fundy'.

3.3.2 Biochemical properties of fruits in SC and TC blueberry plants

3.3.2.1 Phytochemical content in fruits in different growing seasons

The total phenolic content in fruits varied significantly with genotype, propagation method, growing season and genotype × propagation method interaction (**Table 3.5)**. The *F* values for genotype and propagation method were higher than that for the interaction between those factors, indicated that the genotypes reacted similarly to propagation techniques for phenolic synthesis. The *F* ratio for growing season suggested that seasonal variations had profound impact on phenolic content of blueberry as found higher phenolics in 2013 than

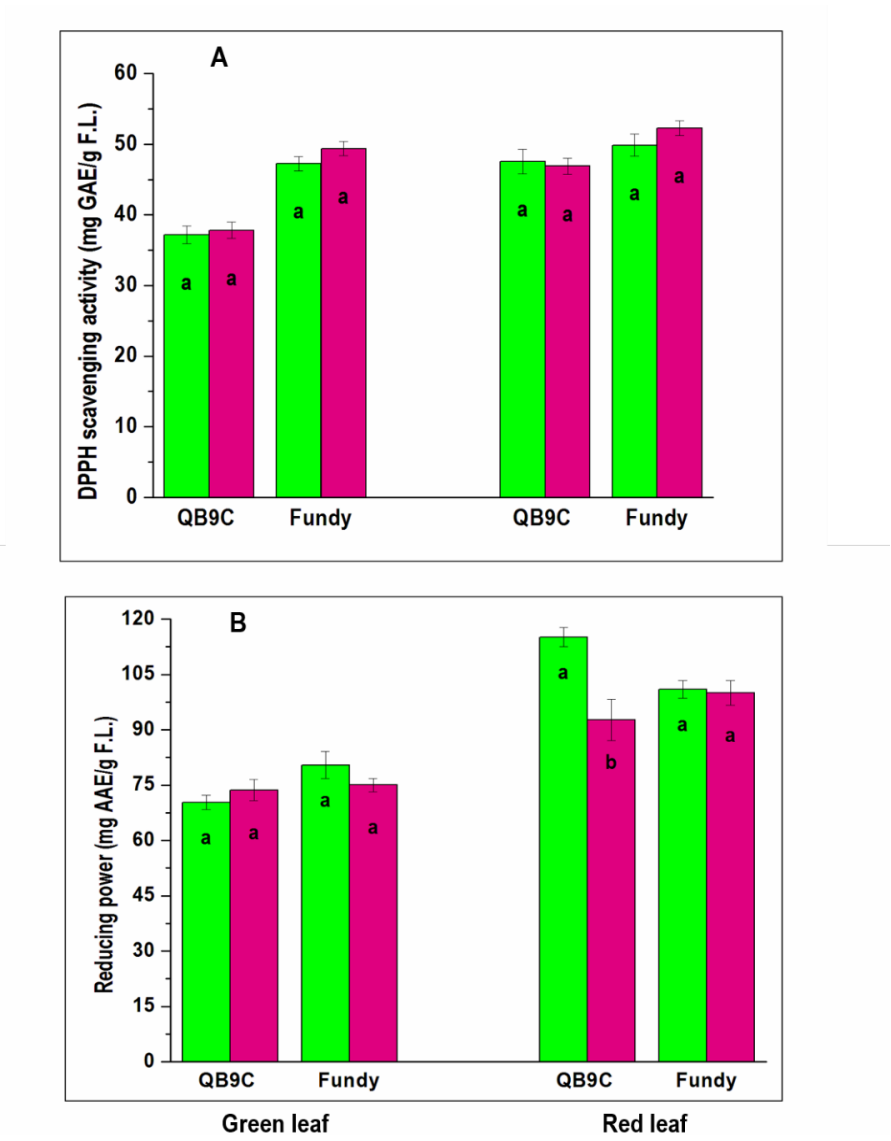

Figure 3.6 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity (A) and reducing power (B) in green and red leaves of blueberry genotypes propagated by softwood cutting (green bars) and tissue culture (red bars). GAE = gallic acid equivalents; AAE = ascorbic acid equivalents; F.L. = fresh leaf. Different letters (a, b) indicate significant differences between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 5).

in 2011 and 2012 (**Table 3.6**). Analysis of variance for combined effect of three factors (genotype × propagation method × growing season) was significant ($P \leq 0.05$) for total flavonoid content in fruit extract (**Table 3.5** & **3.6**).

Interaction between genotype and propagation method was significant for anthocyanin content of fruit extract whereas, propagation method was interacted significantly with growing season for all the phytochemical content studied in fruits except total phenolics. The wild clone 'QB9C' and the cultivar 'Fundy' performed differently for flavonoid and anthocyanin content in fruits in different growing seasons as found in interaction between genotype and growing season for those phytochemical content (**Table 3.6**). The fruit extract of 'QB9C' had higher total polyphenols, flavonoids, anthocyanins and proanthocyanidins compared to those of 'Fundy' across the propagation methods and growing seasons. Across the genotypes and growing seasons, total phenolic and flavonoid content was higher in fruit extract of TC plants than in fruit extract of SC counterparts. The content of total polyphenolics, flavonoids and proanthocyanidins in fruits were significantly higher in the growing season of 2013 than in other seasons. However, anthocyanin content was higher in 2011 compared to in 2012 and 2013.

The levels of phytochemical content in fruits of individual blueberry genotypes propagated by SC and TC are shown in **Figure 3.7A - D**. The wild clone 'QB9C' was influenced more by micropropagation for all the phytochemical content in fruits than the cultivar 'Fundy'. Total phenolic, flavonoid, anthocyanin and proanthocyanidin content were higher in fruits of TC-derived 'QB9C' plants than in SC counterparts at least in two out of three growing

Table 3.5 Analysis of variance for combined effect of genotype, propagation method and growing season on phenolic, flavonoid, anthocyanin and proanthocyanidin content, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DRSA) and reducing power in fruits of lowbush blueberry genotypes measured in three seasons of 2011, 2012 and 2013

Source of variation	df	Phenolic content (mg GAE/g F.F.)		Flavonoid content (mg CE/g F.F.)		Anthocyanin content (mg C3GE/g F.F.)		Proanthocyanidin content (mg CE/g F.F.)	
		F values	*P* values	*F* values	*P* values	*F* values	*P* values	*F* values	*P* values
Genotypes (G)	1	61.3	<0.001	69.6	<0.001	21.3	<0.001	7.09	0.012
Propagation methods (PM)	1	21.2	<0.001	28.3	<0.001	1.37	0.249	0.01	0.972
Growing seasons (GS)	2	24.1	<0.001	5.09	0.012	24.2	<0.001	19.5	<0.001
G×PM	1	17.5	<0.001	0.21	0.651	9.51	0.004	3.43	0.073
G×GS	2	1.72	0.195	10.1	<0.001	9.53	<0.001	1.67	0.203
PM×GS	2	0.49	0.618	12.2	<0.001	4.74	0.016	5.19	0.011
G×PM×GS	2	1.15	0.328	13.7	<0.001	1.69	0.201	1.75	0.189

GAE = gallic acid equivalents; CE = catechin equivalents; C3GE = cyanidine-3-glucoside equivalents; F.F. = fresh fruit.

Table 3.5 cont'd

Source of variation	df	DRSA (mg GAE/g F.F.)		Reducing power (mg AAE/g F.F.)	
		F values	*P* values	*F* values	*P* values
Genotypes (G)	1	16.8	<0.001	41.8	<0.001
Propagation methods (PM)	1	2.38	0.132	7.93	0.008
Growing seasons (GS)	2	19.2	<0.001	1.41	0.257
G×PM	1	5.42	0.026	5.93	0.020
G×GS	2	2.59	0.091	0.59	0.561
PM×GS	2	1.80	0.182	1.87	0.171
G×PM×GS	2	0.48	0.622	5.28	0.010

GAE = gallic acid equivalents; AAE = ascorbic acid equivalents; F.F. = fresh fruit.

Table 3.6 Mean values of the main factors across all the treatments for combined effect of genotype, propagation method and growing season on phenolic, flavonoid, anthocyanin and proanthocyanidin content, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DRSA) and reducing power in fruits of two lowbush blueberry genotypes measured in three growing seasons of 2011, 2012 and 2013

Parameters	Phenolic content (mg GAE/g F.F.)	Flavonoid content (mg CE/g F.F.)	Anthocyanin content (mg C3GE/g F.F.)	Proanthocyanidin content (mg CE/g F.F.)
Genotypes (G)				
QB9C	8.29a	3.07a	2.62a	2.15a
Fundy	6.55b	2.64b	2.24b	1.96b
Propagation methods (PM)				
Softwood cutting	6.91b	2.72b	2.38a	2.05a
Tissue culture	7.93a	2.99a	2.48a	2.06a
Growing seasons (GS)				
2011	7.30b	2.77b	2.66a	2.21a
2012	6.54c	2.83b	2.61a	1.75b
2013	8.41a	2.97a	2.03b	2.22a
Significant effects	G, PM, GS, G×PM	G, PM, GS, G×GS, PM×GS, G×PM×GS	G, GS, G×PM, G×GS, PM×GS	G, GS, PM×GS

a - c Means within columns and factors followed by different letters indicate significant differences at $P \leq 0.05$; GAE = gallic acid equivalents; CE = catechin equivalents; C3GE = cyanidine-3-glucoside equivalents; F.F. = fresh fruit.

Table 3.6 cont'd

Parameters	DRSA (mg GAE/g F.F.)	Reducing power (mg AAE/g F.F.)
Genotypes (G)		
QB9C	2.99a	5.53a
Fundy	2.66b	4.36b
Propagation methods (PM)		
Softwood cutting	2.77a	4.69b
Tissue culture	2.89a	5.20a
Growing seasons (GS)		
2011	3.11a	4.74a
2012	2.51c	4.99a
2013	2.86b	5.11a
Significant effects	G, GS, G×PM	G, PM, G×PM, G×PM×GS

a - c Means within columns and factors followed by different letters indicate significant differences at $P \leq 0.05$; GAE = gallic acid equivalents; AAE = ascorbic acid equivalents; F.F. = fresh fruit.

seasons. None of the above antioxidant metabolites were changed significantly in their content in fruit of 'Fundy' genotype in all the growing seasons when it was propagated by either SC or TC.

3.3.2.2 Antioxidant activities in fruits in different growing seasons

Total antioxidant activities of blueberry extract assessed as scavenging capacity of DPPH radicals and reducing power of ferric (III) ions in ferricyanide complex varied with genotype and genotype × propagation method interaction, with genotype being major influence (**Table 3.5** & **3.6**). For both antioxidant activities, *F* value for genotype was much higher than that for genotype × propagation method interaction. Across the propagation methods and growing seasons, the berry extract of 'QB9C' exhibited higher DPPH radical scavenging capacity and ferric ion reducing power compared to 'Fundy' did **(Table 3.6)**. The significant interactions between two factors (genotype × propagation method) and three factors (genotype × propagation method × growing season) were observed in fruit extract for reducing power. Over the genotypes and growing seasons, micropropagated plants performed better for its' antioxidant activities in fruit extract.

The TC 'QB9C' plants found to have higher antioxidant potential at least in two growing seasons than the fruit extract from SC 'QB9C' plants either in scavenging capacity of DPPH radicals or in reducing power of ferric ions (**Figure 3.8A** & **B**). Fruit extracts from SC and TC plants of 'Fundy' were not significantly different in their antioxidant capacities in any growing season.

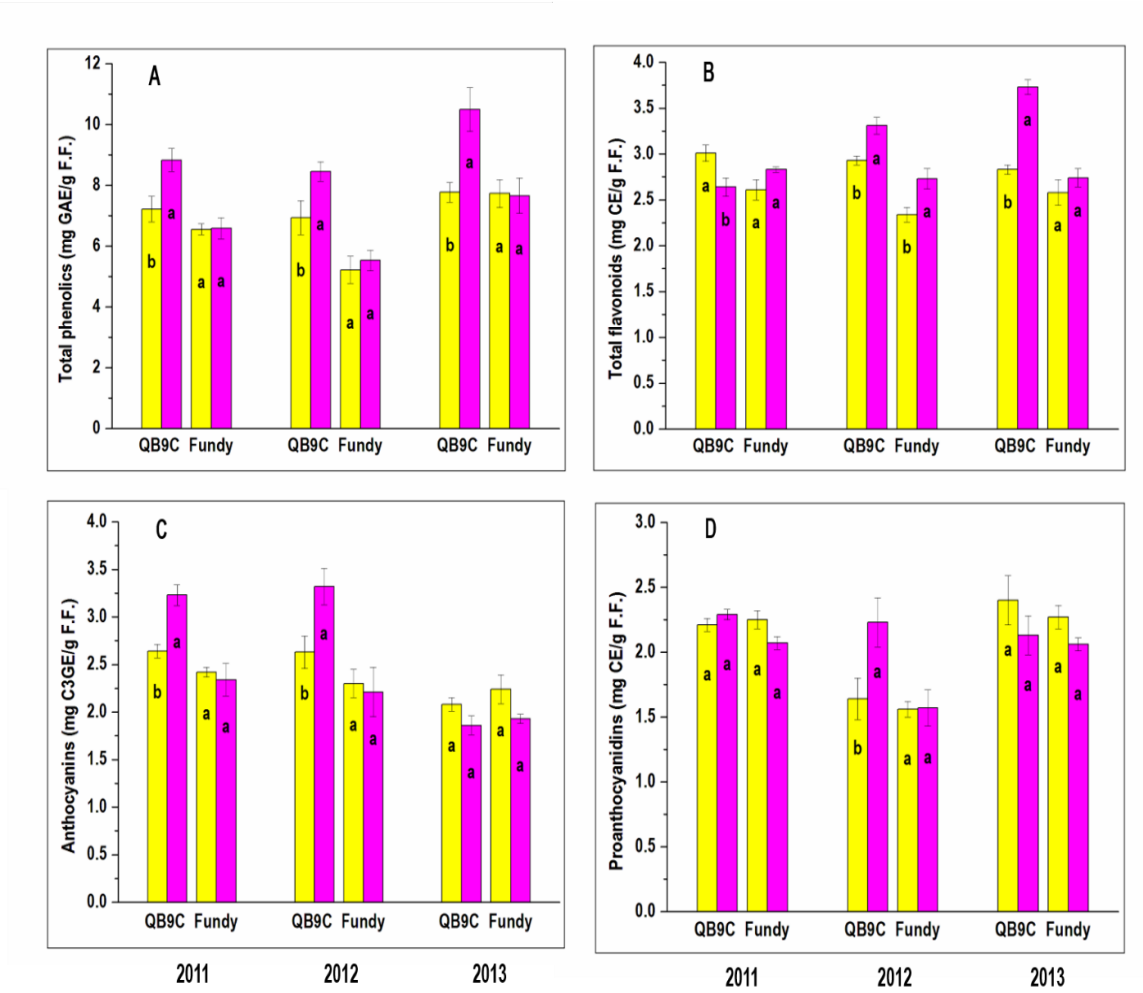

Figure 3.7 Detailed performance of blueberry wild clone 'QB9C' and cultivar 'Fundy' propagated by softwood cutting (yellow bars) and tissue culture (magenta bars) for the content of phenolics (A), flavonoids (B), anthocyanins (C) and proanthocyanidins (D) in fruits measured in 2011, 2012 and 2013. GAE = gallic acid equivalents; CE = catechin equivalents; C3GE = cyanidine-3-glucoside equivalents; F.F. = fresh fruit. Different letters (a, b) indicate significant differences between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 4).

3.3.2.3 Phytochemical content in fruits at different maturity stages

Phenolic and flavonoid content in berry extract exhibited significant variation with maturity stage, propagation method, maturity stage × propagation method interaction and genotype × maturity stage × propagation method interaction (**Table 3.7** & **3.8**). Both *F* ratios for maturity stage and propagation method were much higher for phenolic content than those for the above interactions, revealed that the propagation method responded similar way to maturity stage for phenolic synthesis (**Table 3.7)**. The anthocyanin content revealed significant variation with maturity stage, whereas, proanthocyanidin content in berry extract varied significantly with maturity stage and interaction between maturity stage and propagation method, with maturity stage being the major influence.

Across the genotypes and propagation methods, the extract from green berries had the highest content of phenolics and flavonoids among the berries at different maturity stages (**Table 3.8**). After green stage, those phytochemicals were decreased gradually with the progress of ripening. Conversely, anthocyanin content was gradually increased started at green stage and the content was highest in fully ripe blueberries.

The detailed performance of each genotype for phytochemical content in fruits at different maturity stages under two different propagation methods is shown in **Figure 3.9A - D**. At the green and semi-ripe maturity stages, berries from TC ‘QB9C’ plants had higher phenolic content than berries from SC counterparts (**Figure 3.9A)**. Green berries from TC ‘Fundy’ plants had higher phenolic as well as flavonoid content than green fruits from SC plants of same genotype. Whereas, ripe berries from TC ‘Fundy’ plants had less

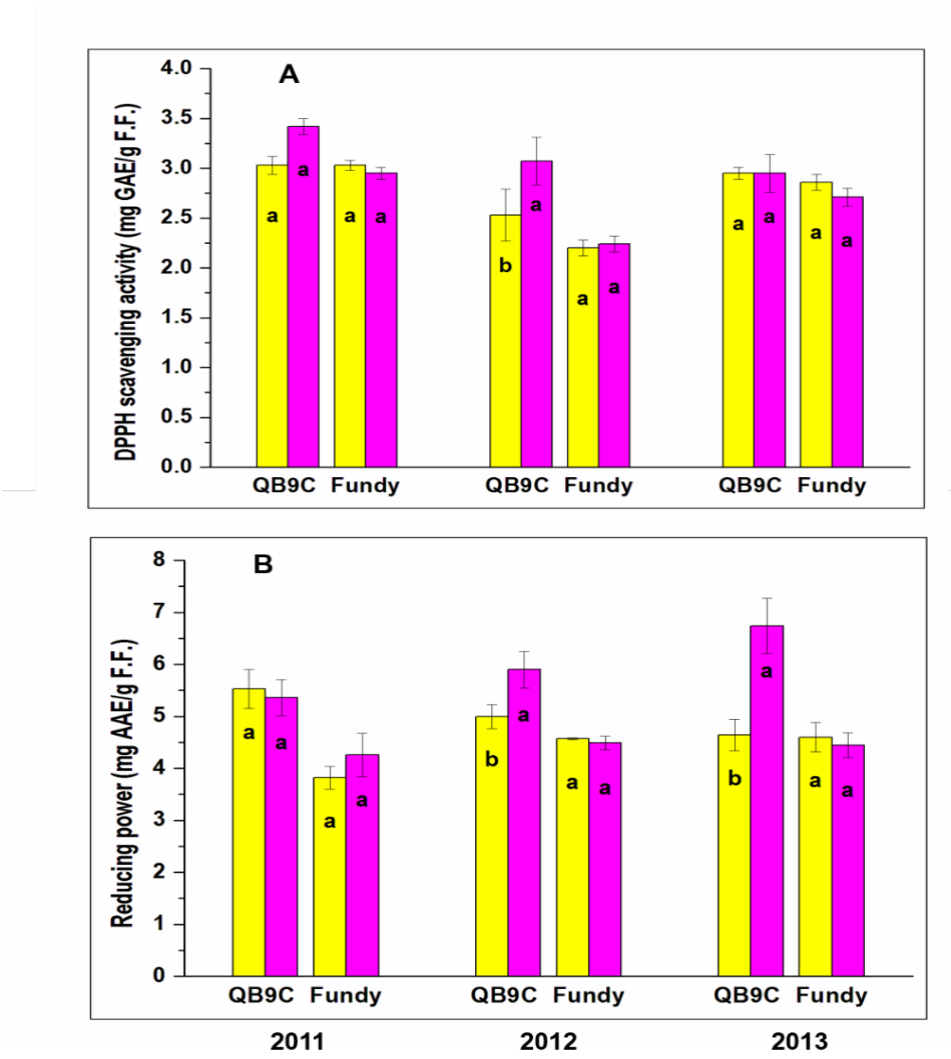

Figure 3.8 Detailed performance of blueberry wild clone 'QB9C' and cultivar 'Fundy' propagated by softwood cutting (yellow bars) and tissue culture (magenta bars) for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (A) and reducing power (B) in fruits measured in 2011, 2012 and 2013. GAE = gallic acid equivalents; AAE = ascorbic acid equivalents; F.F. = fresh fruit. Different letters (a, b) indicate significant difference between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 4).

Table 3.7 Analysis of variance for combined effect of genotype, maturity stage and propagation method on total phenolic, flavonoid, anthocyanin and proanthocyanidin content, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DRSA) and reducing power in green, semi-ripe and ripe fruits of two lowbush blueberry genotypes determined in 2014

Source of variation	df	Phenolic content (mg GAE/g F.F.)		Flavonoid content (mg CE/g F.F.)		Anthocyanin content (mg C3GE/g F.F.)	
		F values	*P* values	*F* values	*P* values	*F* values	*P* values
Genotypes (G)	1	1.30	0.260	1.25	0.270	0.23	0.635
Maturity stages (MS)	2	354	<0.001	337	<0.001	616	<0.001
Propagation methods (PM)	1	21.8	<0.001	16.7	<0.001	2.10	0.155
G×MS	2	0.58	0.566	1.02	0.367	0.91	0.409
G×PM	1	0.49	0.486	6.60	0.014	1.77	0.190
MS×PM	2	5.24	0.009	3.58	0.036	1.09	0.346
G×MS×PM	2	4.07	0.024	16.8	<0.001	1.37	0.266

GAE = gallic acid equivalents; CE = catechin equivalents; C3GE = cyanidine-3-glucoside equivalents; F.F. = fresh fruit.

Table 3.7 cont'd

Source of variation	df	Proanthocyanidin content (mg CE/g F.F.)		DRSA (mg GAE/g F.F.)		Reducing power (mg AAE/g F.F.)	
		F values	*P* values	*F* values	*P* values	*F* values	*P* values
Genotypes (G)	1	5.9	0.019	0.06	0.387	10.8	0.002
Maturity stages (MS)	2	482	<0.001	20.0	<0.001	177	<0.001
Propagation methods (PM)	1	0.04	0.852	2.36	<0.001	36.1	<0.001
G×MS	2	1.62	0.209	0.17	0.136	17.1	<0.001
G×PM	1	3.79	0.058	0.21	0.121	30.7	<0.001
MS×PM	2	7.22	0.002	1.53	<0.001	26.7	<0.001
G×MS×PM	2	1.35	0.269	1.77	<0.001	17.0	<0.001

CE = catechin equivalents; GAE = gallic acid equivalents; AAE = ascorbic acid equivalents; F.F. = fresh fruit.

Table 3.8 Mean values of the main factors across all the treatments for combined effect of genotype, maturity stage and propagation method on total phenolic, flavonoid, anthocyanin and proanthocyanidin content, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DRSA) and reducing power in green, semi-ripe and ripe fruits of two lowbush blueberry genotypes determined in 2014 (n =5).

Parameters	Phenolic content (mg GAE/g F.F.)	Flavonoid content (mg CE/g F.F.)	Anthocyanin content (mg C3GE/g F.F.)
Genotypes (G)			
QB9C	9.94a	5.73a	0.69a
Fundy	9.63a	6.00a	0.71a
Maturity stages (MS)			
Green fruit	14.8a	9.77a	0.06c
Semi-ripe fruit	7.93b	4.41b	0.21b
Ripe fruit	6.66c	3.37c	1.82a
Propagation methods (PM)			
Softwood cutting	9.19b	5.41b	0.73a
Tissue culture	10.4a	6.29a	0.66a
Significant effects	MS, PM, MS×PM, G×MS×PM	MS, PM, G×PM, MS×PM, G×MS×PM	MS

a–c Means within columns and factors followed by different letters indicate significant differences at $P \leq 0.05$; GAE = gallic acid equivalents; CE = catechin equivalents; C3GE = cyanidine-3-glucoside equivalents; F.F. = fresh fruit.

Table 3.8 cont'd

Parameters	Proanthocyanidin content (mg CE/g F.F.)	DRSA (mg GAE/g F.F.)	Reducing power (mg AAE/g F.F.)
Genotypes (G)			
QB9C	1.40b	4.46a	6.23b
Fundy	1.52a	4.52a	6.94a
Maturity stages			
Green fruit	1.02b	5.65a	9.47a
Semi-ripe fruit	0.82c	3.95b	5.25b
Ripe fruit	2.55a	3.88b	5.06b
Propagation methods (PM)			
Softwood cutting	1.47a	4.29b	5.94b
Tissue culture	1.46a	4.69a	7.24a
Significant effects	G, MS, MS×PM	MS, PM, MS×PM, G×MS×PM	G, MS, PM, G×MS, G×PM, MS×PM, G×MS×PM

a–c Means within columns and factors followed by different letters indicate significant differences at $P \leq 0.05$; CE = catechin equivalents; GAE = gallic acid equivalents; AAE = ascorbic acid equivalents; F.F. = fresh fruit.

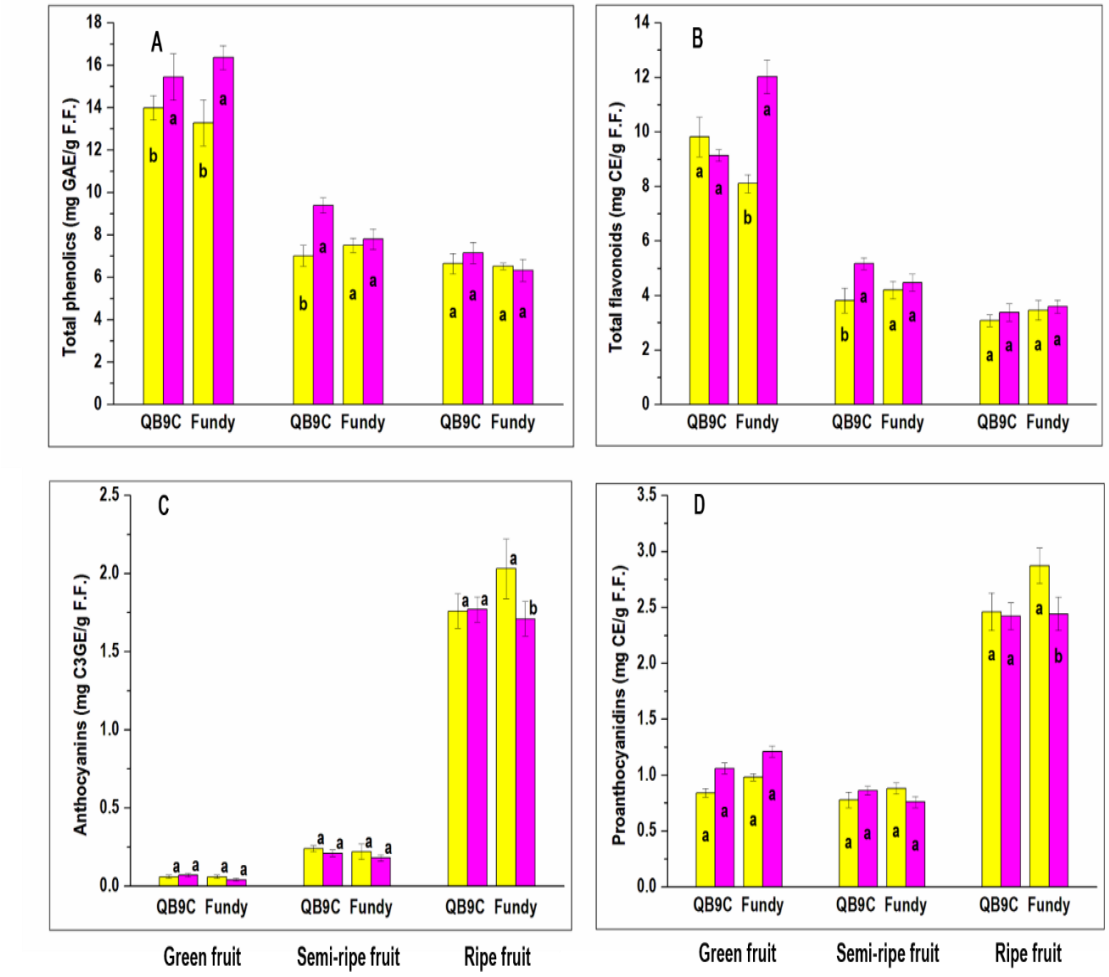

Figure 3.9 The levels of phenolic (A), flavonoid (B), anthocyanin (C) and proanthocyanidin (D) content in fruits at different maturity stages of blueberry genotypes propagated from softwood cutting (yellow bars) and by tissue culture (magenta bars). GAE = gallic acid equivalents; CE = catechin equivalents; C3GE = cyanidine-3-glucoside equivalents; F.F. = fresh fruit. Different letters (a, b) indicate significant differences between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 5).

anthocyanin and proanthocyanidin content than matured fruits of SC ‘Fundy’ plants.

3.3.2.4 Antioxidant activities in fruits at different maturity stages

DPPH radical scavenging activity in blueberry fruits varied significantly with maturity stage, propagation method and with the two-way (maturity stage × propagation method) and three-way (genotype × maturity stage × propagation method) interactions (**Table 3.7** & **3.8)**. The *F* values for maturity stage and propagation method were much higher for DPPH radical scavenging activity and reducing power than those for the interaction between maturity stage and propagation method, suggesting that the propagation methods reacted similarly to variation in maturity stages for antioxidant activities (**Table 3.7**).

Reducing power in fruit extracts showed significant variation with the genotype, maturity stage, propagation method and with all the interactions among those factors. Maturity stage had the highest influence on the variation of reducing power of berry extract. Over the genotypes and propagation methods, green fruits showed the highest DPPH radical scavenging capacity and reducing power among different maturity stages (**Table 3.8**). Whereas across the genotypes and maturity stages, berry from micropropagated plants had higher antioxidant potential than the berry from plants propagated conventionally.

DPPH radical scavenging capacity was higher in semi-ripe and ripe fruits of TC ‘QB9C’ plants than that of SC plants at the same maturity levels (**Figure 3.10A)**. However, green fruits of TC plants of ‘QB9C’ exerted less DPPH radical scavenging capacity and higher reducing power compared to SC counter parts (**Figure 3.10A** & **B)**. In case of ‘Fundy’,

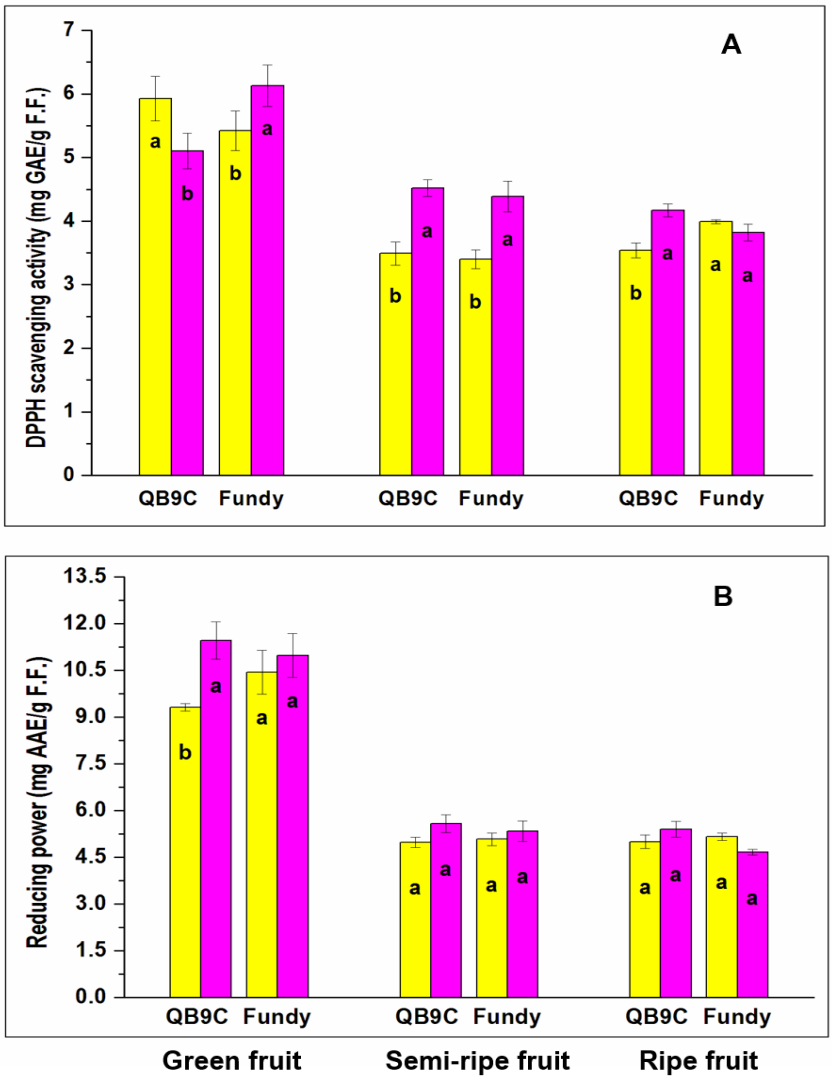

Figure 3.10 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity (A) and reducing power (B) in fruits at different maturity stages of blueberry genotypes propagated by softwood cutting (yellow bars) and tissue culture (magenta bars). GAE = gallic acid equivalents; AAE = ascorbic acid equivalents; F.F. = fresh fruit. Different letters (a, b) indicate significant differences between propagation methods at $P \leq 0.05$ by least significant difference test. Bars indicate mean ± SE (n = 5).

green and semi-ripe fruits of TC plants performed higher DPPH radical scavenging activity compared to berries of SC plants at same maturity stages.

3.3.3 Leaves versus fruits from SC and TC blueberry plants

The overall levels of phenolic based phytochemicals and antioxidant activities were higher in leaves of both genotypes propagated either conventionally or by tissue culture. Polyphenolic content in green leaves were about 4–6 times of fruits from same plants (**Table 3.9)**. The highest content of flavonoid was found in leaves of SC 'QB9C' plants and that was about 8.4 times of fruits from same plants. In each case, flavonoid content in leaves was higher than in fruits. Similar trend was found in case of proanthocyanidin content. The SC 'QB9C' leaves contained about 3.4 times condensed tannins of fruit counterparts. In contrast, DPPH radical scavenging activities in leaves of SC 'Fundy' plants were the highest and that was about 13 times of fruit counterparts. The lowest difference in DPPH radical scavenging activity between leaf and fruit was observed in TC 'QB9C' plants.

3.4. Discussion

As an indication of the importance on the antioxidant phytochemicals in edible fruits especially in blueberries, there is increasing interest on improving their content through advance technology. Plant tissue culture can result in significant changes in secondary metabolism, and hence content of metabolites (Georgiev et al., 2010). The phytochemical content and antioxidant property of blueberry leaves can hasten the selection of the plants

Table 3.9 Mean values of total phenolic, flavonoid, proanthocyanidin content and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DRSA) in green leaves and ripe fruits of two lowbush blueberry genotypes propagated by softwood cutting and tissue culture (n = 4)

Genotypes	Propagation methods	Plant tissue	Phenolic content (mg GAE/g F.W.)	Flavonoid content (mg CE/g F.W.)	Proanthocyanidin content (mg CE/g F.W.)	DRSA (mg GAE/g F.W.)
QB9C	Softwood cutting	Leaf[z]	44.2	24.5	6.99	31.3
		Fruit[y]	7.31	2.92	2.08	2.83
	Tissue culture	Leaf	38.4	20.1	5.99	31.4
		Fruit	9.26	3.22	2.21	3.14
Fundy	Softwood cutting	Leaf	40.5	14.9	5.88	35.3
		Fruit	6.50	2.51	2.03	2.69
	Tissue culture	Leaf	31.0	10.6	3.52	28.1
		Fruit	6.59	2.77	1.90	2.63

[z]Average of 2011 and 2012; [y]Average of 2011, 2012 and 2013; GAE = gallic acid equivalents; CE = catechin equivalents; F.W. = fresh weight.

with high level of fruit antioxidants and establish a potential relationship and understanding its different beneficial activities in human health. There are several reports on the improvement of antioxidant secondary metabolites through plant tissue culture, most of which are on laboratory conditions for medicinal plants (Matkowski, 2008; Amoo et al., 2014; Luczkiewicz et al., 2014). A number of elicitors such as biological (bacteria, fungus), physical (light, temperature) and chemical (amino acids, growth regulators) are used to increase the production of those phytochemicals in controlled laboratory conditions (Dias et al., 2016). Among those factors, the external application of plant growth regulator plays a key role to enhance the phenolic productions. Although the vital role of cytokinins singly or in combination with auxins on phytochemical content in micropropagated shoot during tissue culture has been recognized in a number of plant species (Taveira et al., 2009; Ozden & Karaaslan, 2011; Amoo & Staden, 2013; Aremu et al., 2013), the fate of *in vitro* synthesized phenolic compounds in the plants after an *ex vitro* growth period remains unexplained or speculative. The reports on the phenolic compounds and antioxidant properties of matured plants derived through tissue culture are very few in *Vaccinium* spp. (Vyas et al., 2013a), and the factors either influence or hinder the level of antioxidant phytochemical production are not still clear. Few reports proposed that the left over cytokinins in tissue culture derived plants influence the level of phenolic compounds especially flavonoid, anthocyanin and condensed tannins when those are grown *ex vivo* conditions (Bairu et al., 2011a; Amoo et al., 2012; Lugato et al., 2014; Amoo et al., 2015). Other factors like propagation methods, tissue culture techniques, growth conditions, genotypes, explants and growing seasons significantly affect the total phytochemical

content. In the present study, significant interactions among genotype, propagation method and growing season for flavonoid and proanthocyanidin content showed that the level of flavonoids and proanthocyanidins of blueberry leaves was affected by propagation methods which were genotype and growing season specific. Significantly higher content of phenolics, flavonoids and proanthocyanidins were observed in the leaf extract of SC propagated plants than TC counterparts. The effect of propagation on antioxidant metabolite content was previously reported in leaves, and higher content of phenolics, anthocyanins and proanthocyanidins in leaves was found in *in vivo* derived plants than *in vitro* plants of *V. vitis-idaea* L. ssp. *vitis-idaea* Britton (Vyas et al., 2013a) and *Passiflora alta* (Lugato et al., 2014). Vyas et al. (2013a) also reported that plants regenerated through leaf culture contained higher levels of phenolics, anthocyanins and flavonoids in leaves than the plants derived through node culture. Chavan et al. (2014) reported that the plants of *Ceropegia santapau* species regenerated through indirect shoot regeneration technique had higher level of phenolics and flavonoids in leaves than the plants derived through direct shoot regeneration using nodes as explants.

Higher levels of phenolics, flavonoids and proanthocyanidins in leaves of SC plants than in those of TC plants may be due to differential nutritional levels of two propagation methods. The most abundant class of secondary phenolic compounds in plants are synthesized through the shikimic acid and secondary metabolic pathways from aromatic carboxylic acid and phenylalanine. The reaction catalyzed by phenylalanine ammonia lyase (PAL) is an important regulatory step in the formation of phenolic compounds (Macheix et al., 1990; Dixon & Paiva, 1995). The activity of PAL is influenced by the environmental

factors, such as low nutrient levels and light (Taiz & Zeiger, 2006). The shoot explants experienced readily available nutrient in media *in vitro* conditions than *in vivo*. Low nutrient level faced by source plants during stem cutting may induce a stress in the SC blueberry plants that results in higher levels of bioactive compounds within the leaves of SC plants than those of TC plants. Nutritional stresses, for example, low nitrogen and phosphorus levels in soil enhanced the formation of phenolic and anthocyanin pigments in plants reviewed by Dixon and Paiva (1995) and Zhao et al. (2006). However, increasing nitrogen application decreases the level anthocyanin in grapes (Kliewer, 1977). Excess and readily available nitrogen in TC media may be attributed mainly to reduction in carbohydrate accumulation and an increase in nitrogenous substances like arginine and total free amino acids (Kliewer, 1977) which causes lower phenolic and flavonoid content in leaves of blueberry.

The differences in the levels of polyphenolics and chlorophylls detected in the leaves in the growing season of 2011 from 2012 (**Table 3.2**) may be partially attributed to differing weather conditions especially natural light intensity. Lower light plays a role in triggering the synthesis of phenolic compounds (Taiz & Zeiger, 2006). Less sunshine was observed in St. John's area in the summer of 2011 compared to the summer of 2012. Lower duration and intensity of light in 2011 prompted higher phenolic synthesis in leaves in that growing season than in 2012.

Antioxidant activity is a result of a combination of different compounds and environmental factors having synergistic and antagonistic effects (Hassimotto et al., 2005). The leaves of

the lowbush blueberry may serve as an excellent source of antioxidant metabolites for the nutraceutical industry as they have high level of phytochemicals and antioxidant properties (Heinonen, 2007). Evaluation of antioxidant activity is complex, and no standard antioxidant assay has yet been agreed (Frankel & Meyer, 2000). In the present study, we followed the DPPH radical scavenging method which is more sensitive as well as cheaper than other methods (Giovanelli & Buratti, 2009). The strong antioxidant activity displayed by the leaf extracts of both blueberry genotypes was influenced by propagation methods and growing seasons. Higher antioxidant activities were reported in leaves of SC lingonberry plants than TC derived counterparts (Vyas et al., 2013a). The significantly high level of antioxidant activity in SC blueberry leaves in the present study were consistent with the observed content of total phenolics, flavonoids and proanthocyanidins which were also higher in SC leaf extract. It was confirmed by high correlation coefficients between total phenolic, flavonoid and proanthocyanidin content and DPPH radical scavenging activity (**Table 3.10**). Previous studies showed the significant positive correlation between phenolic compounds and antioxidant capacity in blueberry leaves (Ehlenfeldt & Prior, 2001). Antioxidant activities in blueberry increased with the elevated level of phenolic and anthocyanin.

The DPPH radical scavenging activity in leaf extract of SC 'QB9C' plants was not increased significantly from TC plants in 2011, despite the levels of total phenolic, flavonoid and proanthocyanidin production were significantly higher in leaf extract of SC plants than TC counterparts (**Figure 3.4**). In contrast, in 2012, antioxidant activity in leaf extract of same genotypes were less in TC plants than the leaf extracts of SC ones, although

total flavonoid, proanthocyanidin and chlorophyll content were not changed significantly. This could be due to the interaction either synergistic or antagonistic among the various antioxidant compounds in leaves and environmental factors, or due to the synthesis of new biologically active compounds which are induced by environmental conditions (Skrovankova et al., 2015). It is well known that synergistic effects between phenolic compounds and betalains, leading to significantly increase in biological activities of betalain containing extracts of *Beta vulgaris* (Chavez-Santoscoy et al., 2009; Georgiev et al., 2010). The effectiveness of the antioxidant metabolites is influenced mainly by their chemical composition and their structure, especially the number and position of hydroxyl and methoxyl groups on the phenolic ring of the molecule (Seeram & Nair, 2002). The differences in antioxidant activity in blueberry leaves could be due to differences in concentrations and types of radical in question as well as the molecular structure and kinetic behaviour of the phenolics involved (Naczk et al., 2003). However, DPPH radical scavenging activity in leaves was negatively correlated with vegetative growth (number of stems per plant) and number of branches per plant.

A significant interaction between genotypes and propagation methods in the present study for total phenolic and anthocyanin content in fruit extracts demonstrated that propagation methods could impact the capacity of blueberry plants to synthesize those phytochemicals in fruits and certain genotypes varied in their capacity under different conditions of propagation methods. The higher levels of polyphenols and flavonoids in blueberries of TC plants are agreement with previous studies. Higher phenolic, flavonoid and anthocyanin content were reported in the fruits of *in vitro* derived plants compared to berries of

Table 3.10 Pearson's correlation coefficients for number of stems per plant (NSP), number of branches per plant (NBrP), stem diameter (SD; mm), leaf length (LL; mm), leaf width (LW; mm), leaf area (LA; mm^2), plant vigour (PV), number of flowers per plant (NFP), total phenolic (TPC; mg GAE/g F.L.), flavonoid (TFC; mg CE/g F.L.), proanthocyanidin (PAC; mg CE/g F.L.) and chlorophyll content (CC; SPAD unit) and 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DRSA; mg GAE/g F.L.) in green leaves combined from softwood cutting and micropropagated blueberry plants

Characters	NBrP	SD	LL	LW	LA	PV	NFP	TPC	TFC	PAC	CC	DRSA
NSP	0.98**	-0.95**	0.90**	0.16	0.65	0.60	-0.99**	-0.96**	-0.91**	-0.91**	-0.10	-0.91*
NBrP		-0.93**	0.93**	0.20	0.70	0.48	-0.99**	-0.98**	-0.91**	-0.93**	-0.10	-0.94*
SD			-0.92**	-0.19	-0.68	-0.56	0.96**	0.86**	0.90**	0.81*	-0.10	0.73*
LL				0.46	0.90**	0.33	-0.91**	-0.87**	-0.95**	-0.83*	-0.01	-0.86**
LW					0.79*	-0.30	-0.14	-0.11	-0.36	-0.04	-0.23	-0.33
LA						-0.00	-0.66	-0.61	-0.81*	-0.58	-0.12	-0.72*

* and ** = significant at $P \leq 0.05$ and $P \leq 0.01$, respectively; GAE = gallic acid equivalents; CE = catechin equivalents; F.L. = fresh leaf; correlation coefficient value $|r|$ = 0.3 - 0.5 is moderate correlation and $|r|$ = > 0.5 is strong correlation.

Table 3.10 cont'd

Characters	NBrP	SD	LL	LW	LA	PV	NFP	TPC	TFC	PAC	CC	DRSA
PV							-0.56	-0.53	-0.53	-0.57	0.36	-0.57
NFP								0.97**	0.91**	0.91**	0.03	0.81*
TPC									0.88**	0.96**	0.08	0.86**
TFC										0.90**	-0.06	0.92**
PAC											0.01	0.89**
CC												-0.12

* and ** = significant at $P \leq 0.05$ and $P \leq 0.01$, respectively; GAE = gallic acid equivalents; CE = catechin equivalents; F.L. = fresh leaf; Correlation coefficient value $|r| = 0.3 - 0.5$ is moderate correlation and $|r| = > 0.5$ is strong correlation.

conventionally propagated plants of lingonberry (Foley & Debnath, 2007; Vyas et al., 2013a), strawberry (Debnath, 2009c), bilberry and raspberry (Georgieva et al., 2016). The stimulatory role of micropropagation in increasing phenolic content might be because of plant growth regulators used in media on biosynthesis of phenolic compounds through influencing the expression or up-regulation of genes involved in the biosynthetic pathway of secondary metabolites (Sakakibara et al., 2006). For instance, cytokinin alone or in combination auxin gave a significantly increased amount of total phenolics, flavonoids and condensed tannins in *Aloe arborescens* species, in comparison to plant growth regulator-free medium during *in vitro* propagation through direct shoot proliferation (Amoo et al., 2012). The level transcription of the genes in flavonoid biosynthesis pathway encoding PAL, chalcone synthase (CHS), chalcone isomerase (CHI), and dihydroflavonol reductase (DFR) were shown to increase coordinately with cytokine concentration and thereby enhancing the anthocyanin level in *A. thaliana* (Deikman, 1995). On the other hand, auxins regulate the pool size of active cytokinins by promoting cytokinin glucosylation and oxidative breakdown to others (Nordstrom et al., 2004). The choice of cytokinin and its concentration in tissue culture makes a difference in the production level of secondary metabolites.

Another aspect of increased content of phytochemicals in blueberry propagated *in vitro* is fruit size. The fruits of TC 'QB9C' plants were smaller in size compared to SC plants (**Figure 2.7 B** & **C** in Chapter 2) confirmed higher proportion of berry skin which is enriched with anthocyanin pigments (Gao & Mazza, 1994; Kalt & Dufour, 1997). In 'Fundy', fruit size and phenolic content were not significantly different between SC and

TC plants. This confirmed that different types of epidermal and sub-epidermal layers of peel containing variable amounts of pigments (Allan-Wojtas et al., 2001) and the type, amount and localization of phytochemicals, especially flavonoids, anthocyanins and proanthocyanidins were influenced by genetic differences. Moreover, other factors like fruit size, developmental stages of the fruit, and the specific weather conditions of growing seasons also affect the antioxidant metabolite content (Wang et al., 1996; Kalt & Dufour, 1997; Connor et al., 2002a; Howard et al., 2003). However, Kalt et al. (2001b) 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.

Significant main effects for growing seasons and genotype × growing season interaction for flavonoid and anthocyanin contents showed that seasonal variation could affect flavonoid synthesis and impact of seasonal variation on the accumulations of phenolics, flavonoids, anthocyanins and proanthocyanidins in blueberries was genotype specific. In the present study, the variation in the phytochemical content in fruits of the wild clone 'QB9C' in respect with seasonal variation was prominent compared to the cultivar 'Fundy'. Although the increased levels of polyphenolics in fruits of micropropagated 'QB9C' plants were constant over three years of production, the content of flavonoids, anthocyanins and condensed tannins of TC 'QB9C' plants were varied significantly in different growing seasons. This study is accordance with previous reports in which the total phenolic and anthocyanin content in different blueberry cultivars varied significantly with cultivar and cultivar × year interaction (Scalzo et al., 2013). A multitude of environmental factors are known to influence both the phenolic content of leaves and fruits (Jones & Hartley, 1999)

thus many years of measurements of phenolics including flavonoids, anthocyanins are needed to assess the potential magnitude of seasonal variation in these phytochemicals.

Reductants or antioxidants are capable to reduce the oxidized intermediates by donating electrons. Reductants in the berry extracts reduced the ferric ion to the ferrous form and thus extract served as a good antioxidant. In the present study, antioxidant activity measured as DPPH radical scavenging capacity and reducing power of fruit extract varied in wild clone 'QB9C' from cultivar 'Fundy' (**Table 3.6**) in agreement with previous studies on antioxidant properties of blueberries (Cardeñosa et al., 2016). Lowbush blueberries had significantly higher antioxidant activities compared to cultivated highbush blueberries (Kalt et al., 2001a; Sellappan et al., 2002). Significant interaction between genotype and propagation method for antioxidant activity showed that antioxidant activity was affected by propagation method, and the genotypes responded differently to propagation technique for their antioxidant capacity.

Higher DPPH radical scavenging activity of fruits in 2011 compared to other growing seasons (**Table 3.6)** was attributed to the content of anthocyanins and proanthocyanidins which were also higher in 2011. On the other hand, higher reducing power of fruits in 2013 compared to other growing seasons was attributed to the content of total phenolics, flavonoids and proanthocyanidins which were also higher in 2013. The relationships between phytochemical content and their antioxidant capacity were confirmed by the correlation studies. The phenolic, flavonoid, anthocyanin and proanthocyanidin content in micropropagated fruits were positively correlated with DPPH radical scavenging activity

and reducing power (**Table 3.11**). Significant positive correlations were reported between antioxidant activity and total phenolic, anthocyanin content in blueberries (Koca & Karadeniz, 2009; Wang et al., 2012; Gündüz et al., 2015). Prior et al. (1998) and Connor et al. (2002a) reported that the correlation coefficient of antioxidant capacity and total phenolic content was higher than that of antioxidant capacity and anthocyanin content in fruit of *Vaccinium* species. However, in micropropagated lowbush blueberry in this study, anthocyanin had stronger correlation with antioxidant activity than that of total phenolic content with DPPH radical scavenging activity and reducing power.

It is interesting that both diameter of berry and individual berry weight were negatively correlated with total phenolic, flavonoid, anthocyanin and proanthocyanidin content and reducing power in fruits. Individual berry weight was negatively correlated with DPPH radical scavenging capacity. It means that fruit size and weight play an important role in selecting the genotypes with higher antioxidant properties. Among the phenolic compounds, anthocyanins which are confined principally to the fruit skin, contribute significantly to the high antioxidant activity in blueberry as found in this study with the highest Pearson's correlation coefficients (Prior et al., 1998) and, thus the cultivars with smaller berry size exerted higher antioxidant activity. Connor et al. (2002c) and Gündüz et al. (2015) used berry weight rather than an estimate of surface area and found berry weight negatively correlated with total phenolic and anthocyanin content and antioxidant activity. However, Kalt et al. (1999a) reported that there was no significant correlation between antioxidant capacity and fruit weight, suggesting that larger fruited types can be developed with high antioxidant capacity. Data in this study revealed that the antioxidant activity of

Table 3.11 Pearson's correlation coefficients for berry diameter (BD; mm), individual berry weight (IBW; g), berry weight per plant (BWP; g), total phenolic (TPC; mg GAE/g F.F.), flavonoid (TFC; mg CE/g F.F.), monomeric anthocyanin (MAC; mg C3GE/g F.F.) and proanthocyanidin (PAC; mg CE/g F.F.) content, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DRSA; mg GAE/g F.F.) and reducing power (RP; mg AAE/g F.F.) in fruits from softwood cutting and micropropagated (bold) blueberry plants

Characters	IBW	BWP	TPC	TFC	MAC	PAC	DRSA	RP
BD	0.99**	-0.86**	-0.60	-0.87**	-0.60	-0.51	-0.38	-0.73*
	0.97**	**0.89****	**-0.89****	**-0.87****	**-0.90****	**-0.90****	**-0.90****	**-0.95****
IBW		-0.85**	-0.64	-0.86**	-0.57	-0.45	-0.31	-0.76*
		0.95**	**-0.89****	**-0.88****	**-0.87****	**-0.92****	**-0.94****	**-0.92****
BWP			0.65	0.90**	0.64	0.48	0.14	0.49
			-0.89**	**-0.95****	**-0.87****	**-0.86****	**-0.92****	**-0.87****

* and ** = significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. Correlation coefficient value $|r|$ = 0.3 - 0.5 is moderate correlation and $|r|$ = > 0.5 is strong correlation.

Table 3.11 cont'd

Characters	IBW	BWP	TPC	TFC	MAC	PAC	DRSA	RP
TPC				0.58	0.04	0.53	-0.09	0.10
				0.90**	**0.76***	**0.78***	**0.76***	**0.79***
TFC					0.71*	0.43	0.35	0.64
					0.91**	**0.81***	**0.84****	**0.89****
MAC						0.26	0.20	0.63
						0.84**	**0.83***	**0.93****
PAC							-0.16	-0.05
							0.83*	**0.83***
DRSA								0.43
								0.92**

* and ** = significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. Correlation coefficient value $|r|$ = 0.3 - 0.5 is moderate correlation and $|r|$ = > 0.5 is strong correlation.

fruits increased with the increase in quantity of the secondary metabolites and with the decrease in berry diameter and individual berry weight. Exogenous application of plant growth regulators induced a stress response in the naturally antioxidant-rich wild blueberry and raspberry that results in elevated levels of bioactive compounds within the fruit and significant reductions in berry size (Wang & Zheng, 2005; Percival & MacKenzie, 2007). Connor et al. (2002c) and Yuan et al. (2011) reported similar results in other blueberry species that the antioxidant activity of fruits increased with the increased level of secondary metabolites. Fruit size which was affected by propagation method may be another reason of having higher antioxidant potential in micropropagated blueberries. The general phenomenon in micropropagation of plant is the reversion from mature stage of cell to juvenile characteristics. In the previous chapter, it was found that TC plant showed higher vegetative growth (i.e., higher number of rhizomes, branches and larger leaves) than SC plants.

Higher content of phytochemicals especially polyphenolics, flavonoids and proanthocyanidins, and their antioxidant capacity in leaves compared to fruits in this study (**Table 3.9)** are in accordance with other recent studies on blueberries (Percival & MacKenzie, 2007; Riihinen et al., 2008; Vyas et al., 2013b) and other plant species (Vyas et al., 2013a; Lugato et al., 2014). On the contrary, Alam et al. (2016) reported higher phenolic content and antioxidant capacity in fruits compared to leaves of lingonberry wild populations across Newfoundland and Labrador. The variation in the phenolic and flavonoid content in the different plant parts might be due to the fluctuation in hormonal content, the variation in the distribution of individual phenolic compounds in the plant parts

and/or specific metabolic as well as endogenous physiological changes taking place. For example, blueberry leaves contain higher levels of hydroxycinnamic acids and procyanidins than in fruits, whereas flavonols are in higher level in berry compared to leaves (Riihinen et al., 2008). Hakkim et al. (2007) reported that the phenolic compound eugenol was detected in the leaves but not in inflorescence of *Ocimum sanctum*, whereas ursolic acid detected in inflorescence but not in leaves of same species. The superior phenolic content in leaves of blueberry genotypes compared to fruits confirmed that the biosynthesis of polyphenols is accelerated by light exposure and serves as a filtration mechanism against UV-B radiation (Harborne & Williams, 2000). Higher surface area of leaves exposed to sunshine encouraged their phenolic synthesis.

The significant combined effect of propagation method and growing season was observed for total antioxidant activity in leaves (**Table 3.2**) which was absent for DPPH radical scavenging activity in fruits of the same blueberry species under the same propagation conditions (**Table 3.6**). It may be due to some other external factors or metabolites, which have not been taken in consideration, might have affected the antioxidant capacity. On the other hand, most of the genes and enzymes involved in the flavonoid biosynthesis 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 activities between fruits and leaves.

Differential response of blueberry genotypes for their phytochemical content and antioxidant activities is common. The leaves and fruits of ‘QB9C’ had higher content of

phenolics and flavonoids than those of ‘Fundy’. The genera, species, cultivar and genotypes are varied for their phenolic content in fruits, and wild lowbush blueberries contained more than double antioxidant phenolics than those of the cultivated highbush blueberries (Kalt et al., 2001b; Giovanelli & Buratti, 2009). While, ‘QB9C’ is a wild selection from Quebec, ‘Fundy’ was selected from open-pollinated seedlings of cultivar ‘Augusta’, the first wild clone released as a cultivar (Aalders et al., 1975). Both genotypes ‘QB9C’ and ‘Fundy’ belong to tetraploid *V. angustifolium* species and the proposed origin of this species is allotetraploid of two diploid species either *V. boreale* × *V. palladium* or *V. boreale* × *V. myrtilloides* (Vander Kloet, 1977). However, those are genetically different as proved in DNA based molecular system analysis using expressed sequence tags - simple sequence repeat (EST-SSR) markers (Goyali et al., 2015a). Since both clones originated from open pollinated genotypes and are different at the genetic level, they responded differently to the propagation methods for their metabolite contents and antioxidant activities.

The fruit development is initiated with the cell division just after fertilization and later cell expansion. The final stage of fruit development, ripening is started after seed maturation has been completed (Jaakola et al., 2002). During the ripening phase of fruits, physical and chemical changes especially tissue softening, and pigment accumulation occurred (Gillaspy et al., 1993). Ripe blueberries are characterized by increased fresh weight gain, increased soluble solids, reduced titratable acidity, and well developed blue color (Castrejón et al., 2008). Among the secondary plant metabolites determined in blueberries, the anthocyanin subclass of flavonoids has received the most attention for the pigmentation

of plant tissues and for antioxidant activity. Both genotypes of blueberry in the present study exhibited the same pattern of phenolic compound biosynthesis characterized by increasing anthocyanin content towards maturation and it was the highest in ripe fruits, meanwhile total phenolics and flavonoids were higher in green stage and gradually reduced with the maturity progression. Castrejón et al. (2008) also reported that total phenolics, hydroxycinnamic acids and a flavonoid subclass flavonols decreased from unripe green to ripe blue stage of berry maturation in four highbush blueberry cultivars. However, there was no significant correlation of total phenolics and anthocyanins with maturity of blueberries at bush (Connor et al., 2002c; Wang et al., 2012). Monomeric anthocyanins, which accounted for greater than 85% of the total anthocyanin content in blueberries (Kalt & McDonald, 1996) were substantially higher in the ripe fruit than in green berries (Castrejón et al., 2008). It may be due to synthesis of low number and level of anthocyanin compounds at early developmental stages and increased number as well as high level of compounds in ripening stages. In a previous report, Zifkin et al. (2012) found two anthocyanin compounds in low levels in green fruit of highbush blueberry cultivar 'Rubel' and five in elevated levels in ripe berry. Similarly, Jaakola et al. (2002) detected seven anthocyanins at the half-expanded bilberries, just after coloring began and 13 anthocyanins in expanded berries at red fruit stage. Differences in number and level of individual phenolic compounds are responsible for variation in total phenolic content at different maturity stages.

The content of all the antioxidant metabolites were low in green leaves and those were increased in red leaves. Better performance of red leaves of blueberry than green leaves for

total phenolic and anthocyanin content, and higher content of anthocyanin in ripe fruits compared to red leaves of lowbush blueberry in the present study was in agreement with previous report (Percival & MacKenzie, 2007; Riihinen et al., 2008). They reported that total phenolics and monomeric anthocyanins could be elevated in field grown ripe blueberries and red leaves by applying stress inducing growth regulators. Differential synthesis of phenolic compounds at different maturity stages of leaves and fruits may cause this variation in phenolic content. In previous studies, Riihinen et al. (2008) reported higher content of flavonoid subclasses like quercetin and kaempferol in the red leaves of blueberry compared to the respective green leaves.

The differences in the levels of accumulation of various phenolic compounds in tissue at different maturity stages as found in the present study are the function of enzyme activity, corresponding gene expression and precursor availability in the flavonoid biosynthesis pathway. The enzymes activities involved in the flavonoid biosynthesis pathway are strongly correlated with developmental stage of fruits. Coordinated expression of flavonoid pathway genes encoding PAL, CHS, DFR, flavanone 3-hydroxylase (F3H) and anthocyanidin synthase (ANS) in relation to the accumulation of flavonoids, anthocyanins and proanthocyanidins in developing fruits was reported by Zifkin et al. (2012) in blueberry. Those genes are highly expressed in flowers and in fruit at the ripening stage when anthocyanin is accumulated, and the blue color is developed. Similar trend of anthocyanin biosynthesis was reported in other species of berry plants: bilberry (Jaakola et al., 2002), grape (Boss et al., 1996) and strawberry (Halbwirth et al., 2006). The proanthocyanidin content was not coordinately shifted with developmental progress in this

study. However, in the previous reports, the levels of proanthocyanidins decreased with progression of ripening in blueberry (Zifkin et al., 2012), bilberry (Jaakola et al., 2002) and strawberry (Halbwirth et al., 2006). Flavonoid enzymes activity involved in flavonoid biosynthesis had peaks during fruit ripening at early and late development stages which caused higher proanthocyanidins at early stage and anthocyanins synthesis in ripening stages (Halbwirth et al., 2006). Castrejón et al. (2008) suggested that with the progress of maturation there was a shift in the pool of total phenolics and several flavonoids towards anthocyanin synthesis and for that reason an overall decline in the content of other phenolic components appeared.

Antioxidant activities in leaves were appeared to have similar trend as phytochemical content had. Higher DPPH radical scavenging capacity and reducing power were observed in red leaves than in green counterparts. It means that the differences in phenolic, flavonoid, anthocyanin and proanthocyanidin content between maturity stages reflected differences in the antioxidant activities in blueberry leaves. Higher total phenolic, flavonoid, anthocyanin and proanthocyanidin content in red leaves confirmed higher antioxidant activities in red leaves as found in correlation studies.

Although the anthocyanin and proanthocyanidin content were increased in ripe fruit, the both DPPH radical scavenging capacity and reducing power was less in ripe fruit than in green fruits. It may be due to decrease in the levels of total phenolic and flavonoid content in ripe fruits. The contribution of phenolic compounds other than anthocyanins to overall antioxidant activities in blueberry was also reported by Connor et al. (2002a). However,

Zifkin et al. (2012) proposed that due to reduction in proanthocyanidins with the progress of developmental stages the antioxidant capacity is reduced in blueberries.

In conclusion, this study showed that fruits of lowbush blueberries had substantial level of antioxidants especially polyphenols, flavonoids and proanthocyanidins, however their content in leaves was much higher in comparison with fruits. The response of blueberry genotypes to different propagation methods was not consistent for those antioxidant metabolites. The wild clone 'QB9C' had higher content of antioxidant metabolites than the cultivar 'Fundy'. Although external factors: propagation methods and growing seasons appeared to have clear effects on phenolic biosynthesis, it largely affected by internal factors: plant tissues and maturity stages. Propagation methods contributed more than growing seasons for total flavonoids in both leaves and fruits and for proanthocyanidins in leaves, while growing seasons had higher contribution than propagation methods for total phenolic content in both leaves and fruits. Micropropagated blueberry fruits had higher phenolic and flavonoid content compare to SC plants, meanwhile leaves of SC plants had higher content of phenolics and exhibited higher antioxidant activity than TC plants. The red leaves contained the highest level of phenolic and ripe fruits had the highest level of anthocyanins among the maturity stages studied in leaves and fruits. The enhanced antioxidant activity observed in micropropagated blueberry plants might be beneficial for human health. Growers who wish to collect plants for division and further propagation for health promoting phytochemicals can use TC plant material, but reductions of fruit size and production should be taken into consideration. The leaves, the main waste products in farm and blueberry fruit industry, could be used as an excellent source for phenolic and

proanthocyanidin containing products in nutraceutical, cosmetic or pharmaceutical industries.

CHAPTER 4

Study of Genetic Fidelity in *In vitro* Propagated Lowbush Blueberries Using Molecular Markers

Clonal fidelity of micropropagated lowbush blueberry genotypes 'QB9C' and 'Fundy' will be discussed in this chapter. Parts of the results have been published in the HortScience 50: 888-896 (Goyali et al., 2015a).

4.1 Introduction

In vitro propagation is one of the important components of modern plant improvement programs because of its potential to rapid multiplication of trueness-to-type genotypes. The propagation of wild clones and cultivars of blueberries is generally carried out by stem cuttings, but tissue culture may be a better choice to increase the quantity of clones or selections in short time needed for release as highly productive cultivars, and to fill the gap between demand and supply of planting materials. Micropropagation of lowbush blueberries (*Vaccinium angustifolium* Ait.) has been established through shoot proliferation with juvenile tissues (Debnath, 2009b) and mature explants (Frett & Smagula, 1983; Brissette et al., 1990; Debnath, 2004) as well as adventitious shoot regeneration with leaves (Debnath, 2009a; Debnath, 2011). Shoot proliferation remains the most preferred micropropagation technique in plant being avoid *de novo* morphogenesis, unlike adventitious bud differentiation and somatic embryogenesis (Singh et al., 2013). Blueberry plants propagated by shoot proliferation have been evaluated for their morphological and biochemical performance compared to the mother plants as well as to the plants propagated by other techniques like softwood cutting and seedlings. When propagated by tissue culture

(TC), the blueberry genotypes produce higher number of rhizomes but fewer fruits than plants propagated by stem cutting (Jamieson & Nickerson, 2003; Debnath, 2007b). However, higher phenolic and anthocyanin antioxidants were found in fruits of micropropagated blueberry as well as other berry plants (Debnath, 2009c; Vyas et al., 2013a; Goyali et al., 2015a). The influencial effects of TC on the morphology and phenolic content in blueberry may promote the growers to use micropropagated plants in their farms.

Trueness-to-type propagules and genetic fidelity are prerequisites for *in vitro* propagation. Although tissue culture induces stable phenotypic characteristics in regenerants of many plant species (Skirvin et al., 1993; Hashmi et al., 1997; Salvi et al., 2001; Torres-Morán et al., 2010), past studies have shown that *in vitro* cultures pose somaclonal variation in fruit crops (Biswas et al., 2009; Ge et al., 2015). These deviations from phenotypic stability are usually due to genetic variation, chromosomal rearrangements, point mutations and/or epigenetic aberration such as DNA methylation (Phillips et al. 1994). The factors influencing the loss of genetic fidelity *in vitro* include genotypes, age of donor plant, ploidy level of starting materials, explant source and its pattern of regeneration (somatic embryogenesis, organogenesis or axillary bud multiplication), media composition, types and concentrations of growth regulators in media, auxin-cytokinin balance, cultural conditions (temperature, light, osmolarity and agitation rate of media), duration spent in tissue culture and number of subcultures (Phillips et al., 1994; Rani & Raina, 2000; Debnath et al., 2012b; Krishna et al., 2016). Plant growth regulator by itself or with other factors can affect the rate of somaclonal variation both directly and indirectly by increasing the multiplication rate and inducing adventitious shoots.

It is important to maintain and confirm clonal fidelity or genetic integrity of micropropagated plants at different growth and development stages in *in vitro* as well as in field conditions to ensure reliability of the micropropagation for commercial purpose. 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 tissue culture (TC) derived plants of several fruit species (reviewed in Debnath, 2008). Phenotypic identification based on morphological and biochemical markers is influenced by environmental factors and it requires extensive data recording over years till flowering and fruiting especially in perennials which limit their applicability in large-scale propagation. While karyotype analysis cannot reveal alteration in specific genes or in small DNA segments; isozyme electrophoresis can detect only the genetic changes of DNA segments which are coded for proteins and those are also prone to environmental and developmental variations. Although methods to detect genetic changes have become more streamlined and exhaustive with next-generation sequencing technologies, conventional DNA-based molecular techniques are still reliable and powerful tools for assessing clonal fidelity and sequence variation between source plants and regenerants. Those are rapid, sensitive, more informative, and are not developmentally or environmentally influenced.

Based on the specific requirements, several types of molecular marker systems have been developed, and success of using those molecular markers depends on selection of a marker system and the technique used. For example, polymerase chain reaction (PCR)-based, hybridization-based and sequence-based molecular markers have facilitated species distinction and cultivar identification as well as to assess genetic integrity in

micropropagated plants of *Vaccinium* spp. (Burgher et al., 2002; Debnath, 2005b; Giongo et al., 2006). The sequence-based primers such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), microsatellite or simple sequence repeat (SSR), expressed sequence tag-SSR (EST-SSR) and EST-PCR target specific regions of the genome, while some primers such as random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) select the genomic DNA randomly. SSRs or microsatellites are stretches of DNA which consist of only one or a few (2–6 bp) tandemly repeated nucleotides flanked by unique, conserved DNA sequences and they are repetitive and scattered abundantly in plant genomes (Tautz & Renz, 1984). They occur in both coding and non-coding regions, commonly known as genic or EST-SSRs and genomic SSRs, respectively (Powell et al., 1996). ESTs are unedited short DNA molecules (300 - 500 bp) reverse-transcribed from a cellular mRNA. EST-SSR and EST-PCR markers were developed from EST libraries derived from floral buds of cold acclimated (CA) and non-acclimated (NA) highbush (*V. corymbosum* cv. Bluecrop) blueberry plants and microsatellite-enriched genomic library, constructed from same cultivar (Rowland et al., 2003; Boches et al., 2005). Both markers present a significant improvement over RAPD and ISSR markers which are mostly used for genetic diversity analysis in *Vaccinium* spp. EST-SSR and EST-PCR are co-dominant markers that allow unequivocal distinction of homozygous and heterozygous genotypes, and have been shown very effective for genetic fingerprinting and relationship studies in lowbush blueberries (Bell et al., 2008; Debnath, 2014b). EST-PCR markers have been successfully employed in different studies to assess the genetic stability in regenerated plants of tissue

culture-raised *Vaccinium* spp. including those with no obvious phenotypic alterations (Gajdošová et al., 2006; Debnath, 2011; Debnath, 2017). Use of a combination of two or more marker types has been suggested for genetic fidelity testing of plants so that different regions of the genome under study can be targeted.

EST-SSRs have a higher possibility of being functionally linked with differences in gene expression than the genomic SSRs (Gao et al., 2004). EST-SSRs are considered the markers of choice in ascertaining the clonal fidelity because they are PCR-based, co-dominant, multi-allelic, highly prone to mutation, hyper-variable and randomly dispersed throughout the plant genome (Qureshi et al., 2004). The main limitation of SSR markers is that they have to be isolated *de novo* for new species. Although EST-PCR and microsatellite markers have been used to assess genetic stability of clonal materials of different plant species (Lopes et al., 2006; Agrawal et al., 2014), very few reports have been documented the use of EST-PCR and EST-SSR markers for the assessment of genetic fidelity of micropropagated *Vaccinium* species (Debnath, 2011, 2017). This is the first report on the assessment of genetic fidelity in micropropagated lowbush blueberry using three types of DNA markers, EST-SSR, genomic SSR and EST-PCR. The present investigation was carried out to evaluate the clonal fidelity of *in vitro* derived plants of lowbush blueberry using EST-SSR and EST-PCR markers and to authenticate the reliability of commercial scale application of the micropropagation. The main goal was to assess the possibility of using *in vitro* technique as a sustainable propagation method to produce trueness-to-type propagules.

4.2 Materials and methods

4.2.1 Plant materials

Two lowbush blueberry genotypes (as explained in Chapter 2) were used for this study: a wild clone 'QB9C' collected from Quebec and another was the cultivar 'Fundy' developed in Kentville Research and Development Centre, Nova Scotia (Okie, 2002). Both genotypes were maintained as germplasms in a greenhouse at SJRDC, St. John's, Newfoundland and Labrador, Canada. The plants were propagated from the germplasm stock by conventional softwood cutting (SC) and tissue culture (TC) using nodal explants and grown in greenhouse at SJRDC since 2007. Detailed propagation techniques have been described in Chapter 2. The actively growing young leaves were collected from eleven randomly selected TC-derived plants and two SC plants of each genotypes, and those were shock-frozen in liquid nitrogen immediately after collection and stored at -80 °C until DNA isolation.

4.2.2 Genomic DNA isolation

Genomic DNA was isolated from 80–90 mg of young leaves using DNeasy Plant Mini Kits (Qiagen, 40724 Hilden, Germany) following manufacturer′s instructions with few modifications to adopt the kits for blueberry leaves. The leaf tissues were homogenized with 450 µl buffer AP1 into a 2 ml safe-lock centrifuge tube together with two 8 mm ceramic satellite beads using FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Irvine, CA, USA) at 5.5 m/s speed for 2 times at 45 s each with 5 min rest periods. After

adding 4 µl RNaseA stock solution, the mixture was incubated at 65 °C for 15 min. A 130 µl buffer P3 was added to the lysate and incubated at -20 °C for 8 min. The ceramic beads and leaf-debris was removed by centrifugation at 20,000 *g* for 3 min. The supernatant was transferred to the 1.5 ml Eppendorf tubes and centrifuged at 20,000 *g* for 3 min to remove the rest of cell-debris. The supernatant was pipetted into the QIAshredder mini spin column and centrifuged at 20,000 *g* for 2 min. A mixture of the flow-through fraction from column and AW1 buffer [1:1.5 (v/v)] was transferred into the DNeasy mini spin column and centrifuged for 1 min at 6000 *g*. The DNeasy mini spin column was transferred into a new 2 ml collection tube and 500 µl buffer AW2 was added. It was incubated for 5 min at room temperature and centrifuged for 1 min at 6000 *g*. The flow-through was discarded and the collection tube was reused to wash the membrane of the column with another 500 µl buffer AW2. The DNeasy mini spin column was transferred into a 1.5 ml centrifuge tube after it was centrifuged at 6000 *g* for 1 min. The DNeasy membrane of the column was dried up by centrifuging for 2 min at 20,000 *g*. The DNeasy mini spin column was then transferred into a 1.5 ml microcentrifuge tube and 50 µl buffer AE was pipetted directly onto the DNeasy membrane. The column was centrifuged for 1 min at 6000 *g* to elute the DNA after it had been incubated for 8 min at room temperature. The genomic DNA was visualized through agarose gel electrophoresis (1.6%) to check the impurities (**Figure 4.1**). The concentration and purity of DNA were estimated spectrophotometrically (Ultrospec 2000, Pharmacia Biotech, Cambridge, UK) at 260 nm and the purity was measured by the ratio of the absorbance at 260 nm and 280 nm. The DNA with a concentration of 60–150 ng/µl, and A_{260}/A_{280} and A_{260}/A_{230} absorbance ratio of 1.7 - 1.9 and 2.1 - 2.4, respectively

was used to ascertain clonal fidelity. DNA was diluted with 1× TE buffer (conc. 12.5 ng/µl) to use as template DNA for amplification reactions.

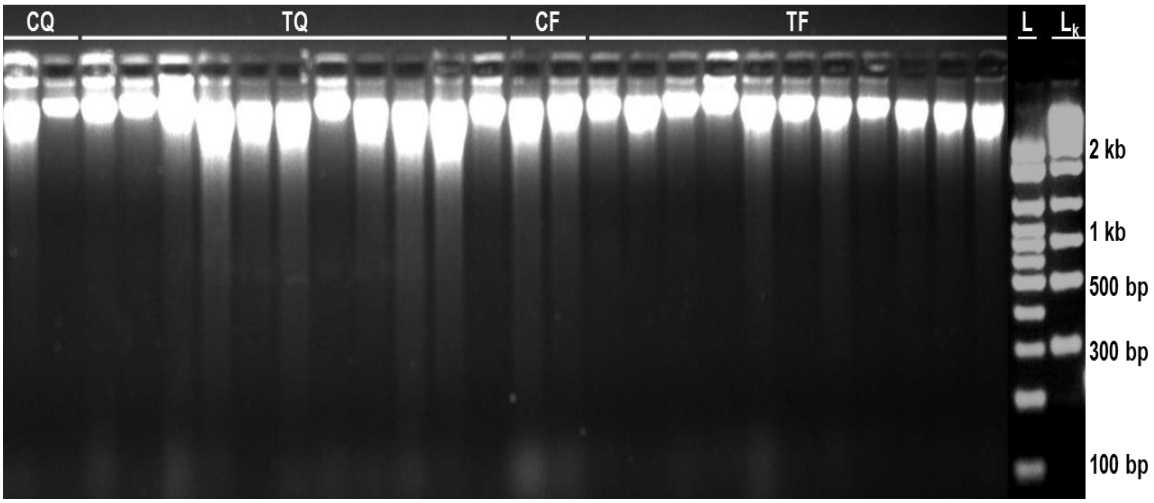

Figure 4.1 Agarose gel electrophoresis of genomic DNA isolated from leaf tissues of softwood cutting 'QB9C' (CQ) and 'Fundy' (CF) plants, and tissue culture-derived 'QB9C' (TQ) and 'Fundy' (TF) plants. L = LowRanger 100 bp DNA ladder and L_K = MidRanger 1 kb DNA ladder (Norgen Bioteck Corp., Thorold, ON, Canada). Size of marker fragments (bp) is indicated at the right.

4.2.3 PCR amplification

A total of 13 EST-SSR (prefix CA or NA) and 7 genomic SSR (prefix VCC) primer pairs (**Table 4.1**) and 13 EST-PCR (prefix CA or NA) primer pairs (**Table 4.2**) synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA) were used to assess the genetic fidelity of micropropagated blueberry plants. Amplification reactions were carried out with DNA samples from eleven TC-regenerated plants and at least one SC plant of both

genotypes following Debnath (2011). The reaction was run in an optimized amplification reaction mixture (25 µL) containing 25 ng of template DNA, 1× PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCL pH 8.7; Qiagen), 200 µM of each dNTP, 0.2 µM of each forward and reverse primers, 0.63 unit of *Taq* DNA polymerase (Qiagen) and PCR grade distilled water (Sigma Chemical Co., St. Louis, MO, USA). 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 °C, followed by 40 cycles of 40 s of denaturation step at 92 °C, 70 s annealing step at the appropriate annealing temperature (**Table 4.1** & **4.2)** and 2 min extension step at 72 °C, followed by a final extension step at 72 °C for 10 min before holding the PCR products at 4 °C. Annealing temperature of EST-PCR and EST-SSR primers was standardized using temperature gradient PCR.

4.2.4 Agarose gel electrophoresis and scoring

Amplified products, along with a LowRanger (100 bp) and a MidRanger (1kb) DNA ladder (Norgen Bioteck Corp., Thorold, ON, Canada) were separated by electrophoresis using 1.6% agarose 3:1 HRB high resolution blend (Ameresco, Solon, OH, USA) gel pre-casted with 2× TBE [tris borate EDTA)] buffer and 1× GelRed nucleic acid stain (Biotium Inc., Hayward, CA, USA) solution and digitally photographed under UV light using the InGenius 3 gel documentation system (Syngene, Beacon House, Cambridge, UK). Scoring and recording of DNA banding patterns were carried out using image analysis software (GeneTools, Syngene). The bands were scored as a dominant binary character, i.e., bands present (1) or bands absent (0). All reactions were run at least twice, and only reproducible

bands were scored.

4.3 Results

4.3.1 Expressed sequence tag-simple sequence repeats (EST-SSR) markers

EST-SSR markers were first time used for genetic fidelity analysis in lowbush blueberries. Two primer pairs from each EST-SSR group of cold acclimated (CA), non-acclimated (NA) EST library, and genomic library (VCC) viz. CA23, CA483, NA398, NA1040 and VCC_I2 and VCC_J1 were tested for the SC plants of both genotypes 'QB9C' and 'Fundy' to determine whether they amplified clear DNA fragments or not, and they were useful to monitor genetic fidelity in lowbush blueberries (**Figure 4.2**). All thirteen EST-SSR and seven genomic SSR primers resulted in successful amplification in SC and TC plants of both genotypes. The number of bands varied from one for CA23, CA112, CA169, CA187, CA855, VCC_I2 and VCC_J3 to five for VCC_I8 with a size ranged from 110 bp to 1751 bp (**Table 4.1**). A total of 44 DNA fragments were scored from 20 SSR primer pairs resulting in an average about two bands per primer pair. EST-SSR analysis showed 100% similarity among 13 randomly selected plants (2 from SC and 11 from TC propagation) from each genotype with monomorphic bands by all primers tested (**Table 4.1**). Representative banding patterns amplified with EST-SSR primers (**Figure 4.3** & **4.4**) and genomic SSR primers (**Figure 4.5** & **4.6**) are illustrated. Four fragments for NA741 (182 bp, 212 bp, 295 bp and 415 bp), one fragment (331 bp) for CA787, three fragments (171 bp, 191 bp and 242 bp) for VCC_K4 and one (216 bp) for VCC_I2 were considered for analysis. Out of thirteen EST-SSR primers tested for genetic fidelity five primer pairs

(CA483, NA398, NA741, NA800 and NA1040) were identified showing polymorphism between wild clone 'QB9C' and cultivar 'Fundy' (**Figure 4.3** & **Table 4.3**). Whereas, four genomic SSR primer pairs out of seven (VCC_I8, VCC_J9, VCC_K4 and VCC_S10) exhibited polymorphism between those two genotypes (**Figure 4.5** & **Table 4.3)**. The rest

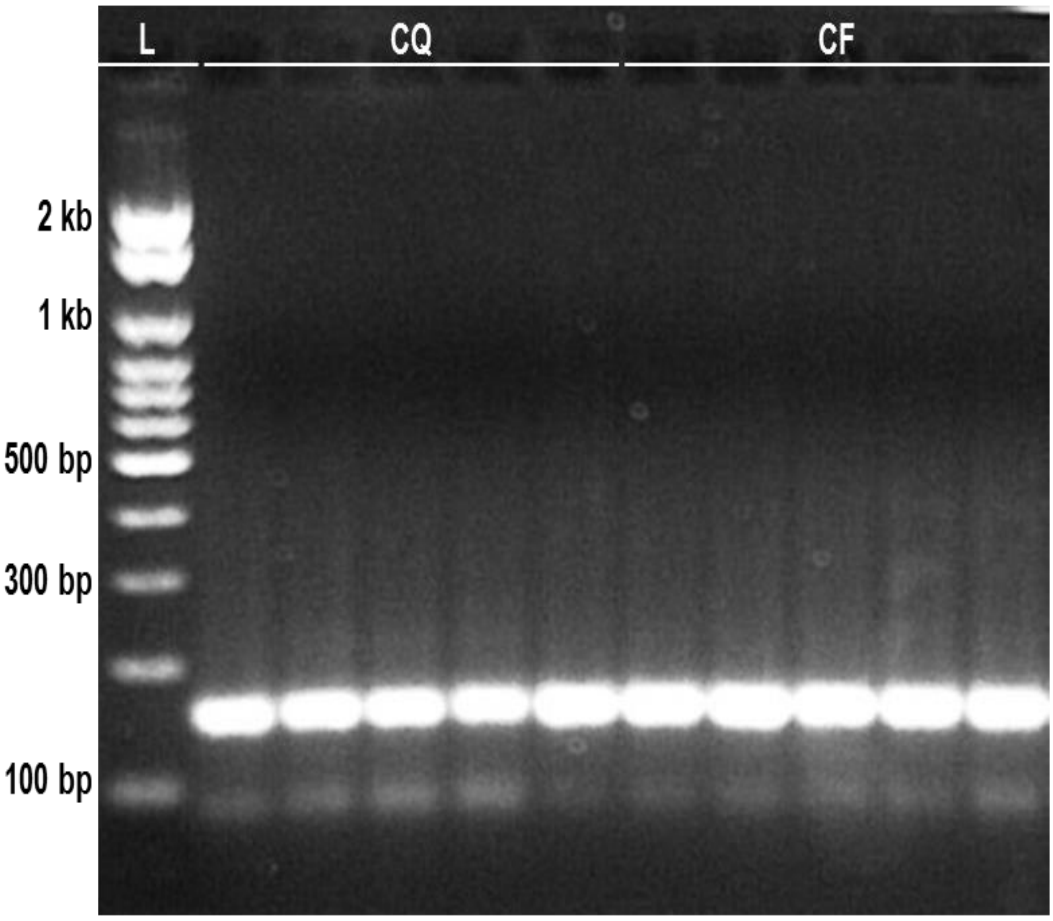

Figure 4.2 Expressed sequence tag-simple sequence repeat (EST-SSR) profiles of conventional softwood cutting 'QB9C' (CQ) and 'Fundy' (CF) plants generated by using primer CA23. L = LowRanger 100 bp DNA ladder (Norgen Bioteck Corp., Thorold, ON). Size of marker fragments (bp) is indicated at the left.

Table 4.1 List of microsatellite markers (EST-SSR and genomic SSR) employed to analyze the clonal fidelity of micropropagated blueberry genotypes, their sequences, annealing temperature (T_A), and number and size of amplified allele(s) per locus. Locus name prefixes reflect origin of GenBank source sequence (CA = cold acclimated EST library; NA = non-acclimated EST library; VCC = enriched genomic library). Bold = polymorphic band between 'QB9C' and 'Fundy'.

Primer ID	Forward (F) and reverse (R) primer sequences (5' to 3')	T_A (°C)	QB9C		Fundy	
			No. of alleles	Size(s) of alleles (bp)	No. of alleles	Size(s) of alleles (bp)
EST-SSR						
CA23	F: GAG AGG GTT TCG AGG AGG AG R: GTT TAG AAA CGG GAC TGT GAG ACG	62	1	152	1	152
CA112	F: TCC ACC CAC TTC ACA GTT CA R: GTT TAT TGG GAG GGA ATT GGA AAC	56	1	110	1	110

Table 4.1 cont'd

Primer ID	Forward (F) and reverse (R) primer sequences (5' to 3')	T_A (°C)	QB9C		Fundy	
			No. of alleles	Size(s) of alleles (bp)	No. of alleles	Size(s) of alleles (bp)
CA169	F: TAG TGG AGG GTT TTG CTT GG R: GTT TAT CGA AGC GAA GGT CAA AGA	62	1	127	1	127
CA236	F: GTT AAG CTT TTA GAT GAG TTG ATG G R: GTT TAA CCA GTC CCA GAC CCA AAT	61	2	209, 1751	2	209, 1751
CA421	F: TCA AAT TCA AAG CTC AAA ATC AA R: GTT TAA GGA TGA TCC CGA AGC TCT	60	2	180, 1084	2	180, 1084
CA483	F: GTC TTC CTC AGG TTC GGT TG R: GAA CGG CTC CGA AGA CAG	61	1	**302**	1	**318**

Table 4.1 cont'd

Primer ID	Forward (F) and reverse (R) primer sequences (5' to 3')	T_A (°C)	QB9C		Fundy	
			No. of alleles	Size(s) of alleles (bp)	No. of alleles	Size(s) of alleles (bp)
CA787	F: TCC TCG TTC TCT CCC TCT CA R: GTT TCG CTG AAG TTG GAG TCC TT	60	1	331	1	331
CA855	F: CGC GTG AAA AAC GAC CTA AT R: GTT TAC TCG ATC CCT CCA CCT G	62	1	266	1	266
NA398	F: TCC TTG CTC CAG TCC TAT GC R: GTT TCC TTC CAC TCC AAG ATG C	61	2	216, **336**	1	216
NA741	F: GCC GTC GCC TAG TTG TTG R: GTT TGA TTT TGG GGG TTA AGT TTG C	58	3	**212,** 295, **415**	2	**182,** 295

Table 4.1 cont'd

Primer ID	Forward (F) and reverse (R) primer sequences (5' to 3')	T_A (°C)	QB9C		Fundy	
			No. of alleles	Size(s) of alleles (bp)	No. of alleles	Size(s) of alleles (bp)
NA800	F: CAA TCC ATT CCA AGC ATG TG	62	3	**130,** 213, 252	2	213, 252
	R: GTT TCC CTA GAC CAG TGC CAC TTA					
NA961	F: TCA GAC ATG ATT GGG GAG GT	61	2	121, 176	2	121, 176
	R: GTT TGG AAT AAT AGA GGC GGT GGA					
NA1040	F: GCA ACT CCC AGA CTT TCT CC	56	3	**200, 376, 664**	1	**182**
	R: GTT TAG TCA GCA GGG TGC ACA A					
Genomic SSR						
VCC_I2	F: AGG CGT TTT TGA GGC TAA CA	62	1	216	1	216
	R: TAA AAG TTC GGC TCG TTT GC					

Table 4.1 cont'd

Primer ID	Forward (F) and reverse (R) primer sequences (5' to 3')	T_A (°C)	QB9C		Fundy	
			No. of alleles	Size(s) of alleles (bp)	No. of alleles	Size(s) of alleles (bp)
VCC_I8	F: TTC AGC ATT CAA TCC ATC CA R: GTT TCT CTT CTC CAA TCT CTT TTC CA	59	2	**153, 270**	3	**121, 182, 225**
VCC_J1	F: CTC ATG GGT TCC CAT AGA CAA R: TGC AGT GAG GCA AAA GAT TG	62	2	227, 507	2	227, 507
VCC_J3	F: TGA TTA CAT TGC CAG GGT CA R: TGG AAA CAA CCG GGT TAC AT	58	1	194	1	194
VCC_J9	F: GCG AAG AAC TTC CGT CAA AA R: GTG AGG GCA CAA AGC TCT C	61	2	216, **392**	2	216, **238**

Table 4.1 cont'd

Primer ID	Forward (F) and reverse (R) primer sequences (5' to 3')	T_A (°C)	QB9C		Fundy	
			No. of alleles	Size(s) of alleles (bp)	No. of alleles	Size(s) of alleles (bp)
VCC_K4	F: CCT CCA CCC CAC TTT CAT TA R: GCA CAC AGG TCC AGT TTT TG	53	2	**171,** 242	2	**191,** 242
VCC_S10	F: ATT TGG TGT GAA ACC CCT GA R: GTT TGC GGC TAT ATC CGT GTT TGT	61	1	**154**	2	**139, 174**
Total			34		31	

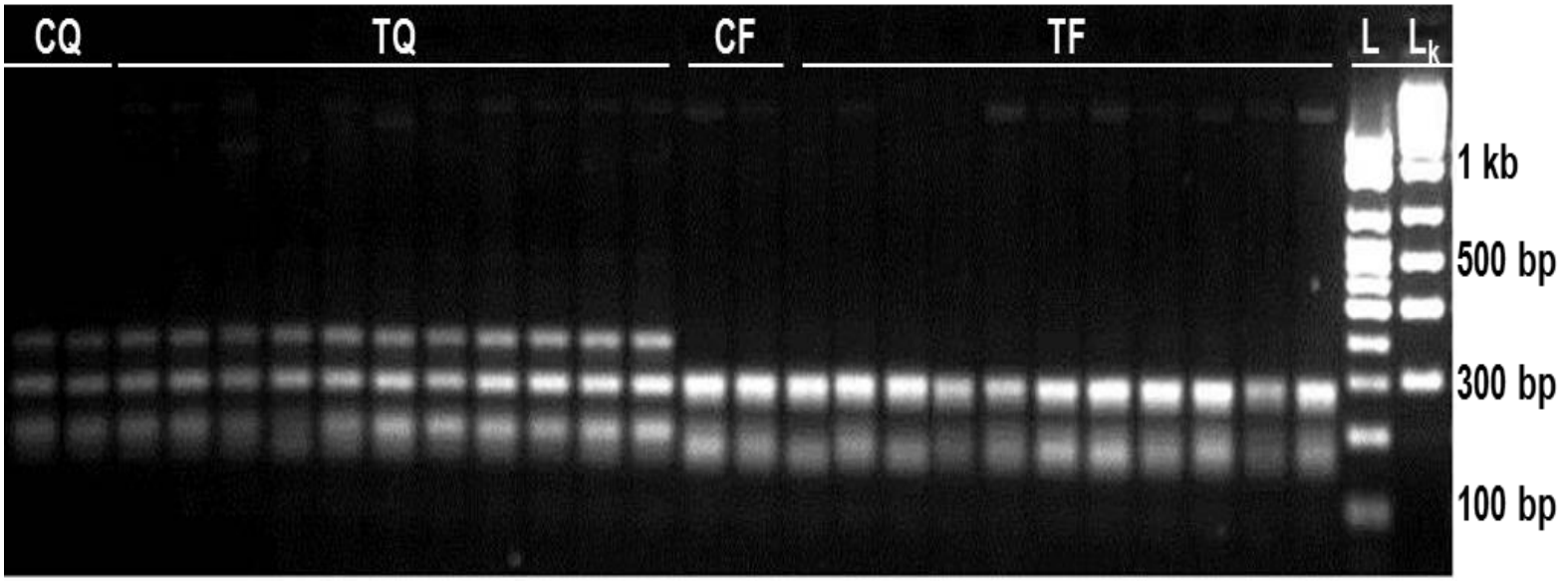

Figure 4.3 Expressed sequence tag-simple sequence repeat (EST-SSR) profiles of lowbush blueberry leaves obtained from softwood cutting plants of 'QB9C' (CQ) and 'Fundy' (CF), and tissue culture derived plants of 'QB9C' (TQ) and 'Fundy' (TF) generated by using primer NA741. L = LowRanger 100 bp DNA ladder and L_K = MidRanger 1 kb DNA ladder. Size of marker fragments (bp) is indicated at the right.

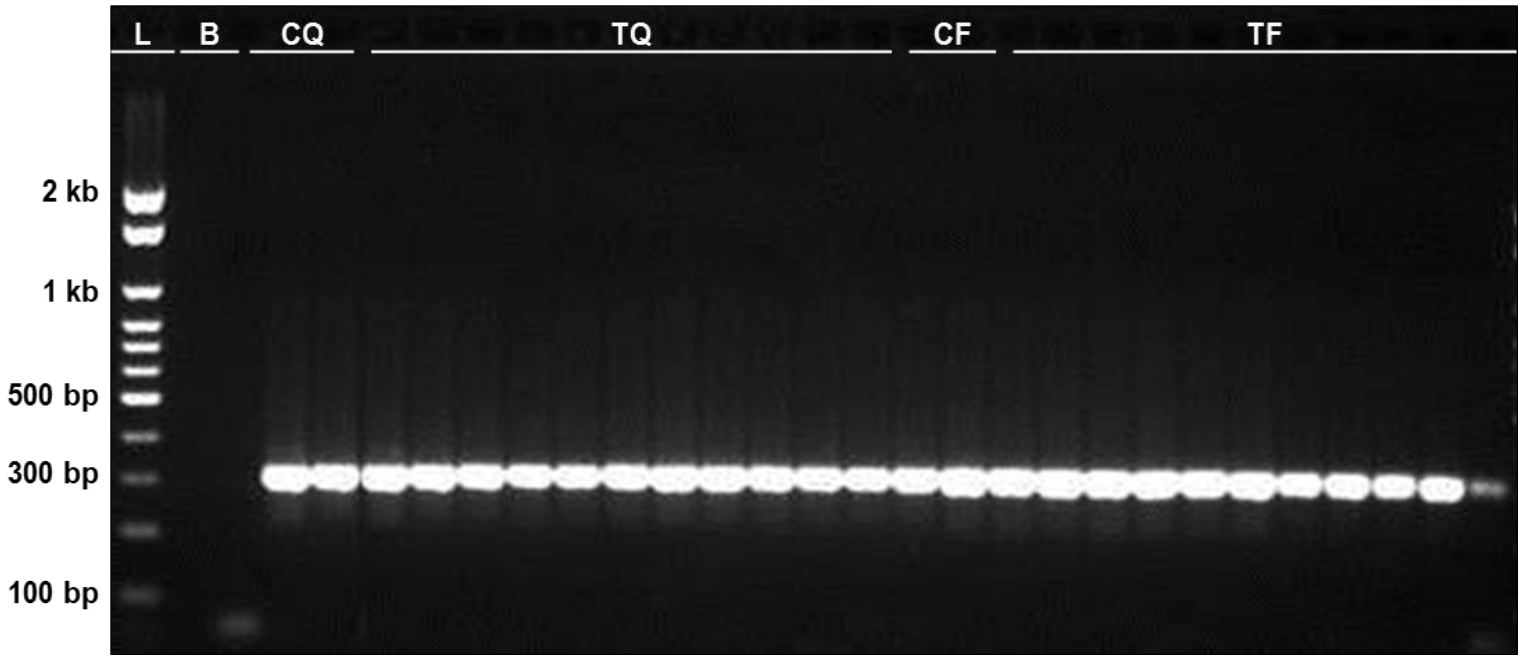

Figure 4.4 Expressed sequence tag-simple sequence repeat (EST-SSR) profiles of lowbush blueberry leaves of softwood cutting 'QB9C' (CQ) and 'Fundy' (CF) plants, and tissue culture derived 'QB9C' (TQ) and 'Fundy' (TF) plants amplified by using primer CA787. L = LowRanger 100 bp DNA ladder; B = blank with PCR mixture except DNA templates. Size of marker fragments (bp) is indicated at the left.

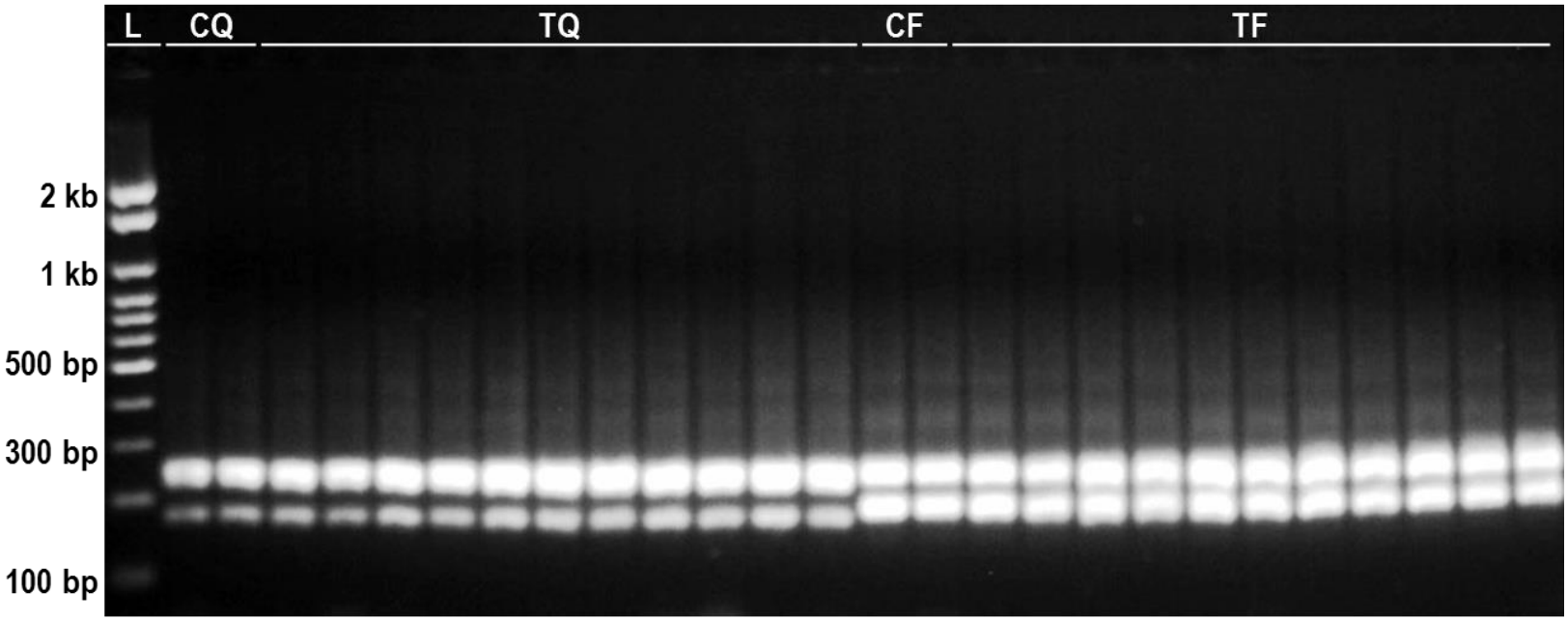

Figure 4.5 Simple sequence repeat (SSR) profiles of lowbush blueberry leaves of softwood cutting 'QB9C' (CQ) and 'Fundy' (CF) plants, and tissue culture derived 'QB9C' (TQ) and 'Fundy' (TF) plants generated by using genomic SSR marker VCC_K4. L = LowRanger 100 bp DNA ladder. Size of marker fragments (bp) is indicated at the left.

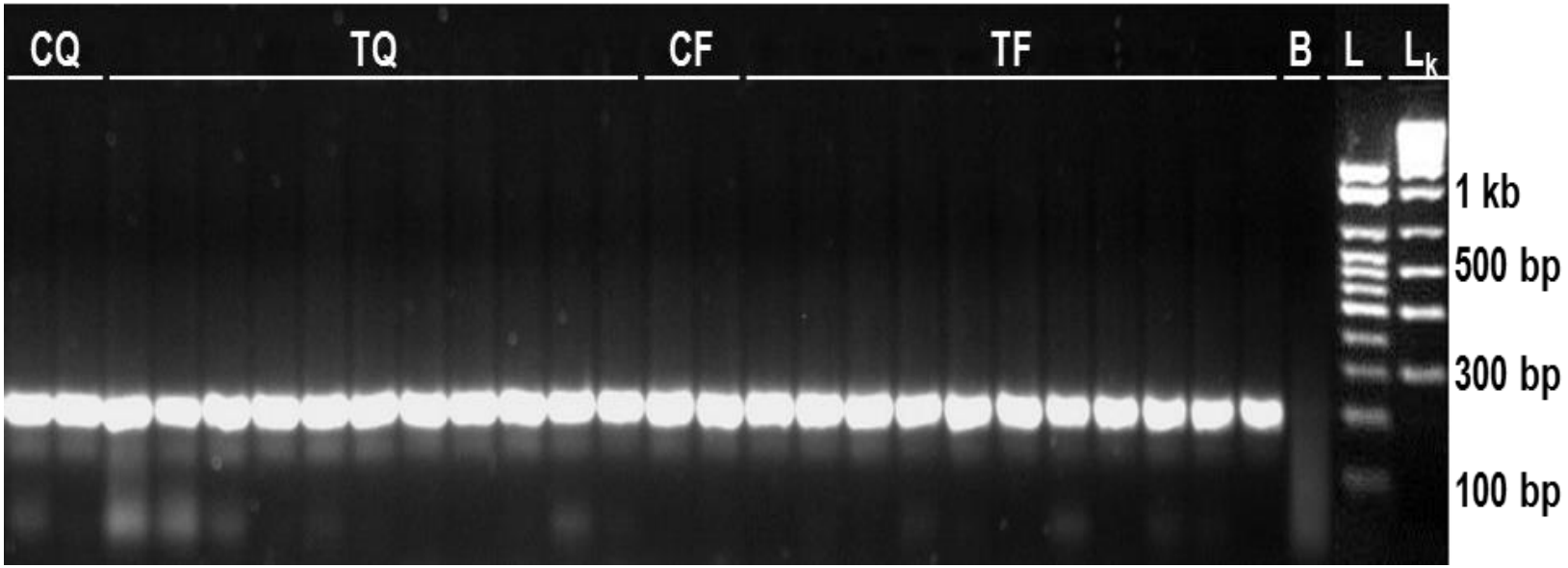

Figure 4.6 Simple sequence repeat (SSR) profiles of lowbush blueberry leaves of softwood cutting 'QB9C' (CQ) and 'Fundy' (CF) plants, and tissue culture derived 'QB9C' (TQ) and 'Fundy' (TF) plants generated by using genomic enriched SSR primer VCC_I2. B = blank with PCR mixture except DNA templates; L = LowRanger 100 bp DNA ladder, and L_K = MidRanger 1 kb DNA ladder (Norgen Bioteck Corp., Thorold, ON). Size of marker fragments (bp) is indicated at the right.

of the primers produced monomorphic banding pattern across both of the genotypes as well as the plants propagated by SC and TC (**Figure 4.4** & **4.6**). The amplification of alleles was consistent between repeated runs of PCR.

4.3.2 Expressed sequence tag-polymerase chain reaction (EST-PCR) markers

All thirteen EST-PCR primers considered for genetic analysis produced reproducible and intense good quality banding patterns in one SC and eleven TC derived plants of 'QB9C' clone and 'Fundy' cultivar. Annealing temperatures ranged from 45 °C to 58 °C (**Table 4.2**) which was similar as those reported by Debnath (2011) in the clonal fidelity analysis of lowbush blueberry plants derived through adventitious shoot regeneration system. Each primer generated a set of amplification products ranging from 255 bp to 4229 bp in size (**Table 4.2**). Out of thirteen primer pairs, four (CA287, CA1029, NA27 and NA353) amplified one DNA fragment, three (CA227, CA1105 and CA1590) amplified two bands, CA1785 detected three bands, two (CA21 and CA791) detected four bands, CA16 detected five bands, CA231 detected six bands and CA54 detected nine DNA bands. In the present study, thirteen EST-PCR primer pairs produced 41 alleles with an average of about 3 per locus. Representative amplified banding pattern produced by primer CA21 in SC and TC plants of 'QB9C' and 'Fundy' is illustrated in **Figure 4.7** and four fragments (959 bp, 1056 bp, 1124 bp and 1166 bp) were considered for analysis. The entire fragment pattern of TC plants appeared as bands in 'QB9C' 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). Out of thirteen EST-PCR primers tested, six EST-PCR primer pairs (CA16, CA21, CA54,

Table 4.2 List of expressed sequence tag-polymerase chain reaction (EST-PCR) markers used to analyze the clonal fidelity of micropropagated blueberry genotypes, their sequences, annealing temperature (T_A), and number and size of amplified allele(s) per locus. Locus name prefixes reflect origin of GenBank source sequence (CA = cold acclimated EST library; NA = non-acclimated EST library). Bold = polymorphic band between 'QB9C' and 'Fundy'.

Primer ID	Forward (F) and reverse (R) primer sequences (5' to 3')	T_A (°C)	QB9C		Fundy	
			No. of alleles	Size(s) of alleles (bp)	No. of alleles	Size(s) of alleles (bp)
CA16	F: CCA ATG CCA CAA ACG AGA TT R: AGC CCC CAA CTT TCG TTC T	45	5	285, **380**, 798, 1342, **2208**	3	285, 798, 1342
CA21	F: TCC GAT AAC CGT TAC CAA GC R: TAT ACA GCG ACA CGC CAA AA	54	2	**959, 1124**	2	**1056, 1166**
CA54	F: CCG GTG AAC TTC CAC TTG TT R: AGA TAC TAC TGG GGG TGG GG	58	7	521, 731, 855, 1208, **1383**,**2435**, 4229	7	521, 731, 855, 1208, **1635**, **2790**,4229

Table 4.2 cont'd

Primer ID	Forward (F) and reverse (R) primer sequences (5' to 3')	T_A (°C)	QB9C		Fundy	
			No. of alleles	Size(s) of alleles (bp)	No. of alleles	Size(s) of alleles (bp)
CA227	F: TGG AGA CTG GAG TGA TGC AA R: TTT GCA AGA ACC ATG CTG AG	56	2	314, 512	2	314, 512
CA231	F: CCA AAA TGC CCA AAC TCA TC R: AAG GAA AAG GAA ACG GGA AA	54	4	255, 325, **475, 621**	4	255, 325, **545, 693**
CA287	F: AGG GCT TTC CCT CAA TCA CT R: CCT TGT TGT TCC TTC CTT CG	58	1	970	1	970
CA791	F: AGA GCC AAA AGA AGG GGA AG R: TCA AAA GTT TTC CGG ACC AG	56	3	605, 2000, **2551**	3	605, **930,** 2000

Table 4.2 cont'd

Primer ID	Forward (F) and reverse (R) primer sequences (5' to 3')	T_A (°C)	QB9C		Fundy	
			No. of alleles	Size(s) of alleles (bp)	No. of alleles	Size(s) of alleles (bp)
CA1029	F: GAA GTT TTC CGT TCT CTG CAA	48	1	1150	1	1150
	R: CTG CAG CTA GGA CCG AAG AG					
CA1105	F: TGG TGC TTT CAT CCT GCT AA	58	2	329, 525	2	329, 525
	R: GCT TGC TTC TTG GGT GAC TC					
CA1590	F: AAC CCA GCA CCT CCT TTC TT	56	2	295, 605	2	295, 605
	R: CTC TGT TGC TGG CTG TGT GT					
CA1785	F: CAC CACCAC TGT CGT ACA CC	58	3	498, **1160,** 1330	2	498, 1330
	R: GCA TGA GCC GAA CAT AAT CA					

Table 4.2 cont'd

Primer ID	Forward (F) and reverse (R) primer sequences (5' to 3')	T_A (°C)	QB9C		Fundy	
			No. of alleles	Size(s) of alleles (bp)	No. of alleles	Size(s) of alleles (bp)
NA27	F: CGC TCG CTC CAT TGT TTC R: TAT GCA TGA AGC TTG CCG TA	56	1	457	1	457
NA353	F: GGA AGG GTA TGC TGA GCT TG R: CAG AAT CAT GAG GCC CAC TT	56	1	2208	1	2208
Total			34		31	

Table 4.3 The total number of loci and polymorphic bands between two genotypes ('QB9C' and 'Fundy') obtained from EST-SSR and genomic SSR markers

Primer ID	Total scorable bands	Monomorphic band(s)	Polymorphic band(s)	Polymorphisms (%)
EST-SSR				
CA483	2	0	2	100
NA398	2	1	1	50
NA741	4	1	3	75
NA800	3	2	1	33.3
NA1040	4	0	4	100
Genomic SSR				
VCC_I8	5	0	5	100
VCC_J9	3	1	2	66.7
VCC_K4	3	1	2	66.7
VCC_S10	3	0	3	100
Total	29	6	23	79.3

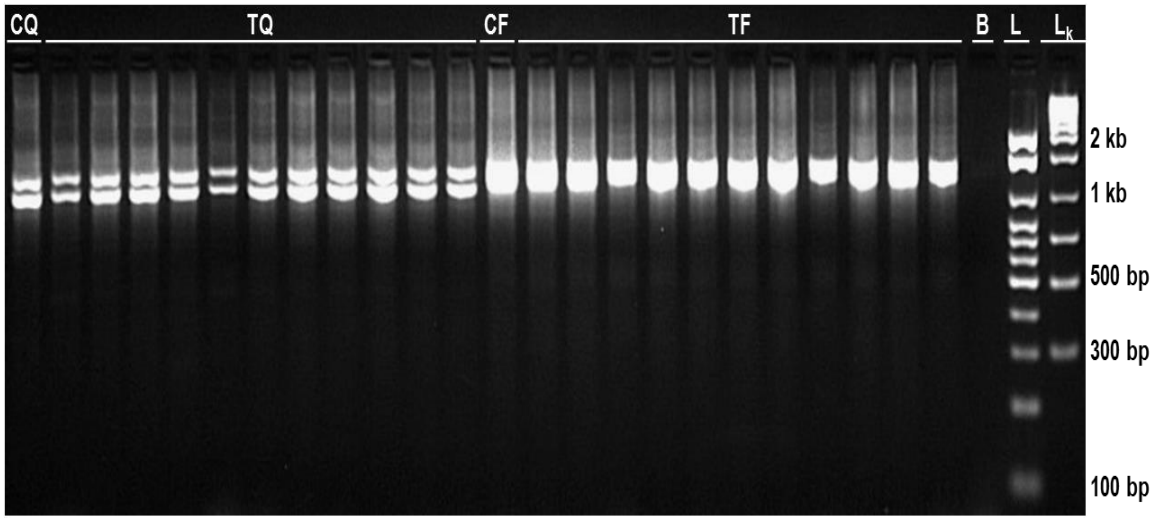

Figure 4.7 Expressed sequence tag-polymerase chain reaction (EST-PCR) banding pattern of softwood cutting 'QB9C' (CQ) and 'Fundy' (CF) plants, and tissue culture-derived 'QB9C' (TQ) and 'Fundy' (TF) plants generated by using primer CA21. B = blank with PCR mixture except DNA template; L = 100 bp DNA ladder and L_K = 1 kb DNA ladder (Norgen Bioteck Corp., Thorold, ON, Canada). Size of marker fragments (bp) is indicated at the right.

CA231, CA791 and CA1785) were identified as polymorphic markers between 'QB9C' and 'Fundy' genotypes (**Table 4.4**). In total 17 polymorphic DNA fragments, out of 41 were observed between two genotypes. The rest of the primers were monomorphic across both genotypes as well as among the plants propagated by two different methods.

4.4 Discussion

From an evolutionary point of view, genomic stability and variability of a eukaryotic plant need to be complicatedly balanced for the sake of adaptation and survival (Joyce et al.,

2003). Thus, it is not unexpected that minor changes occurred in a substantial number of cellular genes to perform a function for the strict control of genomic integrity under normal or favorable environments, and in relaxing the control to allow mutations to be carried out in stressful conditions (Boyko & Kovalchuk, 2008; Zhang et al., 2010). Plant tissue culture imposes a stressful environment for the plant cells (McClintock, 1984) and concurrently induce various types of genetic and/or epigenetic instability which are consistent with the idea that tissue culture-induced somaclonal variation is self-imposed as a consequence of disrupted normal cellular controls (Kaeppler et al., 2000; Joyce et al., 2003; Madlung & Comai, 2004). There is an increasing interest in *in vitro* propagation of blueberry plants

Table 4.4 The total number of loci and polymorphic bands between two genotypes ('QB9C' and 'Fundy') obtained by using EST-PCR markers

Primer ID	Total scorable bands	Monomorphic band(s)	Polymorphic band(s)	Polymorphisms (%)
CA16	5	3	2	40
CA21	4	0	4	100
CA54	9	5	4	44.4
CA231	6	2	4	66.7
CA791	4	2	2	50
CA1785	3	2	1	33.3
Total	31	14	17	54.8

due to its potential to multiplication of trueness-to-type clones which are characterized by vigorous growth and fast spreading capacity, and for releasing a highly productive and quality cultivar in short time (El-Shiekh et al., 1996; Goyali et al., 2015a). Literature reports that somaclonal variations may be escalated under certain stress conditions *in vitro* which are controlled by many factors including plant genotype, medium, explant type and origin, plant growth regulator, duration and number of sub-cultures, and mutagens, and those variations are obvious morphologically, genetically and/or epigenetically (Rani & Raina, 2000). Trueness-to-type regenerated plants and their genetic uniformity are essential for the commercial application of micropropagation in blueberries.

Microsatellites or genomic SSRs, being abundant components of eukaryotic genomes, undergo frequent changes in their sequence length by several mechanisms, including replication slippage, DNA repair and recombination, causing either contractions or expansions of the number of repeat units in different developmental/metabolic cues or under stressed environmental conditions (Nag et al., 2004; Varshney et al., 2005). Hence, they are ideal markers for monitoring genome stability status under various circumstances especially under *in vitro* conditions. Since the EST-SSR markers are derived based on the available sequence data for ESTs, transcribed regions, genes and complementary DNA clones, it indicates that changes in the repeats may also alter the expression pattern and/or function of a cellular gene, depending on the position of the SSR tracts (Nag et al., 2004). Therefore, genic SSRs are not only a reliable technique for assaying the genetic, functional and phenotypic diversities among the species, cultivars or clones of *Vaccinium* spp. (Boches et al., 2006; Gajdošová et al., 2006; Česonienė et al., 2013; Debnath, 2014b), but

also studying the mutagenic basis of tissue culture-induced somaclonal variations. In general, EST-SSR primers are less polymorphic compared with genomic SSRs in crop plants (Rungis et al., 2004). In the present study, 5 out of 13 EST-SSR (**Table 4.3** & **Figure 4.3**), and 4 out of 7 genomic-SSR (**Table 4.3** & **Figure 4.5**) showed polymorphism between 'QB9C' and 'Fundy' genotypes. It is due to greater DNA sequence conservation in transcribed regions (Chabane et al., 2005). Polymorphic banding pattern at nine out of twenty SSR primer pairs showing over all 52.3% polymorphism between 'QB9C' and 'Fundy' confirmed the diversification between the wild clone and named cultivar studied, and confirmed the utility of using EST-SSR markers to check the clonal fidelity of micropropagated blueberry plants. Although only two SSR primer pairs are enough to analyse diversity among genotypes, thirteen genic and seven genomic SSR primers have been used in this study to increase the polymorphism and thus reduced the probability of false assessment regarding genetic fidelity in the TC plants. The absence of any variation in the banding pattern at twenty microsatellite loci clearly indicated the genetic integrity among the blueberry TC plants of both 'QB9C' and 'Fundy' genotypes. The confirmed genetic fidelity of bioreactor-derived micropropagated plants in four raspberry cultivars was reported by Debnath (2014c) using microsatellite markers. Although SSR technique has been proved to be reliable technique to assess genetic alterations generated by *in vitro* conditions in different plant species (Ryu et al., 2007; Rathore et al., 2011; Debnath, 2014c; Regalado et al., 2015), only one technique cannot guarantee the genetic purity of tissue-cultured plants due to most of the cases only a very small fraction of the genome (0.001–1%) is analyzed (Benson et al., 2013). In the present study, EST-SSR and genomic SSR

markers detected the clonal fidelity in micropropagated blueberries. However, a number of reports suggested that SSRs were not powerful tools either for detection of clones of a specific cultivar or for the identification of phenotypic variation among somaclones in *Vitis vinifera* species (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 DNA marker and methylation sensitive amplification polymorphism (MSAP) techniques. Moreover, SSR technique could not detect the point mutation in the length of a microsatellite product. Thus, if SSR analysis shows no genetic variations, as found in the present study, this does not necessarily mean that there are none (Mallón et al., 2010). Therefore, use of more than one DNA amplification technique has been suggested advantageous. EST-PCR has been employed to assess the reliability of results of microsatellite electrophoretic profiling.

EST-PCR markers selected for this study were used to analyse genetic diversity or phylogenetic relationships among the species, interspecific hybrids, cultivars and clones of the genus *Vaccinium* (Bell et al., 2008, 2009; Debnath, 2014b; An et al., 2015; Tailor et al., 2017), and to study the clonal fidelity of lowbush (Debnath, 2011) and other blueberries (Debnath, 2017). This is the first report on using EST-PCR molecular markers to monitor trueness-to-type of greenhouse grown mature micrpropagated plants in two blueberry genotypes together, a wild clone and a cultivar. EST-PCR markers differentiated the wild clone ‘QB9C’ from the cultivar ‘Fundy’ (**Table 4.4** & **Figure 4.7**). On an average, EST-PCR markers amplified higher number of DNA fragments per primer pair as well as larger

size of bands compared with EST-SSR markers, thus revealed more coverage of the blueberry genome. None of the EST-PCR primers used in the present study exerted polymorphism (i.e., 100% genetic similarity) among the plants regenerated *in vitro* via axillary shoot proliferation. Using EST-PCR molecular marker, Debnath (2011) reported complete similarity among the micropropagated plants of a wild clone 'QB1' of lowbush blueberry originated from leaves via adventitious shoot regeneration. The EST-PCR analysis confirmed the reliability of results in the present study obtained by using SSR markers that *in vitro* derived lowbush blueberry plants had maintained clonal fidelity. Debnath (2017) used combination of EST-SSR, genomic SSR and EST-PCR markers to assess true-to-type propagules in highbush, half-high, and hybrids between half-high/highbush and lowbush blueberries derived from nodal explants via shoot proliferation in semi-solid and liquid media. The author reported that each type of marker produced a monomorphic DNA banding pattern among the regenerants, and between regenerants and donor plants which confirmed the clonal fidelity in *V. corymbosum* species and their hybrids. Landey et al. (2015) used AFLP, MSAP and sequence specific amplification polymorphism (SSAP) molecular markers to detected somaclonal variation in somatic seedlings of coffee (*Coffea arabica* L.) and found no polymorphic fragments between the electrophoretic profiles of mother plants and those of the *in vitro* progenies in AFLP and SSAP molecular markers analyses. Similarly, the genetic stability was confirmed in all developmental stages of *Ocotea catharinensis* and *Coffea arabica* derived in *in vitro* cultures using RAPD and AFLP marker analysis (Hanai et al., 2010; Landey et al., 2015). However, the modifications to some extend in DNA methylation were observed between

mother plants and their *in vitro* progenies.

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 or true-to-type clone to source plants (Goyali et al., 2015a). Biotic challenge experienced by cutting plants is available in conventional blueberry propagation where trueness-to-type is maintained (Debnath, 2011). Despite the plants of lowbush blueberries derived through micropropagation have been distinguished from the plants propagated conventionally by SC based on the morphological and biochemical characteristics as discussed in Chapter 2, Chapter 3 and Goyali et al. (2013; 2015a), the genetic patterns of TC plants in EST-SSR, genomic SSR and EST-PCR molecular marker systems are the same as their SC counterparts. It represents that the micropropagated progenies are of the same genotype and maintain identical genetic features as the SC plants do. Debnath (2014c) reported stable genetic constituents in the plants propagated conventionally with root cuttings and by tissue culture in raspberry. In another study, Debnath (2009c) found identical genetic constituent between strawberry plant propagated using runner cuttings and through adventitious shoot regeneration system using sepals, leaf disks and petiole. However, difference between filed grown plants of *Agave tequilana* propagated by rhizome cutting, shoot proliferation and somatic embryogenesis were distinct in a cluster analysis using inverse ISTR (sequence-tagged repeat) molecular markers (Torres-Morán et al., 2010). It is not unexpected given the fact that organized tissues such as nodes, apical and axillary buds or meristems used as explants tend to produce micropropagated progeny with reduced or no phenotypic variation among them (Pierik, 1991; Rahman & Rajora, 2001),

because the totipotent cells of those tissues perform dedifferentiation or redifferentiation in *in vitro* conditions with few or none genomic aberrations and consequently maintain genetic stability in *in vitro* derived plants. The plants used in this study were derived through axillary shoot proliferation using young shoot as an explant. Therefore, TC plants might have shown genetic stability. Although genetic variation is appeared mostly in plant regeneration from unorganized callus (Piola et al., 1999; Biswas et al., 2009), variations have also been reported in plantlets derived from axillary bud proliferation in apple and pineapple (Soneji et al., 2002; Modgil et al., 2005). Despite phenotypic similarities in plantlets produced directly from axillary bud proliferation and indirectly from adventitious shoot regeneration via an intermediate callus phase, the differences in genomic constituents of plantlets produced through intermediate callus phase have been effectively distinguished by molecular markers which are absent in the plants derived from direct shoot organogenesis (Biswas et al., 2009; Rathore et al., 2011; Chavan et al., 2014). Whereas no polymorphism in DNA banding pattern or genetic variation was reported among the plants of strawberry, raspberry, lingonberry and blueberry derived through adventitious shoot regeneration techniques (Gajdošová et al., 2006; Debnath, 2009c, 2011, 2014c). TC progenies of berry crops have predominantly stable genetic features.

Micropropagated plants derived through axillary shoot proliferation exhibited morphological and biochemical variation from SC plants (data shown in Chapter 2 and Chapter 3). The deviations are probably due to the synergetic effect of genetic and epigenetic modifications, as well as due to the artificial stress of TC. The present study demonstrated that the *in vitro* derived plants maintained the genetic fidelity, and they had

same genetic constituent as in conventionally propagated plants, although tissue culture induced variations were reported in micropropagules derived through axillary bud proliferation techniques (Modgil et al., 2005). *In vitro* techniques established the feasibility of using propagation directly to improve some important agro-morphological characteristics without undergoing any genetic change in blueberry. Hence the shoot proliferation system is a reliable propagation method to produce trueness-to-type plant with vigorous growth and fruit quality. Further studies will be carried out to find the possible cause of the difference in morphological characteristics between two sets of plants derived from two propagation methods. Epigenetic variations in the plants propagated SC and TC will be studied through DNA methylation polymorphism technique.

CHAPTER 5

DNA Methylation in Lowbush Blueberry (*Vaccinium angustifolium* Ait.) Propagated by Softwood Cutting and Tissue Culture

5.1 Introduction

DNA methylation by 5´-methylcytosine is a relatively stable (inheritable) epigenetic mechanism existing in a range of eukaryotic organisms, and particularly abundant in higher plants. In angiosperms, approximately 20–55% of all cytosine residues in the genomic DNA are methylated (Messeguer et al., 1991; Lister et al., 2008) which are much higher than in animal with 2.5–11.6% (Belanger & Hepburn, 1990). This covalent modification of DNA in cytosine-guanine (CG) dinucleotide sequence is commonly found in plants, animal and some fungi (Finnegan & Kovac, 2000; Bird, 2002). In addition, plants have significant levels of cytosine methylation in asymmetrical CH, CHG and CHH sequences (where H is A, T or C) (Gruenbaum et al., 1981). Unmethylated CG and CHG sites are mainly clustered in the CG rich sequence of the gene promoter region, termed as CG island (Ng & Adrian, 1999). Those sites are also randomly distributed throughout the plant genome.

DNA methylation plays a key role in all eukaryotes in gene expression which is essential for development and stress response. It is primarily involved in maintaining genomic integrity by controlling the activity of transposable elements, minimizing occurrence of ectopic recombination, formatting and perpetuating heterochromatin, reducing

transcriptional noise, and in controlling genomic imprinting (Rangwala & Richards, 2004; Tariq & Paszkowski, 2004; Ibarra et al., 2012; Jaligot et al., 2014). Genome imprinting in which the differential expression of genes inherited from maternal and paternal genomes is mediated by differential methylation of the two genomes in endosperm (Xiao et al., 2006; Gehring et al., 2009). Consequently, changes in inherent DNA methylation patterns may have structural and functional effects to the organisms with this epigenetic code (Tariq & Paszkowski, 2004). In *Arabidopsis*, drastic reduction of cytosine methylation of the *Met* 1 gene produces pleiotropically defective phenotypes and developmental abnormalities including reduced apical dominance, plant and seed size, variation in leaf shape and size, low fertility and altered flowering time (Finnegan et al., 1996; Xiao et al., 2006). In banana and oil palm, DNA methylation pattern has been found to be associated with well characterized phenotypic somaclonal variants (Jaligot et al., 2000; Peraza-Echeverria et al., 2001). DNA methylation controls the expression of several genes involved in development.

Differences in methylation state (level and/or pattern) of cytosine have been observed in response to various endogenous and exogenous factors in both *in vivo* and *in vitro* conditions, which are tissue and developmental stage specific, and might be adapted to deal with a particular stress (Richards, 2006). DNA methylation/demethylation is affected by *in vitro* propagation, which is comprised of a de-differentiation (callus formation) process and a re-differentiation (plant regeneration) course (Huang et al., 2012a; Rathore & Jha, 2016). Although, *in vitro* propagation techniques allow all the year-round production of pathogen free horticultural crops, in tissue culture propagation process, the normal cellular control is disrupted (Phillips et al., 1994; Kaeppler et al., 2000). Subsequently, cytological changes

and/or genetic and epigenetic modifications in DNA could be exhibited (Kaeppler et al., 2000; Peraza-Echeverria et al., 2001; Chakrabarty et al., 2003; Park et al., 2009), which may have phenotypic consequences collectively called somaclonal variation (Larkin & Scowcroft, 1981). In *Pinus radiata*, for example, the increase in plant vigour and rejuvenation, decrease in organogenesis capability have been reported due to DNA methylation altered in *in vitro* propagation (Valledor et al., 2007, 2010). The changes in DNA methylation patterns in maize and apple are induced by tissue culture conditions (Kaeppler et al., 2000; Li et al., 2002). On the other hand, significant differences in cytosine methylation among various tissue types in many plant species such as tomato (Messeguer et al., 1991), maize (Lu et al., 2008), sorghum (Zhang et al., 2011), rice (Dhar et al., 1990) and cauliflower (Li et al., 2014) have been reported.

Blueberry, a perennial deciduous shrub, is highly valued for its health benefits and antioxidant activities (Neto, 2007). Generally, blueberries are propagated by seed or stem cutting. Due to genetic variation in blueberry plants developed from seed propagation, and slow spreading capacity of cutting plants, it is difficult for large-scale commercial cultivation by conventional stem cutting propagation. Micropropagation is an easy and inexpensive alternative method that allows huge number of clonal plants in a short period of time and helps to cope with high demand of blueberry plants for establishing a new farm and filling up bare area of an established farm quickly (Debnath, 2007c). However, *in vitro* conditions induce phenotypic variations which may have linkage with genetic and/or epigenetic changes. There is evidence of differential phenotypic changes and of having higher health beneficial phytochemicals like total phenolics, flavonoids and antioxidant

capacity in micropropagated berry crops compared to *in vivo* propagation (Goyali et al., 2013; Vyas et al., 2013a; Goyali et al., 2015a). Apparently, to elucidate the basis of these differential properties in morphology and biochemical content, it is meaningful to screen the tissue culture-induced genetic and/or epigenetic variations. Molecular marker analysis using expressed sequence tag-simple sequence repeat (EST-SSR) and the expressed sequence tag-polymerase chain reaction (EST-PCR) DNA markers confirmed the genetic fidelity in the tissue culture (TC) plants. Micropropagated plants have same genetic constituent as softwood cuttings (SC) plants (Goyali et al., 2015a). The variation originated in the *in vitro* originated lowbush blueberry clones might be due to the epigenetic changes. In this chapter, the epigenetic variation especially global DNA methylation will be discussed.

Several methods are available to evaluate the methylation levels especially the distribution of 5´-methylcytosines. To assess global DNA methylation, complete enzymatic DNA degradation, followed by high performance liquid chromatography (HPLC) of nucleotides or derivatives (Rival et al., 2013) are used which has very low sensitivity, and it is impossible to locate the genomic localization of the methylated cytosine (Baurens et al., 2004). The sequence-specific method is based on either bisulfite modifications of the DNA (Cokus et al., 2008), or immunoprecipitation (Thomas et al., 2008). The drawbacks of these methods are that they are entirely dependent on detailed knowledge of the target sequence and involve a time-consuming and complicated set up for plants (Fulneček et al., 1998). Methylation sensitive amplification polymorphism (MSAP) approach, an adaptation of the amplified fragment length polymorphism (AFLP) technique (Vos et al., 1995), is easy to

assess the epigenetic stability in *in vitro* propagated non-model plants like grape (Baránek et al., 2010), banana (Schellenbaum et al., 2008), rose (Xu et al., 2004), coffee (Landey et al., 2015), cauliflower (Li et al., 2014) and *Doritaenopsis* (Park et al., 2009). This method is based on the sensitivity of restriction endonucleases to site-specific methylation (McClelland et al., 1994). Digestion with methylation sensitive restriction endonucleases followed by amplification of restriction fragments is independent on the availability of prior genome sequence information other than the approximate genome size. Moreover, the high number of methylation events can be detected using a relatively small number of primer combinations and the additional ability to clone and characterize novel methylated sequences. Therefore, MSAP is a suitable technique to evaluate epigenetic changes at the level of DNA methylation in the blueberry plants. The objective of the present study is to investigate the levels of epigenetic variations in the form of cytosine methylation in lowbush blueberry propagated conventionally with SCs and micropropagation through node culture. DNA methylation in somatic callus of blueberry clones have been reported by Ghosh et al. (2017). To the best of our knowledge, this is the first report on the global DNA methylation in greenhouse grown matured plants of a micropropagated wild clone and a cultivar of lowbush blueberry plants.

5.2 Materials and methods

5.2.1 Plant materials

Two lowbush blueberry genotypes ‘QB9C’ and ‘Fundy’ maintained at St. John’s Research and Development Centre (SJRDC), St. John’s, Newfoundland were used for this study.

The plants were propagated by conventional SC and micropropagation through node culture from the stock plants, and those were grown in a greenhouse at SJRDC since 2007. Detailed propagation techniques have been described in 'Materials and Methods' in Chapter 2 and in Goyali et al. (2013, 2015a). Actively growing young leaves were collected from ten SC and TC plants which had been used for clonal fidelity analysis in both genotypes (Chapter 4). The leaves were shock-frozen in liquid nitrogen immediately after collection and stored at -80 °C until DNA isolation.

5.2.2 DNA isolation

Genomic DNA was isolated from 90 - 160 mg of young leaves using DNeasy Plant Mini Kits (Qiagen, 40724 Hilden, Germany) following manufacturer′s instructions with few modifications. In brief, 500 µl buffer AP1 was added to leaf tissues placed into a 2 ml safe-lock centrifuge tube, together with two 8 mm ceramic satellite beads, and homogenized using FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Irvine, CA, USA). A 4.5 µl RNaseA stock solution (100 mg/ml) was added with the mixture and vortexed vigorously. The mixture was incubated at 65 °C for 15 min and mixed 2 - 3 times during incubation by inverting the tubes. After cooling down at room temperature, a 165 µl buffer P3 was added to the lysate, mixed and incubated at -20 °C for 8 min. The lysate was centrifuged at 20,000 *g* for 3 min to remove the ceramic beads and leaf-debris. Following steps are same as described in Materials and Methods in Chapter 4. The concentration and purity of DNA were estimated spectrophotometrically and by running 1.8% agarose gel (**Figure 5.1**). The DNA with a concentration of 60 - 190 ng/µl, and A_{260}/A_{280} and A_{260}/A_{230}

absorbance ratio of 1.8 - 1.9 and 2.1 - 2.4, respectively was used directly to assess epigenetic variation (global DNA methylation) of SC and TC blueberry plants.

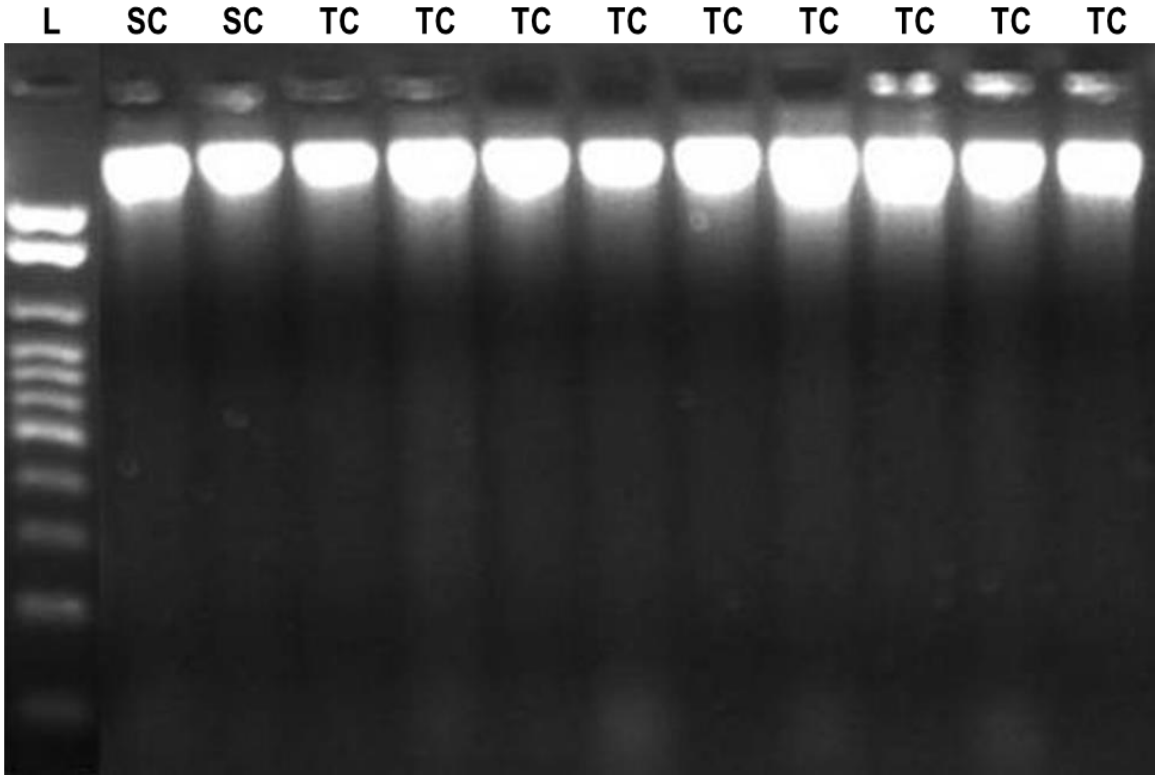

Figure 5.1 Agarose gel electrophoresis of genomic DNA isolated from leaf tissues of softwood cutting (SC) and tissue culture (TC) derived blueberry clone 'QB9C'. L = LowRanger 100 bp DNA ladder (Norgen Bioteck Corp., Thorold, ON, Canada).

5.2.3 Methylation sensitive amplification polymorphism (MSPA) assay

Before assessing DNA methylation, the genetic stability of *in vitro* regenerates of both genotypes was confirmed using EST-SSR (Goyali et al., 2015a) and EST-PCR (Chapter 4) markers. The MSAP technique was adapted by Reyna-López et al. (1997) who modified

the original AFLP technique for DNA fingerprinting (Vos et al., 1995) to incorporate methylation sensitive restriction enzymes. In the adapted protocol, the isoschizomer pair *Msp*I and *Hpa*II was used instead of *Mse*I as the 'frequent cutter' enzymes, while the 'rare/hexa cutter' was *Eco*RI. *Msp*I and *Hpa*II endonucleases are specific to the same recognition sites 5′-CCGG-3′ but respond differently when any cytosine of the site is methylated. MSAP analysis was carried out following the modified method by Fulneček and Kovařík (2014) who recommended to add a set of reaction mixture of three restriction enzymes of *Eco*RI+*Msp*I+*Hpa*II to cleave DNA of each sample in addition to *Eco*RI+*Msp*I and *Eco*RI+*Hpa*II digestions. The sequences of double stranded DNA fragments, referred to as the adapters, and pre-amplification and selective amplification primers for the *Eco*RI and *Msp*I/*Hpa*II ends were the same as those were described in (Baurens et al., 2008; Schellenbaum et al., 2008; Agboola et al., 2012) (**Table 5.1**). All the adapter and primer sequences were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

5.2.3.1 Restriction of DNA

For each plant sample, 900 - 1100 ng DNA was cleaved with 3 U of *Eco*Rl restriction endonuclease (#FD0274, Thermo Scientific, Waltham, MA, USA) in a 75 µl reaction volume containing 3× FastDigest buffer for 1.5 h at 37 °C. *Eco*Rl enzyme was inactivated by incubating at 65 °C for 10 min. Restricted DNA was then divided into three separate aliquots. One aliquot was treated with 2 U *Msp*I (#FD0544, Thermo Scientific), second aliquot with 2 U *Hpa*II (#FD0514) and third aliquot was cleaved with 2 U each of *Msp*I and *Hpa*II restriction endonucleases in a total volume of 50 µl containing 1× corresponding

Table 5.1 Name and sequence of adapters, pre-amplification and selective amplification primers used in methylation sensitive amplification polymorphism analysis of softwood cutting and micropropagated 'QB9C' and 'Fundy' blueberry genotypes (the overhanging nucleotides of the adapters are indicated in italics)

Name	Nucleotide sequences
*Eco*RI adapter	5′-*CT*GTAGACTGCGTACC-3′ 3′-CATCTGACGCATGG*TTAA*-5′
*Msp*I-*Hpa*II adapter	5′-*GA*TCATGAGTCCTGCT-3′ 3′-AGTACTCAGGACGA*GC*-5′
Pre-amplification primers	
*Eco*R1 (E)	5 ′-GAC TGC GTA CCA ATT CA-3′
*Msp*I-*Hpa*II (MH)	5′-ATC ATG AGT CCT GCT CGG-3′
Selective amplification primers	
E-TT (E1)	5′-GAC TGC GTA CCA ATT CAT T-3′
E-TG (E2)	5′-GAC TGC GTA CCA ATT CAT G-3′
MH-ATG (MH1)	5′- ATC ATG AGT CCT GCT CGG ATG-3′
MH-AAC (MH2)	5′-ATC ATG AGT CCT GCT CGG AAC-3′
MH-AAG (MH3)	5′- ATC ATG AGT CCT GCT CGG AAG-3′
MH-ACA (MH4)	5′- ATC ATG AGT CCT GCT CGG ACA-3′
MH-ATT (MH5)	5′- ATC ATG AGT CCT GCT CGG ATT-3′
MH-TCC (MH6)	5′- ATC ATG AGT CCT GCT CGG TCC-3′
MH-AAT (MH7)	5′- ATC ATG AGT CCT GCT CGG AAT-3′
MH-TCG (MH8)	5′- ATC ATG AGT CCT GCT CGG TCG-3′

buffer for 3 h at 37 °C. Denaturation of enzymes was carried out after digestion by incubating at 65 °C for 15 min. The tubes were centrifuged briefly and let them cool to room temperature. An 8 µl cleaved DNA mix was transferred to check restriction status by agarose (1.8%) gel electrophoresis (**Figure 5.2**).

5.2.3.2 Ligation of adapter

DNA fragments were then ligated to *Eco*RI and *Msp*I-*Hpa*II adapters by following Ghosh et al. (2017) with few modifications. Briefly, the following components were added with 42 µl restricted DNA mixture: 10 pmol *Eco*RI and 100 pmol *Msp*I-*Hpa*II oligonucleotide adapters (**Table 5.1)**, 2.5 Weiss units T4 DNA ligase (#EL0014, Thermo Scientific), 10 µl 10× T4 DNA ligase buffer, 2 µl 50% (w/v) polyethylene glycol solution and sterile water to the volume of 100 µl. Ligation was carried out at 23 °C for 5 h before inactivation of enzymes at 65 °C for 10 min.

5.2.3.4 Pre-amplification

The resulting ligation products were used as templates for the pre-amplification reactions. A 4 µl of template DNA fragments was amplified by polymerase chain reaction (PCR) with the *Eco*RI (E; forward) and *Msp*I-*Hpa*II (MH; reverse) primers, which were complementary to the *Eco*RI and *Msp*I-*Hpa*II adapters, in a volume of 50 µl containing a final concentration of 1× PCR buffer (1.5 mM $MgCl_2$, pH 8.7; Qiagen), 200 µM of each dNTP, 0.2 µM of each primer and 1 U of Top *Taq* DNA polymerase (Qiagen). DNA was amplified in a Mastercycler ep Gradient S (Eppendorf AG, 22331 Hamburg, Germany)

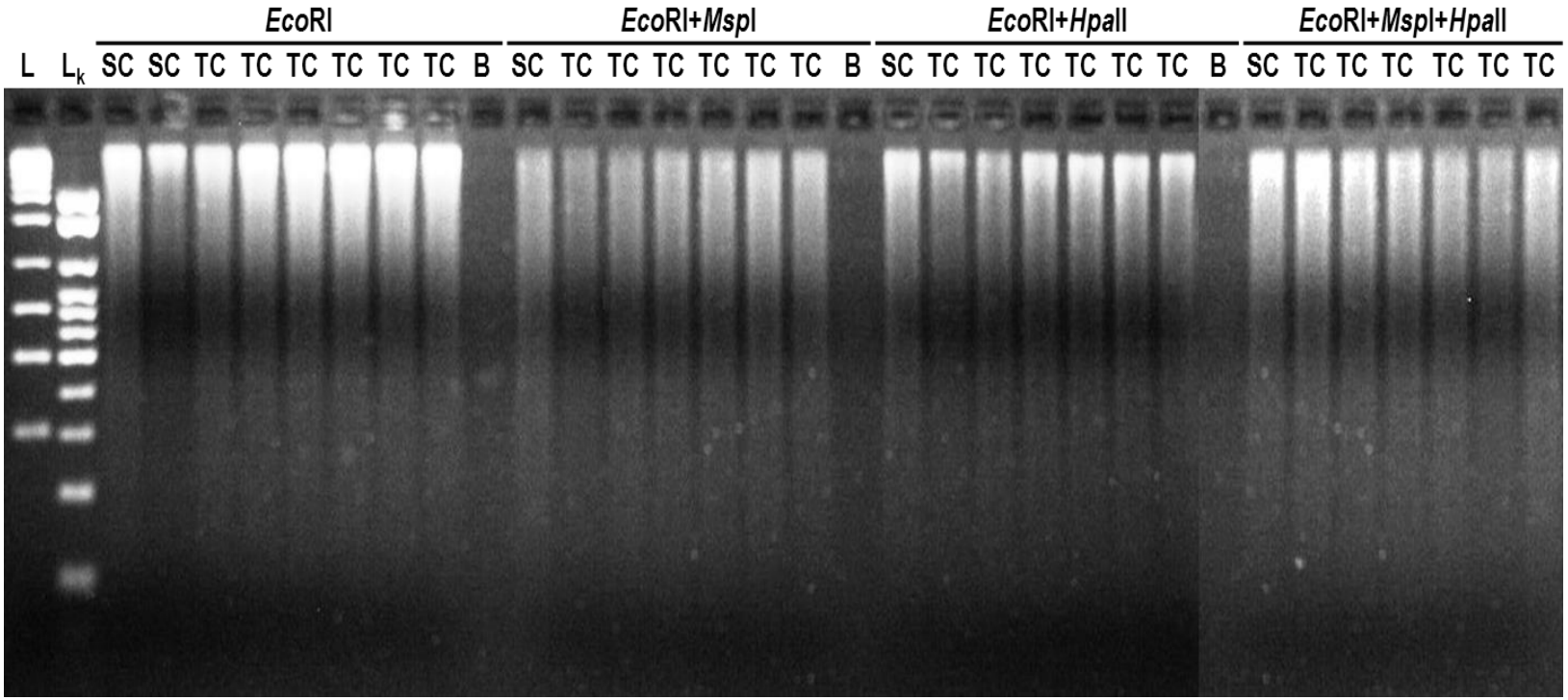

Figure 5.2 Agarose gel electrophoresis of DNA fragments of softwood cuttings (SC) and tissue culture (TC) derived plants of 'QB9C' blueberry clone restricted with *Eco*RI, combinations of *Eco*RI+*Msp*I, *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II endonuclease enzymes. B = blank with PCR master mix and water instead of DNA templates; L = LowRanger 100 bp DNA ladder and L_k = MidRanger 1 kb DNA ladder (Norgen Bioteck Corp., Thorold, ON).

with the following cycling parameters: 65 °C for 2 min, 94 °C for 5 min followed by 25 cycles of 30 s denaturing at 94 °C, 70 s annealing at 56 °C and 2 min extension at 72 °C, ending with 10 min at 72 °C to complete extension. Pre-selective PCR products were checked by electrophoresis in 1.8% agarose gels (visible as a smear from 100 to 1000 bp; **Figure 5.3**) before those were diluted to 7 times with 0.1× TE buffer and stored at -20 °C before using for selective amplification.

5.2.3.5 Selective amplification

Selective amplifications of the diluted pre-amplified products were conducted using a total of 16 primer combinations obtained with two *Eco*RI primers having two selective bases as forward primers and eight *Msp*I-*Hpa*II primers having three selective bases as reverse primers (**Table 5.1**). A 4 µl pre-amplified product was amplified in a 25 µl total reaction volume containing 1× PCR buffer, 0.4 µM of each selective primer, 400 µM of dNTPs and 1 U of Top *Taq* DNA polymerase (Qiagen). The amplification reactions were performed using the touch-down cycles with the following profile: 94 °C for 5 min; 13 cycles of 94 °C for 30 s, 65 °C for 1 min reduced by 0.7 °C per cycle, and 72 °C for 2 min followed by 23 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 2 min with a final extension step of 10 min at 72 °C (Ghosh et al., 2017).

5.2.3.6 MSAP electrophoresis

The selective PCR product (8 µl) was checked by 1.8% agarose [3:1 HRB high resolution

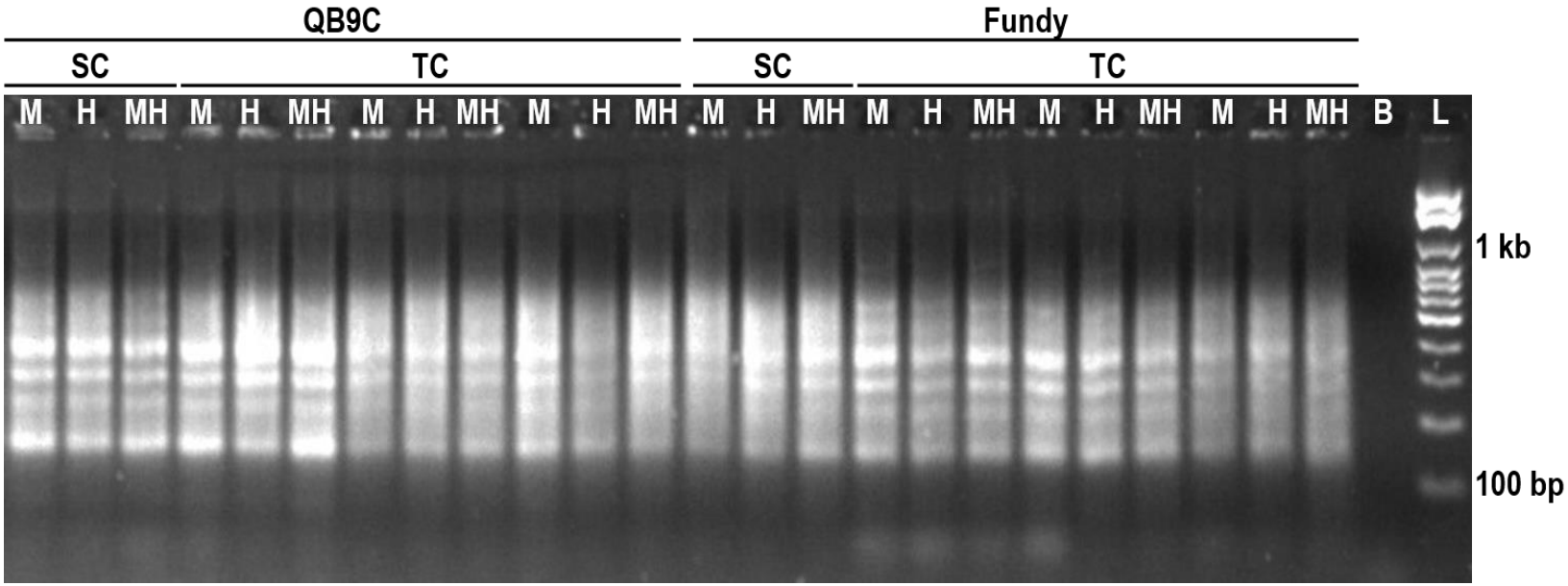

Figure 5.3 Visualization of pre-selective amplification products using agarose gel electrophoresis. DNA samples were isolated from leaf tissues of softwood cutting (SC) and micropropagated (TC) 'QB9C' and 'Fundy' blueberry genotypes. B = blank with PCR master mix and water instead of DNA templates; L = LowRanger 100 bp DNA ladder. M, H and MH refer to digestion with *Eco*RI+*Msp*I, *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II, respectively. The size of fragment smear is indicated on the right in base pair (bp).

blend (Ameresco, Solon, OH, USA)] gel electrophoresis for the presence of amplification (**Figure 5.4**) before final analysis using 6% denaturing polyacrylamide gel electrophoresis following Portis et al. (2004) with some modifications. Polyacrylamide gels (10 cm × 18 cm × 0.75 mm) were prepared by adding 225 µl 20% (w/v) ammonium persulfate (APS) and 45 µl TEMED to 50 ml polyacrylamide sequencing gel solution [6% (w/v) of acrylamide/bis-acrylamide (37.5:1), 8 M urea in 1× TBE buffer] which was mixed and immediately dispensed between glass plates held between assemblies. After polymerization (about 35–40 min after), running buffer (1× TBE) was poured to submerge the gels. Equal volume of final selective PCR product was mixed with denaturing formamide dye [98% formamide, 10 mM EDTA (pH 8), 0.01% (w/v) bromophenol blue and 0.01% (w/v) xylene cyanol] and denatured at 95 °C for 4 min and immediately cooled by keeping at -20 °C for 5 min. The gels were pre-run at 85 V for about 45 min and the wells were cleaned before 10 µl of the mixture was loaded. Gels were run at 65 V for about 4 h, and visualized via silver staining method adopted from Brant and Peter (2007)**.**

5.2.3.7 Silver staining

The amplified DNA fragments were fixed in the gel with 7.5% (v/v) acetic acid for 15 min and the gels were washed thrice for 3 min each with a large quantity of deionized double distilled water collected from Barnstead Mega-Pure D2 system (Thermo Scientific). Following fixation, the gels were pretreated with 15% (v/v) freshly prepared formaldehyde solution for 10 min. This step is important for stain sensitivity and maximum image contrast. The gels were submerged in a silver impregnation solution [0.1% (w/v) silver

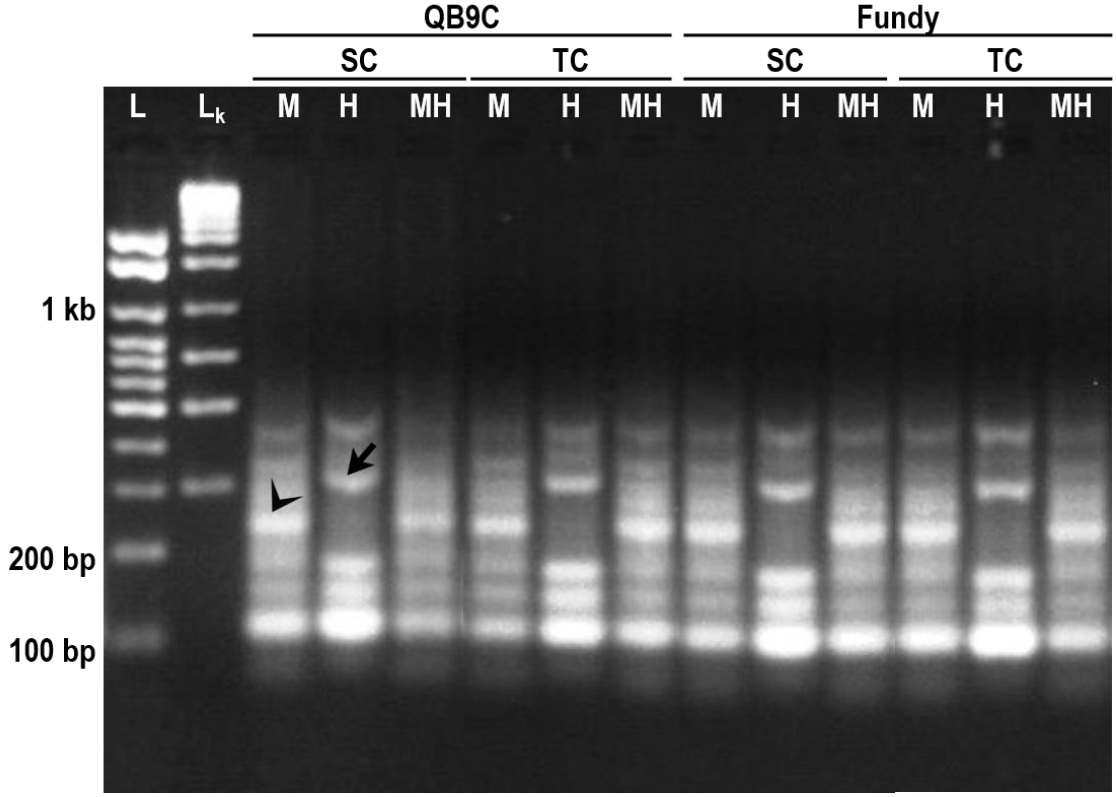

Figure 5.4 Banding pattern of selective amplification products amplified by using E-TT/MH-ATG primer combination and visualized in agarose gel electrophoresis. DNA samples were isolated from leaf tissues of softwood cutting (SC) and tissue culture originated (TC) plants of 'QB9C' and 'Fundy' blueberry genotypes. L = LowRanger 100 bp DNA ladder and L_k = MidRanger 1 kb DNA ladder. M, H and MH refer to restriction with *Eco*RI+*Msp*I, *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II, respectively. DNA bands (marked by arrowhead) found in M digestion lane but not in H and vice versa indicate cytosine methylation.

nitrate] for 25 min after the formaldehyde solution was decanted. The gels were briefly rinsed with 200 ml deionized double distilled water for 5–10 s to remove residual silver solution from the gel surfaces. All the above steps were performed on Rocker 25 shaker (Labnet International, Edison, NJ, USA) with slow agitation (once very 2–3 s). Following silver impregnation, image development was carried out in freshly prepared cold (≤ 10 °C) developing solution (32 g/L sodium carbonate and 4 g/L sodium thiosulfate) with manual agitation for 1–3 min until the image development was begun. To stop development and fix the gels, 7.5% (v/v) cold (≤ 10 °C) acetic acid was added after decanting the developing solution and incubated with shaking for 10 min. The gels were then rinsed briefly with deionized water and digitally photographed using the InGenius 3 gel documentation system (Syngene, Frederick, MD, USA). The 50 bp ladder (New England Biolabs Ltd. Whitby, ON, Canada) was used as molecular size marker. The fragments at the upper part (above 1000 bp) and the lower part (below 50 bp) of the gels with poor resolution were not used for band scoring because those were beyond template size. The reproducibility of the methylation patters was confirmed by repeating the experiments twice. The DNA fragments showing reproducible results between replicates were scored for MSAP data analysis.

5.2.4 Profiling, scoring and data analysis

The methylation status was detected by comparing the DNA profiles of tetranucleotide restriction sites (5′-CCGG-3′) at *Eco*RI+*Msp*I and *Eco*RI+*Hpa*II reaction lanes based on the presence or absence of DNA bands in those lanes. To facilitate comparison, the

amplified fragments of a single plant obtained from DNA restricted with the combinations of endonucleases *Eco*RI+*Msp*I, *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II were loaded in three lanes in a gel next to each other and analyzed the banding pattern according to Park et al. (2009). The isoschizomers *Msp*I and *Hpa*II cleaved their restriction sites when the sites were non-methylated, and similar DNA fragment profiles were appeared in all three lanes (Type I). *Msp*I cleaved hemi- (mC in one DNA strand only) or fully- methylated internal cytosine (5′-CmCGG-3′) but not outer cytosine (5′-mCCGG-3′) sequences (Reyna-López et al., 1997). The band(s) was present in *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II lanes but absent in *Eco*RI+*Msp*I when the external cytosine (5′-mCCGG-3′) of one strand was methylated (Type II). DNA band(s) was detected in the *Eco*RI+*Msp*I reaction lane but disappeared from the *Eco*RI+*Hpa*II reaction lane (Type III) when the internal cytosine was methylated. However, any extra *Hpa*II bands present in *Eco*RI+*Hpa*II but absent in other two lanes was not detected for the digestion of hemi-methylated external cytosine (5′-mCCGG-3′). It indicated the presence of an internal cytosine methylation (5′-CmCGG-3′) between the cleaved distal 5′-CCGG-3′ site and the *Eco*RI site (Fulneček & Kovařík, 2014). A DNA methylation event was considered to be polymorphic when a band was present in *Eco*RI+*Msp*I reaction lane in one plant but was not found in *Eco*RI+*Msp*I lane in other plants; or a band was present in *Eco*RI+*Hpa*II in one plant but was not found in *Eco*RI+*Hpa*II lane in other plants. Percentage methylation and methylation polymorphism were calculated as below:

$$\text{Methylation (\%)} = \frac{\text{number of methylated bands}}{\text{total number of bands}} \times 100$$

Methylation polymorphisim (%)

$$= \frac{\text{number of polymorphic methylated bands}}{\text{total number of methylated bands}} \times 100$$

However, in any sample, if no bands were detected in both digestions, this was not considered as a polymorphism.

5.3 Results

In the present study, sixteen combinations of selective *Eco*RI and *Msp*I/*Hpa*II primers were used and the number of non-methylated, hemi-methylated and fully methylated cytosine at 5′-CCGG-3′ restriction sites were calculated in SC and TC plants of a wild clone 'QB9C' and the cultivar 'Fundy'. The DNA methylation profiles were explained regarding fragment polymorphism patterns among three adjacent digestion lanes for each plant. A total of 106 fragments of different sizes were amplified from the leaf tissues of ten SC 'QB9C' plants, and 105 fragments from the same number of TC 'QB9C' plants; each of the fragments represented a recognition site cleaved by one or both of the *Msp*I/*Hpa*II (**Table 5.2**). Twenty six fragments (24.5%) from SC 'QB9C' plants were differentially amplified due to methylation of the internal cytosine of restriction sites (5′-CmCGG-3′) – resulting in cleavage by *Msp*I, but not by *Hpa*II, or due to hemi-methylation of external cytosine – leading to restriction by *Hpa*II, but not by *Msp*I. Methylation event detected in TC 'QB9C' plants was 30 (28.6%). In the cultivar 'Fundy', total of 107 and 109 fragments were detected from SC plants and TC plants, respectively. The DNA from SC and TC plants of 'Fundy' showed a similar degree of methylation as 'QB9C' wild clone: 20 out of

107 differentially amplified fragments were detected in SC and 22 out of 109 observed in TC plants, respectively (**Table 5.2**). Due to the differential recognition by two isoschizomers, 18.7% of 5′-CCGG-3′ sites were methylated in SC and 20.2% TC in plants of 'Fundy' cultivar, respectively. The 'QB9C' TC plants produced the highest level of cytosine methylation at 5′-CCGG-3′ restriction sites and SC 'Fundy' presented the lowest level of cytosine methylation. Representative global DNA methylation events in SC and TC plants of 'QB9C' and 'Fundy' genotypes are shown in **Figure 5.4** (visualized in agarose gel) and **Figure 5.5** (visualized in polyacrylamide gel). The recognition sites (5′-CCGG-3′) were cleaved by *Msp*I (lane M; marked by arrowhead) and DNA fragments were amplified (present band) but those were not cleaved by *Hpa*II (absent band). Conversely, the recognition sites were cleaved by *Hpa*II (lane H; marked by arrow) and fragments were appeared as clear band which were absent in lane M. Primer pair-wise detailed methylation events were shown in **Table 5.3** for 'QB9C' and **Table 5.4** for 'Fundy'.

Compared with the SC plants, micropropagated plants showed higher percentage of methylation alterations in both blueberry genotypes studied. Of the total 26 methylated fragments in SC 'QB9C' plants, 11 (42.3%) fragments were generated due to hemi-methylation of external cytosine of recognition sites (5′-mCCGG-3′) and 15 (57.7%) were due to fully methylation of internal cytosine (5′-CmCGG-3′). Eleven fragments (36.7%) were generated from cleavage only by *Hpa*II due to the hemi-methylation of external cytosine (5′-mCCGG-3′) in the TC 'QB9C' plants. Nineteen (63.3%) fragments were produced from cleavage by *Msp*I but not by *Hpa*II indicating full methylation of the internal cytosine (5′-CmCGG-3′). In SC and TC plants of 'Fundy', 30.0% and 31.8% of

Table 5.2 Summary of total number of bands, number and percentage (%) of DNA methylation events detected by methylation sensitive amplification polymorphism (MSAP) technique using sixteen selective primer combinations in ten lowbush blueberry plants each from softwood cutting (SC) and tissue culture (TC) propagation methods

Genotypes	Number of bands in SC plants					Number of bands in TC plants				
	Total bands	[a]Type I (%)	Type II (%)	Type III (%)	Total methylation (Type II+Type III)	Total bands	Type I (%)	Type II (%)	Type III (%)	Total methylation (Type II+Type III)
QB9C	106	80 (76.5)	11 (42.3)	15 (57.7)	26 (24.5)	105	75 (71.4)	11 (36.7)	19 (63.3)	30 (28.6)
Fundy	107	87 (81.3)	6 (30.0)	14 (70.0)	20 (18.7)	109	87 (79.8)	7 (31.8)	15 (68.2)	22 (20.2)

[a]Bands present in all three lanes of *Eco*RI+*Msp*I, *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II (Type I), in *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II lanes but not in *Eco*RI+*Msp*I lane (Type II), and in *Eco*RI+*Msp*I and *Eco*RI+*Msp*I+*Hpa*II lanes but not in *Eco*RI+*Hpa*II lane (Type III). Type I - non-methylated 5′-CCGG-3′ sites, Type II - hemi-methylated external cytosine of recognition sites (5′-mCCGG-3′) and Type III - fully-methylated internal cytosine of recognition sites 5′-CmCGG-3′ sites.

The recognition sites, respectively, were hemi-methylated at the external cytosine whereas, fully methylated at the internal cytosine was observed for 70.0% and 68.2%, respectively. However, for any plant studied, no extra *Hpa*II band was detected in *Eco*RI+*Hpa*II lane which was absent in other two lanes (*Eco*RI+*Msp*I and *Eco*RI+*Msp*I+*Hpa*II) of same plant. It proved absence of recognition site having a methylated internal cytosine (5′-CmCGG-3′) between the cleaved distal 5′-CCGG-3′ site and the *Eco*RI site (Fulneček & Kovařík, 2014). TC plants of both genotypes showed slightly higher level of methylation at the internal and external cytosines of the 5′-CCGG-3′ recognition sites compared to those in SC plants (**Table 5.2**). Overall the level of DNA methylation of blueberry genome due to the full methylation of the internal cytosine of the recognition sites was relatively higher than those of due to hemi-methylation of external cytosine.

The same methylation sites among the plants within same treatment were characterized as monomorphic with regards to cytosine methylation which were detected in both SC and TC derived plants of both genotypes 'QB9C' and 'Fundy'. However, differential methylation patterns were observed among the TC plants of both genotypes. Individual primer pair-wise methylation polymorphisms among the TC plants of 'QB9C' and 'Fundy' detected by differentially amplified fragment(s) in *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I digestions are shown in **Table 5.5.** 5 out of 16 primer combinations detected 5 methylation polymorphic sites in 'QB9C' wild clone and those were 3 in 'Fundy' cultivar. Representative methylation polymorphism detected by using the primer combination of E-TT/MH-ATG is shown in **Figure 5.6**. Polymorphism was detected in TC plant #1 from 'QB9C' at three sites (encircled): polymorphic three bands between 100 and 50 bp were

Table 5.3 Primer pair-wise DNA methylation events detected by methylation sensitive amplification polymorphism (MSAP) technique in ten plants of 'QB9C' blueberry clone each from softwood cutting (SC) and tissue culture (TC) propagation methods

Primer combinations	No. of methylated 5′-CCGG-3′ sites in SC plants			No. of methylated 5′-CCGG-3′ sites in TC plants		
	Type I	Type II	Type III	Type I	Type II	Type III
E1-MH1	7	2	1	6	3	4
E1-MH2	4	0	0	4	0	0
E1-MH3	4	2	1	4	2	1
E1-MH4	8	0	1	8	0	1
E1-MH5	8	0	2	7	1	0
E1-MH6	2	0	1	2	0	1
E1-MH7	6	1	1	6	1	1
E1-MH8	6	0	1	5	0	2
E2-MH1	4	0	0	4	0	0
E2-MH2	6	1	1	6	1	1
E2-MH3	2	2	2	2	2	2
E2-MH4	4	0	0	4	0	0
E2-MH5	4	0	2	4	0	2
E2-MH6	3	3	1	3	1	2
E2-MH7	6	0	1	4	0	2
E2-MH8	6	0	0	6	0	0
Total	80	11	15	75	11	19

[a]Bands present in all three lanes of *Eco*RI+*Msp*I, *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II (Type I), in *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II lanes but not in *Eco*RI+*Msp*I lane (Type II) and in *Eco*RI+*Msp*I and *Eco*RI+*Msp*I+*Hpa*II lanes but not in *Eco*RI+*Hpa*II lane (Type III). Type I - non-methylated sites, Type II - hemi-methylated external cytosine and Type III - fully-methylated internal cytosine.

Table 5.4 Primer pair-wise DNA methylation events detected by methylation sensitive amplification polymorphism (MSAP) technique in ten plants of blueberry cultivar 'Fundy' each from softwood cutting (SC) and tissue culture (TC) propagation methods

Primer combinations	No. of methylated 5′-CCGG-3′ sites in SC plants			No. of methylated 5′-CCGG-3′ sites in TC plants		
	Type I	Type II	Type III	Type I	Type II	Type III
E1-MH1	9	1	0	11	1	2
E1-MH2	4	0	0	4	0	0
E1-MH3	5	1	2	5	1	2
E1-MH4	9	0	2	9	0	2
E1-MH5	8	0	2	7	1	0
E1-MH6	2	0	1	2	0	1
E1-MH7	6	1	1	6	1	1
E1-MH8	8	0	1	8	0	2
E2-MH1	4	0	0	4	0	0
E2-MH2	3	1	0	3	1	0
E2-MH3	5	0	0	5	0	0
E2-MH4	4	0	0	4	0	0
E2-MH5	4	0	2	4	0	2
E2-MH6	6	2	2	5	2	1
E2-MH7	6	0	1	4	0	2
E2-MH8	4	0	0	6	0	0
Total	87	6	14	87	7	15

[a]Bands present in all three lanes of *Eco*RI+*Msp*I, *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II (Type I), in *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II lanes but not in *Eco*RI+*Msp*I lane (Type II) and in *Eco*RI+*Msp*I and *Eco*RI+*Msp*I+*Hpa*II lanes but not in *Eco*RI+*Hpa*II lane (Type III). Type I - non-methylated sites, Type II - hemi-methylated external cytosine and Type III - fully-methylated internal cytosine.

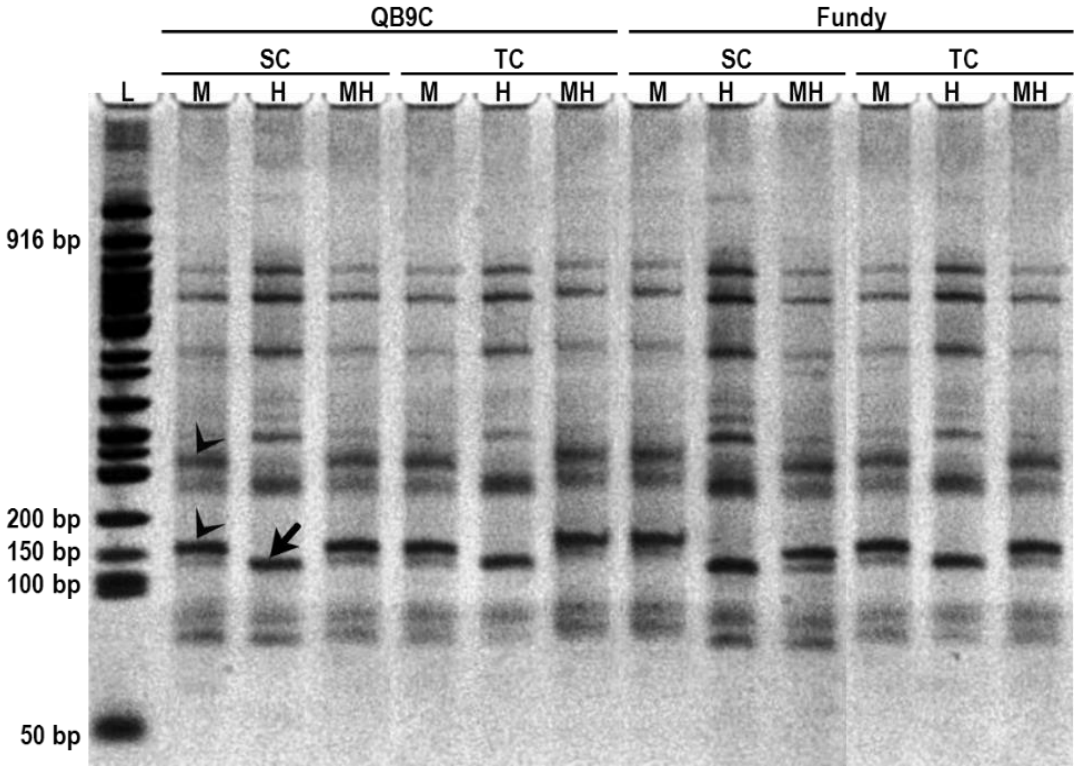

Figure 5.5 Example of DNA methylation pattern observed in 'QB9C' and 'Fundy' blueberry genotypes propagated by softwood cutting (SC) and tissue culture (TC). Selective amplification was carried out using E-TG/MH-TCC primer combination. DNA bands (marked by arrowheads) present in M digestion lanes but not in H lanes indicate fully methylated internal cytosine at 5′-CCGG-3′ recognition site, and DNA bands (marked by arrows) present in H digestion lanes but not in M lanes indicate hemi-methylated external cytosine of 5′-CCGG-3′ sites in genomic DNA. L = 50 bp DNA ladder (New England Biolabs Ltd. Whitby, ON). M, H and MH refer to DNA fragments originated from digestion with the combinations of *Eco*RI+*Msp*I, *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II, respectively (detailed in Table 5.3 and Table 5.4).

disappeared from the lane H of one TC plant (3rd lane from left) but those were present in other TC 'QB9C' plants. A total of 4.76% DNA methylation polymorphisms were detected in TC 'QB9C' plants and those were 2.75% in TC 'Fundy' plants (**Table 5.5**). DNA methylation polymorphism was not detected either in the SC plants of 'QB9C' or those of 'Fundy' while same primer combinations were used for same number of SC plants of both genotypes.

5.4 Discussion

Tissue culture-induced variations, including morphological, biochemical and genetic/epigenetic alterations, have been frequently reported in different plant species (Biswas et al., 2009; Bairu et al., 2011b; Krishna et al., 2016). However, the mechanism behind this variation is still unclear. Alterations in DNA methylation was detected in many plant species indicating that epigenetic variations play a vital role (Schellenbaum et al., 2008). Recent works pointed out the possible interactions of both genetic and epigenetic changes induced by the plant tissue culture process (Guo et al., 2007; Li et al., 2007; Linacero et al., 2011). These changes may or may not affect the phenotypic variations.

In the present study, MASP analysis based on the sensitivity of *Msp*I and *Hpa*II isoschizomer pair to differential cytosine methylation state on their recognition site, 5′-CCGG-3′ enabled investigation of global DNA methylation of blueberry genome. *Msp*I/*Hpa*II endonucleases produced different scorable DNA fragments while selective amplification was carried out using sixteen primer combinations. According to principle of MSAP (Fulneček & Kovařík, 2014), DNA digested with the combinations of *Eco*RI and

Table 5.5 Polymorphisms in cytosine methylation at 5′-CCGG-3′ recognition site detected by methylation sensitive amplification polymorphism (MSAP) technique in leaves of micropropagated lowbush blueberry plants (*n* = 10)

Primer combinations	Digestion with	Total number of methylated sites		Methylation polymorphism	
		QB9C	Fundy	QB9C	Fundy
E1-MH1	*Eco*RI+*Msp*I	4	2	0	0
	*Eco*RI+*Hpa*II	3	1	3	0
E1-MH4	*Eco*RI+*Msp*I	1	2	0	0
	*Eco*RI+*Hpa*II	0	0	1	0
E1-MH6	*Eco*RI+*Msp*I	1	1	0	1
	*Eco*RI+*Hpa*II	0	0	0	0
E2-MH5	*Eco*RI+*Msp*I	2	2	0	0
	*Eco*RI+*Hpa*II	0	0	0	1
E2-MH6	*Eco*RI+*Msp*I	2	1	0	0
	*Eco*RI+*Hpa*II	1	2	1	1
Total		14	11	5	3

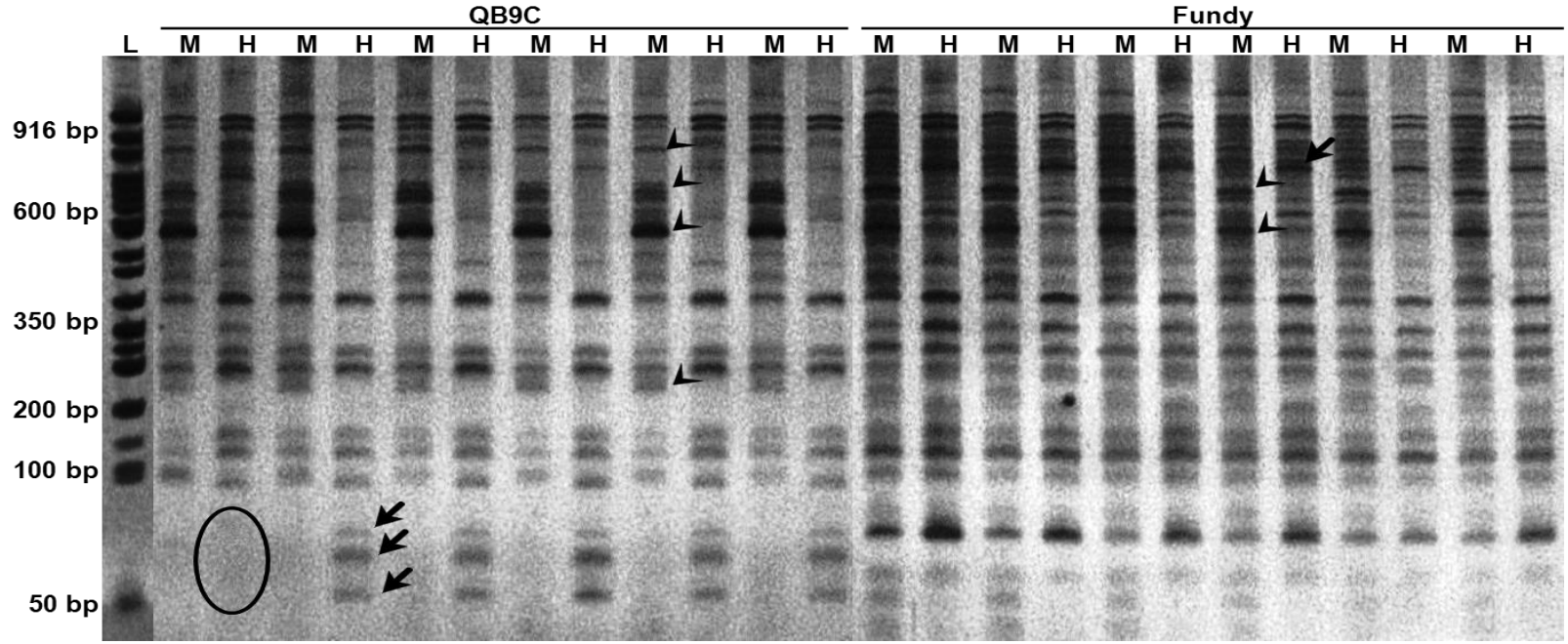

Figure 5.6 Example of methylation sensitive amplification polymorphism (MSAP) profiles in micropropagated plants of 'QB9C' and 'Fundy' blueberry genotypes obtained by using the primer combination of E-TT/MH-ATG. The 50 bp ladder (L) was used as molecular size marker. M and H refer to digestion with *Eco*RI+*Msp*I and *Eco*RI+*Hpa*II, respectively. DNA bands (marked by arrowheads) present in M digestion lanes but not in H lanes indicate cytosine methylation. Banding patterns (marked by arrows) present in H lanes which is absent in one plant (encircled) indicate DNA methylation polymorphisms in micropropagated 'QB9C' plants (detailed in Table 5.5).

one or both of the isoschizomers *Msp/Hpa*II produced three classes of DNA fragments: i) *Msp*I and *Hpa*II recognized non-methylated restriction site (5′-CCGG-3′) for which identical DNA fragments appeared in all three lanes (Type I), ii) *Hpa*II specific fragments attributed to represent hemi-methylated external cytosine (5′-mCCGG-3′) were present in both *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I+*Hpa*II lanes (Type II); and iii) *Msp*I specific fragments resulted from digestion of internal cytosine (5′-CmCGG-3′) in the recognition sites were appeared in *Eco*RI+*Msp*I and *Eco*RI+*Msp*I+*Hpa*II lanes (Type III). Cytosines at the recognition sites in blueberry genome was mostly non-methylated, roughly from 71% (TC 'QB9C' plants) to 81% (SC 'Fundy' plants) which is agreement with Baurens et al. (2003) report in banana with about 80% cytosines of the 5′-CCGG-3′ recognition sites were non-methylated. The higher levels of cytosine methylation in micropropagated blueberry plants in both genotypes compared to conventionally propagated plants (**Table 5.2),** have similarity with the previous reports on established micropropagated banana (Peraza-Echeverria et al., 2001) and orchid (*Doritaenopsis*) (Park et al., 2009) plants. In TC regenerated maize plants hypomethylation was detected (Kaeppler & Phillips, 1993). In blue agave (*Agave tequilana* L.), however, both increase and decrease of methylation was found in micropropagated plants (Díaz-Martínez et al., 2012), while in carrot root culture, *de novo* methylation was taken place (Arnholdt-Schmitt, 1993). Low level of methylation at the external cytosine and high level of methylation at internal cytosine in blueberry genome is agreement with previous studies in grape (Schellenbaum et al., 2008), banana (Peraza-Echeverria et al., 2001) and Barbados nut (Rathore & Jha, 2016). The comparison of mother plant and TC-regenerated grape vines using MSAP technique

revealed that higher cytosine methylation level in regenerants compared to mother clones was mainly due to a high level in full methylation of the internal cytosines (Schellenbaum et al., 2008). Conversely, Baurens et al. (2003) reported low internal cytosine methylation and high external cytosine methylation at 5′-CCGG-3′ sites in micropropagated banana plants.

Programmed gene expression is crucial for the normal development of all plant species. The cells with same DNA play different role and identity during developmental processes (Rathore et al., 2015). The specific interaction between external stimuli and plant developmental program coordinates the gene expression which determines the adaptability of a plant species under the usual environmental conditions. Developmental process could be affected by misregulation of a key regulatory gene or through the epigenetic modification of many genes within the same pathway by cytosine methylation resulting changes in gene expression (Attwood et al., 2002). During tissue culture process, explants changes its differentiation status in the media supplemented with plant growth regulators. Generally, plant cells are dedifferentiated from organized tissues such as the nodal region, shoot or root tip or leaf to totipotent cells to grow as callus, and followed by re-differentiation of callus is carried out to produce tissues or organs (Huang et al., 2012a; Rathore & Jha, 2016). Since the process bypasses the normal developmental events in a stressful environment, it is possible to be occurred numerous genetic and epigenetic alterations involving single gene mutations, chromosome breakages and transposable element activations and modifications of normal DNA methylation patterns which is account for gene expression and phenotypic differences in regenerated plants (Kaeppler et

al., 2000). Differential expression levels of DNA methyltransferase genes under *in vitro* conditions, difference in sensitivity of DNA modification or methylation site to phytohormones used in culture media are reported for the methylation changes in regenerated plants (Vlasova et al., 1995; Taskin et al., 2015). In the present study, TC plants showed higher methylation that could be the effect of growth regulator zeatin used in media (Debnath, 2007b). LoSchiavo et al. (1989) and Huang et al. (2012a) reported that the global DNA methylation was increased with higher concentration of auxin 2,4-D in culture media in carrot and apple micropropagation. Bucherna et al. (2001) reported that DNA methylation is higher in suspension cultures of eggplant when these cultures were maintained in the presence of cytokinins rather than auxins. Conversely, Ghosh et al. (2017) reported higher concentration of cytokinin (thidiazuron) in culture media decreased the global DNA methylation in callus of blueberries.

Plant growth hormones play a significant role in mediating the signal transduction cascade leading to the reprogramming of gene expression which involved an epigenetic variation especially cytosine methylation in stressful tissue culture environment. The growth regulator causes changes in chromatin and chromosome structure (Pavlica et al., 1991). DNA hyper-methylation in this study may be the characteristic of constitutive heterochromatin. Alterations in chromatin compaction change transcriptional machinery from accessing DNA, thereby affecting gene regulation and silencing the genes in heterochromatic regions (Grant-Downton & Dickinson, 2005). DNA methylation may lead to changes in recombination rates, and variations in the timing or initiation of DNA replication, perhaps leading to chromosome breakage (Phillips et al., 1994). The alteration

in fully methylated and hemi-methylated sequences suggests that many coding regions may be affected through changes in promoter regions. Some plant promoter sequences contain clusters of CG dinucleotides (CG islands) and the methylation of CG dinucleotides in the promoter region has been shown to influence the expression of a reporter gene (Pradhan et al., 1999). The present study has provided further evidence that methylation changes occur in tissue culture originated matured plants which are genotype specific. The cultivar 'Fundy' expressed lower cytosine methylation in genomic DNA in leaf tissue than the wild clone 'QB9C' did. Ghosh et al. (2017) reported that global DNA methylation in somatic callus of blueberry varied significantly among the genotypes. Nimmakayala et al. (2011) and Mastan et al. (2014) found genotype wise epigenetic especially methylation specific diversity in watermelon and *Jatropha curcas*, respectively.

The occurrence of DNA methylation polymorphism in micropropagated blueberry plants clearly indicated alternation in degree and pattern of DNA methylation in TC regenerants of both genotypes from the SC plants. Although genetic fidelity detected by the EST-SSR (Goyali et al., 2015a) and EST-PCR (Chapter 3) manifested genetic stability among the TC plants, the MSAP technique detected polymorphism in methylation state of regenerants. The results are agreement with those found in banana where about 3% DNA methylation events were polymorphic in micropropagated plants with no methylation polymorphism was detected in the plants propagated conventionally with sucker cuttings (Peraza-Echeverria et al., 2001). However, Ghosh et al. (2017) reported methylation polymorphism in SC-propagated greenhouse grown blueberry plants which was much lower compared to the respective callus cultures. The polymorphism in cytosine methylation among in the TC

plants might be one of the sources of tissue culture-induced variations (González et al., 2013b). Genome stability is not the “default position” but is the result of "active checks and balances" within each cell (Pardue, 1991). In regeneration process of tissue culture, a plant is regenerated from a cell and the “checks and balances” in each cell is somehow disrupted by the tissue culture process which seems to involve DNA methylation alterations. The reasons for these kinds of alterations in the genomic DNA of the regenerants might be due to significant cell re-programming and start of de novo production of hormones during tissue culture (Rathore & Jha, 2016). This kind of polymorphism could be related with activation of the transposable elements. Transposons are activated by the tissue culture process via changing methylation state of cytosine residues. Brettell and Dennis (1991) found activation of transposable element AC in *Zea mays* that was associated with methylation changes of cytosine residues. When transposition occurs, the inserted element can change the epigenetic status of the flanking sequences, modifying their expression or interrupting gene sequences (Kashkush et al., 2002; Valledor et al., 2007). Thereby gene silencing may occur which can lead to genotypic and phenotypic variations.

Plants propagated by two different methods, SC and TC in present case exhibit different degree of methylation. Naturally matured plant tissues showed the higher DNA methylation in compare to juvenile plant parts (Fraga et al., 2002). In the sense of this suggestion, TC plants might have lower methylation, since micropropagated blueberry plants having higher vegetative growth potential with higher number of rhizomes are in younger state compare to SC plants (Goyali et al., 2013). However, those plants exhibited

higher cytosine methylation in genomic DNA. Alteration in DNA methylation pattern of the nucleotide sequences in leaf tissues suggest possibility of involvement of these fragments in the dynamic processes regulating plant growth and development under prevailing growth conditions (Rathore et al., 2015). Characterization of fragments representing differentially methylated sequences could be informative to explain the hyper methylation of TC plants as it may lead to the differentially methylated genes. Changes in methylation patterns of specific genes may uncover details of the underlying regulatory mechanisms of tissue culture effect on the DNA methylation of greenhouse grown blueberry plants.

CHAPTER 6

Summary and Future Direction

Lowbush blueberries (*Vaccinium angustifolium* Ait.) are well-known for their nutritive and antioxidant metabolite content which have high potential to prevent several degenerative diseases. Not only *in vitro* but also *in vivo* research identified blueberry as a health-promoting super table fruit. Despite the high demand of lowbush blueberry due to its health benefits, its major portion is commercially harvested from wild stands and conventionally propagated farms. Micropropagation of lowbush blueberries is well-established which could be an alternative method of propagation to fulfill blueberry demand. Although tissue culture (TC) plants have enhanced morphological potential in berry crops, the development of somaclonal variation may inhibit acceptance of TC plants for commercial production. This study investigated the morphological characteristics, and the secondary metabolite content and antioxidant capacity in fruits and leaves of 7 year old softwood cutting (SC) and TC blueberry plants grown in greenhouse. A wild clone 'QB9C' and the cultivar 'Fundy' propagated by conventional SC and shoot proliferation technique using node-culture were used for this study. The antioxidant metabolites were further studies in several growing seasons and in different maturity stages of fruits. The genetic integrity of TC plants was evaluated using molecular markers. The epigenetic variation especially the global DNA methylation was detected in the plants propagated by both methods.

The TC plants were morphologically superior with higher number of rhizomes, branches and larger leaves compared to those propagated conventionally using SCs. However,

micropropgated plants are adversely affected for number of flowers and fruits, and fruit yield which were genotype specific. Morphological characters studied in three consecutive years exhibited that growing season had significant effects on those characteristics in greenhouse conditions. Although light and temperature were not controlled in greenhouse, the other environmental factors such as wind speed, incidence of snowfall, insect were different in greenhouse compared to field conditions. Therefore, further study needs to be carried out in replicated field trials over few more years to confirm the propagation potential of TC with respect to morphological characteristics in lowbush blueberries.

There is ample literature dealing with several factors, such as genotype, year of production, maturity stage and type of plant tissue which effect biochemical characteristics of plants. The effect of *in vitro* propagation on the phenolic content of blueberries is rare. In the present study, micropropagation influenced the synthesis of phenolic and flavonoid compounds, and their antioxidant activities in lowbush blueberries. However, those effects were genotype specific. Overall, wild clone 'QB9C' was highly influenced by micropropagation for the phytochemical content and its antioxidant capacity. The estimation of antioxidant metabolite content in two different plant tissues demonstrated that leaves contained substantially higher levels of polyphenolics, flavonoids and proanthocyanidins than those in the berries. Moreover, the leaves of SC plants contained all the above-mentioned phytochemicals in higher level and performed greater 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity than the leaves of micropropagated plants did. This study proved that *in vitro* propagation had tissue specific effect for phytochemical characteristics in blueberry. Micropropagated blueberry leaves

contained less antioxidants than SC leaves whereas tissue culture fruits have higher level secondary metabolites compare to SC fruits. The antioxidant metabolites estimated in three years exhibited significant effect of growing seasons on the total phenolic, flavonoid, anthocyanin and proanthocyanidin content and antioxidant activity.

Maturity stage plays an important role in phenolic synthesis. In case of leaf tissue, red leaves had higher bioactive phytochemicals and antioxidant potential than the green leaves, and both genotypes reacted similarly to the maturity stages for their phytochemical content. Green fruits had significantly higher phenolic and flavonoid content and antioxidant activity compare to semi-ripe and fully ripe berries and those were gradually decreased with the progression of ripening. In contrary, anthocyanin content increased with the advancement of fruit maturity. The propagation method responded similarly to the maturity stage for phenolic content. Green fruits from TC plants of both genotypes had higher content of phenolics than those from propagated by SC and that difference between two propagation methods lower when fruits were fully ripe. Several genes involved in biosynthesis pathways of phenolics are expressed differently at various maturity stages of blueberris. Further investigation of individual genes in flavonoid synthesis pathways of micropropagated plants will help to understand the effect of propagation methods on phytochemical content under different maturity stages of blueberry.

The genetic analysis of the micropropagated blueberry plants using EST-SSR and EST-PCR molecular marker systems showed that DNA profiles of TC plants were similar to SC plants in both genotypes which proved the genetic integrity among the plants propagated

by two different methods. Nine out of twenty EST-SSR and six out of thirteen EST-PCR primer pairs distinguished the wild clone ‘QB9C’ from the cultivar ‘Fundy’, while the monomorphic banding pattern in entire DNA profiles of all micropropagated plants of each genotype confirmed their clonal fidelity. Diverse types of DNA markers cover different sometime common sequences of genomic DNA. As many as molecular markers are used, more parts of genomic DNA will be covered. Although two DNA marker systems were used in this study, more types of marker may be used to confirm the genetic stability among micropropagated blueberry plants. Morphological variation but stable genetic feature of blueberry plants propagated by two different methods has driven to study the epigenetic variation between two propagation methods as well as among TC derived plants.

Although epigenetic variations especially global DNA methylation have been reported in several plant species which are triggered by tissue culture, DNA methylation of micropropagated blueberry plants is unavailable. Present investigation on global DNA methylation suggested that tissue culture had sustainable effect on cytosine methylation in blueberry. Micropropagated blueberry plants of both genotypes demonstrated higher global cytosine methylation compared to SC plants. Discrete methylation polymorphism was observed among the tissue culture regenerated plants in both genotypes, while no polymorphism was detected in MSAP profiles among conventionally propagated blueberry plants. Although MSAP technique detects global cytosine methylation pattern in blueberry based on the recognition sites of isoschizomer pairs, the methylation status in a specific gene or loci is undermined in MSAP analysis. In addition to global methylation analysis, methylation status of specific gene provides more information in phenotypic changes.

Bisulfite modification and characterization of the genes involved in metabolite synthesis pathways under tissue culture system will help to better understanding the correlation between DNA methylation and changes in phytochemical synthesis in blueberry plants.

CHAPTER 7

References

Aalders, L. E., Hall, I. V., & Brydon, A. C. (1979). A comparison of fruit yields of lowbush blueberry clonal lines and related seedling progenies. *Canadian Journal of Plant Science, 59*(3), 875-877. doi:10.4141/cjps79-135

Aalders, L. E., Ismail, A. A., Hall, I. V., & Hepler, P. R. (1975). Augusta lowbush blueberry. *Canadian Journal of Plant Science, 55*(4), 1079. doi:10.4141/cjps2012-307

Adams, L. S., Phung, S., Yee, N., Seeram, N. P., Li, L., & Chen, S. (2010). Blueberry phytochemicals inhibit growth and metastatic potential of MDA-MB-231 breast cancer cells through modulation of the phosphatidylinositol 3-kinase pathway. *Cancer Research, 70*(9), 3594-3605. doi:10.1158/0008-5472.CAN-09-3565

Agboola, R., Kimatu, J., Liao, Y. C., & Liu, B. (2012). Morphological and cytosine DNA methylation changes induced by a combined effect of boron (B) and salttoxicity in sorghum bicolor inbred line. *African Journal of Biotechnology, 11*(48), 10874-10881. doi:10.5897/AJB11.3534

Aggarwal, R., Hendre, P., Varshney, R., Bhat, P., Krishnakumar, V., & Singh, L. (2007). Identification, characterization and utilization of EST-derived genic microsatellite markers for genome analyses of coffee and related species. *Theoretical and Applied Genetics, 114*(2), 359-372. doi:10.1007/s00122-006-0440-x

Agrawal, A., Sanayaima, R., Singh, R., Tandon, R., Verma, S., & Tyagi, R. K. (2014). Phenotypic and molecular studies for genetic stability assessment of cryopreserved banana meristems derived from field and *in vitro* explant sources. *In vitro Cellular*

and Developmental Biology - Plant, 50(3), 345-356. doi:10.1007/s11627-014-9606-4

Al-Zahim, M. A., Ford-Lloyd, B. V., & Newbury, H. J. (1999). Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. *Plant Cell Reports, 18*(6), 473-477. doi:10.1007/s002990050606

Alam, Z., Morales, H. R., & Roncal, J. (2016). Environmental conditions affect phenolic content and antioxidant capacity of leaves and fruit in wild partridgeberry (*Vaccinium vitis-idaea*). *Botany, 94*(7), 509-521. doi:10.1139/cjb-2016-0041

Albert, T., Starast, M., Karp, K., Kaldmäe, H., Vool, E., & Paal, T. (2009). The influence of propagation method on growth of the half-highbush blueberry 'Northblue'. *Acta Horticulturae, 812,* 141-146. doi:10.17660/ActaHortic.2009.812.14

Alizadeh, M., & Singh, S. K. (2009). Molecular assessment of clonal fidelity in micropropagated grape (*Vitis* spp.) rootstock genotypes using RAPD and ISSR markers. *Iranian Journal of Biotechnology, 7*(1), 37-44.

Allan-Wojtas, P. M., Forney, C. F., Carbyn, S. E., & Nicholas, K. U. K. G. (2001). Microstructural indicators of quality-related characteristics of blueberries-an integrated approach. *LWT - Food Science and Technology, 34*(1), 23-32. doi:http://dx.doi.org/10.1006/fstl.2000.0738

Amoo, S., Aremu, A., Moyo, M., Sunmonu, T., Plíhalová, L., Doležal, K., & Staden, J. (2015). Physiological and biochemical effects of a tetrahydropyranyl-substituted meta-topolin in micropropagated *Merwilla plumbea*. *Plant Cell, Tissue and Organ Culture, 121*(3), 579-590. doi:10.1007/s11240-015-0728-0

Amoo, S., & Staden, J. (2013). Influence of plant growth regulators on shoot proliferation and secondary metabolite production in micropropagated *Huernia hystrix*. *Plant Cell, Tissue and Organ Culture, 112*(2), 249-256. doi:10.1007/s11240-012-0230-x

Amoo, S. O., Aremu, A. O., Moyo, M., Szüčová, L., Doležal, K., & Van Staden, J. (2014). Physiological effects of a novel aromatic cytokinin analogue in micropropagated *Aloe arborescens* and *Harpagophytum procumbens*. *Plant Cell, Tissue and Organ Culture, 116*(1), 17-26. doi:10.1007/s11240-013-0377-0

Amoo, S. O., Aremu, A. O., & Van Staden, J. (2012). *In vitro* plant regeneration, secondary metabolite production and antioxidant activity of micropropagated *Aloe arborescens* Mill. *Plant Cell, Tissue and Organ Culture, 111*(3), 345-358. doi:10.1007/s11240-012-0200-3

An, D., Bykova, N. V., & Debnath, S. C. (2015). EST-PCR, EST-SSR and ISSR markers to identify a set of wild cranberries and evaluate their relationships. *Canadian Journal of Plant Science, 95*(6), 1155-1165. doi:10.4141/CJPS-2015-158

Andersen, J. R., & Lübberstedt, T. (2003). Functional markers in plants. *Trends in Plant Science, 8*(11), 554-560. doi:10.1016/j.tplants.2003.09.010

Anderson, W. C. (1975). Propagation of rhododendrons by tissue culture. 1. Development of a culture medium for multiplication of shoots. *Proceedings of the International Plant Propagator's Society, 25*, 129-135.

Andreotti, C., Castagnoli, M., Maltoni, M. L., Magnani, S., Fontanari, M., Carlini, A., Marchesini, A., Pititto, A., Faedi, W., & Baudino, M. (2014). Quality and phenolic composition of blueberries cultivated in Italy. *Acta Horticulturae, 1017*, 427-432. doi:10.17660/ActaHortic.2014.1017.52

Aremu, A., Moyo, M., Amoo, S., Gruz, J., Šubrtová, M., Plíhalová, L., Doležal, K., & Staden, J. (2014). Effect of a novel aromatic cytokinin derivative on phytochemical levels and antioxidant potential in greenhouse grown *Merwilla plumbea*. *Plant Cell, Tissue and Organ Culture, 119*(3), 501-509. doi:10.1007/s11240-014-0550-0

Aremu, A. O., Gruz, J., Šubrtová, M., Szüčová, L., Doležal, K., Bairu, M. W., Finnie, J. F., & Van Staden, J. (2013). The role of cytokinins on the antioxidant capacity and phenolic acid content during tissue culture and acclimatization of *Merwilla plumbea*. *South African Journal of Botany, 86,* 172. doi:10.1016/j.sajb.2013.02.126

Arnholdt-Schmitt, B. (1993). Rapid changes in amplification and methylation pattern of genomic DNA in cultured carrot root explants (*Daucus carota* L.). *Theoretical and Applied Genetics, 85*(6), 793-800. doi:10.1007/BF00225021

Arnholdt-Schmitt, B., Herterich, S., & Neumann, K. (1995). Physiological aspects of genome variability in tissue culture. I. Growth phase-dependent differential DNA methylation of the carrot genome (*Daucus carota* L.) during primary culture. *Theoretical and Applied Genetics, 91*(5), 809-815. doi:10.1007/BF00220964

Ashapkin, V. V., Kutueva, L. I., & Vanyushin, B. F. (2002). The gene for domains rearranged methyltransferase (DRM2) in *Arabidopsis thaliana* plants is methylated at both cytosine and adenine residues. *FEBS Letters, 532*(3), 367-372. doi:10.1016/S0014-5793(02)03711-0

Attwood, J. T., Yung, R. L., & Richardson, B. C. (2002). DNA methylation and the regulation of gene transcription. *Cellular and Molecular Life Sciences, 59*(2), 241-257. doi:10.1007/s00018-002-8420-z

Bairu, M. W., Amoo, S. O., & Van Staden, J. (2011a). Comparative phytochemical analysis of wild and *in vitro*-derived greenhouse-grown tubers, *in vitro* shoots and callus-like basal tissues of *Harpagophytum procumbens*. *South African Journal of Botany, 77*(2), 479-484. doi:10.1016/j.sajb.2010.09.009

Bairu, M. W., Aremu, A. O., & Van Staden, J. (2011b). Somaclonal variation in plants: causes and detection methods. *Plant Growth Regulation, 63*(2), 147-173. doi:10.1007/s10725-010-9554-x

Baránek, M., Čechová, J., Raddová, J., Holleinová, V., Ondrušíková, E., & Pidra, M. (2015). Dynamics and reversibility of the DNA methylation landscape of grapevine plants (*Vitis vinifera*) stressed by *in vitro* cultivation and thermotherapy. *PLoS ONE, 10*(5), e0126638. doi:10.1371/journal.pone.0126638

Baránek, M., Křižan, B., Ondrušíková, E., & Pidra, M. (2010). DNA-methylation changes in grapevine somaclones following *in vitro* culture and thermotherapy. *Plant Cell, Tissue and Organ Culture, 101*(1), 11-22. doi:10.1007/s11240-009-9656-1

Barker, W. G., & Collins, W. B. (1963). The blueberry rhizome: *in vitro* culture. *Canadian Journal of Botany, 41*(9), 1325-1329. doi:10.1139/b63-113

Bassam, B. J., & Gresshoff, P. M. (2007). Silver staining DNA in polyacrylamide gels. *Nature Protocols, 2*(11), 2649-2654. doi:10.1038/nprot.2007.330

Basu, A., Du, M., Leyva, M. J., Sanchez, K., Betts, N. M., Wu, M., Aston, C. E., & Lyons, T. J. (2010). Blueberries decrease cardiovascular risk factors in obese men and women with metabolic syndrome. *The Journal of Nutrition, 140*(9), 1582-1587. doi:10.3945/jn.110.124701

Basu, B. (2017). Biomaterials for Musculoskeletal Regeneration: Concepts. Singapore: Springer.

Baurens, F.-C., Bonnot, F., Bienvenu, D., Causse, S., & Legavre, T. (2003). Using SD-AFLP and MSAP to assess CCGG methylation in the banana genome. *Plant Molecular Biology Reporter, 21*(4), 339-348. doi:10.1007/BF02772583

Baurens, F.-C., Causse, S., & Legavre, T. (2008). Methylation-sensitive amplification polymorphism (MSAP) protocol to assess CpG and CpNpG methylation in the banana genome. *Fruits, 63*(2), 117-123. doi:10.1051/fruits:2007054

Baurens, F. C., Nicolleau, J., Legavre, T., Verdeil, J. L., & Monteuuis, O. (2004). Genomic DNA methylation of juvenile and mature *Acacia mangium* micropropagated *in vitro* with reference to leaf morphology as a phase change marker. *Tree Physiology, 24*(4), 401-407.

Beckman, C. H. (2000). Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants. *Physiological and Molecular Plant Pathology, 57*(3), 101-110. doi:10.1006/pmpp.2000.0287

Bednarek, P. T., Orłowska, R., Koebner, R. M. D., & Zimny, J. (2007). Quantification of the tissue-culture induced variation in barley (*Hordeum vulgare* L.). *BMC Plant Biology, 7*, 10. doi:10.1186/1471-2229-7-10

Belanger, F., & Hepburn, A. (1990). The evolution of CpNpG methylation in plants. *Journal of Molecular Evolution, 30*(1), 26-35. doi:10.1007/BF02102450

Bell, D. J., Rowland, L. J., & Drummond, F. A. (2012). Does pollen 'Neighborhood' affect berry yield in lowbush blueberry (*Vaccinium angustifolium* Ait)? *International Journal of Fruit Science, 12*(1-3), 65-74. doi:10.1080/15538362.2011.619441

Bell, D. J., Rowland, L. J., Polashock, J. J., & Drummond, F. A. (2008). Suitability of EST-PCR markers developed in highbush blueberry for genetic fingerprinting and relationship studies in lowbush blueberry and related species. *Journal of the American Society for Horticultural Science, 133*(5), 701-707.

Bell, D. J., Rowland, L. J., Stommel, J., & Drummond, F. A. (2010). Yield variation among clones of lowbush blueberry as a function of genetic similarity and self-compatibility. *Journal of the American Society for Horticultural Science, 135*(3), 259-270.

Bell, D. J., Rowland, L. J., Zhang, D., & Drummond, F. A. (2009). Spatial genetic structure of lowbush blueberry, *Vaccinium angustifolium*, in four fields in Maine. *Botany, 87*(10), 932-946. doi:10.1139/B09-058

Benson, E. E., Harding, K., Betsou, F., Kofanova, O., & Fuller, B. J. (2013). Translating cryobiology principles into trans-disciplinary storage guidelines for biorepositories and biobanks: a concept paper. *Cryo-Letters, 34*(3), 277-312.

Berdasco, M., Alcázar Hernández, R., García - Ortiz, M. V., Ballestar Tarín, E., Fernández, A. F., Roldán - Arjona, T., Fernández Tiburcio, A., Altabella Artigas, T., Buisine, N., Quesneville, H., Baudry, A., Lepiniec, L., Alaminos, M., Rodríguez, R., Lloyd, A., Colot, V., Bender, J., Canal, M. J., Esteller, M., & Fraga, M. F. (2008). Promoter DNA hypermethylation and gene repression in undifferentiated *Arabidopsis* cells. *PLoS ONE, 3*(10), e33063. doi:10.1371/journal.pone.0003306

Bidabadi, S., Meon, S., Wahab, Z., & Mahmood, M. (2010). Study of genetic and phenotypic variability among somaclones induced by BAP and TDZ in micropropagated shoot tips of banana (*Musa* spp.) using RAPD markers. *Journal of Agricultural Science, 2*(3), 49-60. doi:10.5539/jas.v2n3p49

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes and Development, 16*(1), 6-21. doi:10.1101/gad.947102

Biswas, M. K., Dutt, M., Roy, U. K., Islam, R., & Hossain, M. (2009). Development and evaluation of *in vitro* somaclonal variation in strawberry for improved horticultural traits. *Scientia Horticulturae, 122*(3), 409-416.

Bitonti, M. B., Cozza, R., Chiappetta, A., Giannino, D., Castiglione, M. R., Dewitte, W., Mariotti, D., Van Onckelen, H., & Innocenti, A. M. (2002). Distinct nuclear organization, DNA methylation pattern and cytokinin distribution mark juvenile, juvenile-like and adult vegetative apical meristems in peach (*Prunus persica* (L.)

Batsch). *Journal of Experimental Botany, 53*(371), 1047-1054. doi:10.1093/jexbot/53.371.1047

Boches, P. S., Bassil, N. V., & Rowland, L. J. (2005). Microsatellite markers for *Vaccinium* from EST and genomic libraries. *Molecular Ecology Notes, 5*(3), 657-660. doi:10.1111/j.1471-8286.2005.01025.x

Boches, P. S., Bassil, N. V., & Rowland, L. (2006). Genetic diversity in the highbush blueberry evaluated with microsatellite markers. *Journal of the American Society for Horticultural Science, 131*(5), 674-686.

Bomser, J. L., Madhavi, D. A. L., Singletary, K. A. L., & Smith, M. A. L. (1996). *In vitro* anticancer activity of fruit extracts from *Vaccinium* species. *Planta Medica, 62*(3), 212-216. doi:10.1055/s-2006-957862

Boss, P. K., Davies, C., & Robinson, S. P. (1996). Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv Shiraz grape berries and the implications for pathway regulation. *Plant Physiology, 111*(4), 1059-1066. doi:10.1104/pp.111.4.1059

Botstein, D., White, R. L., Skolnick, M., & Davis, R. W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics, 32*(3), 314-331.

Bouharmont, J. (1994). Application of somaclonal variation and *in vitro* selection to plant improvement. *Acta Horticulturae, 355*, 213-218. doi:10.17660/ActaHortic.1994.355.23

Bouman, H., & De Klerk, G. J. (2001). Measurement of the extent of somaclonal variation in begonia plants regenerated under various conditions. Comparison of three assays. *Theoretical and Applied Genetics, 102*(1), 111-117. doi:10.1007/s001220051625

Boxus, P. H. (1974). The production of strawberry plants by *in vitro* micro-propagation. *Journal of Horticultural Science, 49*(3), 209-210. doi:10.1080/00221589.1974.11514571

Boxus, P. H., Jemmali, A., Terzi, J. M., & Arezki, O. (2000). Drift in genetic stability in micropropagation: the case of strawberry. *Acta Horticulturae, 530*, 155-161. doi:10.17660/ActaHortic.2000.530.16

Boyko, A., & Kovalchuk, I. (2008). Epigenetic control of plant stress response. *Environmental and Molecular Mutagenesis, 49*(1), 61-72. doi:10.1002/em.20347

Brar, D. S., & Jain, S. M. (1998). Somaclonal variation: mechanism and applications in crop improvement. In S. M. Jain, D. S. Brar, & B. S. Ahloowalia (Eds.), *Somaclonal Variation and Induced Mutations in Crop Improvement* (pp. 15-37). Dordrecht, The Netherlands: Springer

Brettell, R., & Dennis, E. (1991). Reactivation of a silent *Ac* following tissue culture is associated with heritable alterations in its methylation pattern. *Molecular and General Genetics, 229*(3), 365-372. doi:10.1007/BF00267457

Brissette, L., Tremblay, L., & Lord, D. (1990). Micropropagation of lowbush blueberry from mature field-grown plants. *HortScience, 25*(3), 349-351.

Brodowska, K. M. (2017). Natural flavonoids: Classification, potential role, and application of flavonoid analogues. *European Journal of Biological Research*, 7(2), 108-123. doi:10.5281/zenodo.545778

Bucherna, N., Szabó, E., Heszky, L., & Nagy, I. (2001). DNA methylation and gene expression differences during alternative *in vitro* morphogenetic processes in eggplant (*Solanum melongena* L.). *In vitro Cellular and Developmental Biology - Plant, 37*(5), 672-677. doi:10.1007/s11627-001-0117-8

Burgher, K. L., Jamieson, A. R., & Lu, X. (2002). Genetic relationships among lowbush blueberry genotypes as determined by randomly amplified polymorphic DNA analysis. *Journal of the American Society for Horticultural Science, 127*(1), 98-103.

Busslinger, M., Boer, E. D., Wright, S., Grosveld, F. G., & Flavell, R. A. (1983). The sequence GGCmCGG is resistant to *Msp*I cleavage. *Nucleic Acids Research, 11*(11), 3559-3569. doi:10.1093/nar/11.11.3559

Calò, R., & Marabini, L. (2014). Protective effect of *Vaccinium myrtillus* extract against UVA- and UVB-induced damage in a human keratinocyte cell line (HaCaT cells). *Journal of Photochemistry and Photobiology B: Biology, 132*, 27-35. doi:10.1016/j.jphotobiol.2014.01.013

Camp, W. H. (1945). The North American blueberries with notes on other groups of Vacciniaceae. *Brittonia, 5*(3), 203-275. doi:10.2307/2804880

Cao, X., & Hammerschlag, F. A. (2000). Improved shoot organogenesis from leaf explants of highbush blueberry. *HortScience, 35*(5), 945-947.

Cao, X., Hammerschlag, F. A., & Douglass, L. (2002). A two-step pretreatment significantly enhances shoot organogenesis from leaf explants of highbush blueberry cv. Bluecrop. *HortScience, 37*(5), 819-821.

Cardeñosa, V., Girones-Vilaplana, A., Muriel, J. L., Moreno, D. A., & Moreno-Rojas, J. M. (2016). Influence of genotype, cultivation system and irrigation regime on antioxidant capacity and selected phenolics of blueberries (*Vaccinium corymbosum* L.). *Food Chemistry, 202*, 276-283. doi:10.1016/j.foodchem.2016.01.118

Cassells, A., & Curry, R. (2001). Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. *Plant Cell, Tissue and Organ Culture, 64*(2), 145-157. doi:10.1023/A:1010692104861

Cassells, A., Deadman, M., Brown, C., & Griffin, E. (1991). Field resistance to late blight (*Phytophthora infestans* (Mont.) De Bary) in potato (*Solanum tuberosum* L.) somaclones associated with instability and pleiotropic effects. *Euphytica, 56*(1), 75-80. doi:10.1007/BF00041746

Cassells, A., Kowalski, B., Fitzgerald, D., & Murphy, G. (1999). The use of image analysis to study developmental variation in micropropagated potato (*Solanum tuberosum* L.) plants. *Potato Research, 42*(3), 541-548. doi:10.1007/BF02358170

Castrejón, A. D. R., Eichholz, I., Rohn, S., Kroh, L. W., & Huyskens-Keil, S. (2008). Phenolic profile and antioxidant activity of highbush blueberry (*Vaccinium corymbosum* L.) during fruit maturation and ripening. *Food Chemistry, 109*(3), 564-572. doi:10.1016/j.foodchem.2008.01.007

Catania, J., Keenan, B. C., Margison, G. P., & Fairweather, D. S. (1987). Determination of 5- methylcytosine by acid hydrolysis of DNA with hydrofluoric acid. *Analytical Biochemistry, 167*(2), 347-351. doi:10.1016/0003-2697(87)90175-8

Cedar, H., Solage, A., Glaser, G., & Razin, A. (1979). Direct detection of methylated cytosine in DNA by use of the restriction enzyme *Msp*I. *Nucleic Acids Research, 6*(6), 2125-2132. doi:10.1093/nar/6.6.2125

Česonienė, L., Daubaras, R., Paulauskas, A., Žukauskienė, J., & Zych, M. (2013). Morphological and genetic diversity of European cranberry (*Vaccinium oxycoccos* L., Ericaceae) clones in Lithuanian reserves. *Acta Societatis Botanicorum Poloniae, 82*(3), 211-217. doi:10.5586/asbp.2013.026

Chabane, K., Ablett, G., Cordeiro, G., Valkoun, J., & Henry, R. (2005). EST versus genomic derived microsatellite markers for genotyping wild and cultivated barley. *Genetic Resources and Crop Evolution, 52*(7), 903-909. doi:10.1007/s10722-003-6112-7

Chakrabarty, D., Yu, K. W., & Paek, K. Y. (2003). Detection of DNA methylation changes during somatic embryogenesis of Siberian ginseng (*Eleuterococcus senticosus*). *Plant Science, 165*(1), 61-68. doi:10.1016/S0168-9452(03)00127-4

Chandrasekara, A., & Shahidi, F. (2010). Content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. *Journal of Agricultural and Food Chemistry, 58*(11), 6706-6714. doi:10.1021/jf100868b

Chattopadhyay, S., Farkya, S., Srivastava, A., & Bisaria, V. (2002). Bioprocess considerations for production of secondary metabolites by plant cell suspension cultures. *Biotechnology and Bioprocess Engineering, 7*(3), 138-149. doi:10.1007/BF02932911

Chavan, J., Gaikwad, N., Umdale, S., Kshirsagar, P., Bhat, K., & Yadav, S. (2014). Efficiency of direct and indirect shoot organogenesis, molecular profiling, secondary metabolite production and antioxidant activity of micropropagated *Ceropegia santapaui*. *Plant Growth Regulation, 72*(1), 1-15. doi:10.1007/s10725-013-9830-7

Chavez-Santoscoy, R., Gutierrez-Uribe, J., & Serna-Saldívar, S. (2009). Phenolic composition, antioxidant capacity and *in vitro* cancer cell cytotoxicity of nine prickly pear (*Opuntia* spp.) juices. *Plant Foods for Human Nutrition, 64*(2), 146-152. doi:10.1007/s11130-009-0117-0

Chee, P. W., Rong, J., Williams-Coplin, D., Schulze, S. R., & Paterson, A. H. (2004). EST derived PCR-based markers for functional gene homologues in cotton. *Genome, 47*(3), 449-462. doi:10.1139/g04-002

Chen, Q., Zhang, X.-N., Yu, H.-W., Yan-Wang, & Tang, H.-R. (2012). Changes of total anthocyanins and proanthocyanidins in the developing blackberry fruits. *International Journal of ChemTech Research, 4*(1), 129-137.

Cheplick, S., Sarkar, D., Bhowmik, P., & Shetty, K. (2015). Phenolic bioactives from developmental stages of highbush blueberry (*Vaccinium corymbosum*) for hyperglycemia management using *in vitro* models. *Canadian Journal of Plant Science, 95*(4), 653-662. doi:10.4141/CJPS-2014-352

Chinnusamy, V., & Zhu, J.-K. (2009). Epigenetic regulation of stress responses in plants. *Current Opinion in Plant Biology, 12*(2), 133-139. doi:10.1016/j.pbi.2008.12.006

Choi, C.-S., & Sano, H. (2006). Abiotic-stress induces demethylation and transcriptional activation of a gene encoding a glycerophosphodiesterase-like protein in tobacco plants. *Molecular Genetics and Genomics*, 277(5), 589–600. Doi:10.1007/s00438-007-0209-1

Christie, P., Alfenito, M., & Walbot, V. (1994). Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta, 194*(4), 541-549. doi:10.1007/BF00714468

Clark, S. J., Harrison, J., Paul, C. L., & Frommer, M. (1994). High sensitivity mapping of methylated cytosines. *Nucleic Acids Research, 22*(15), 2990-2997. doi:10.1093/nar/22.15.2990

Clark, S. J., Statham, A., Stirzaker, C., Molloy, P. L., & Frommer, M. (2006). DNA methylation: bisulphite modification and analysis. *Nature Protocols, 1*(5), 2353-2364. doi:10.1038/nprot.2006.324

Cline, M., Wessel, T., & Iwamura, H. (1997). Cytokin/auxin control of apical dominance in *Ipomoea nil*. *Plant and Cell Physiology, 38*(6), 659-667. doi:10.1093/oxfordjournals.pcp.a029218

Cloutier, S., & Landry, B. S. (1994). Molecular markers applied to plant tissue culture. *In vitro Cellular and Developmental Biology - Plant, 30*(1), 32-39. doi:10.1007/BF02632117

Cohen, D. (1980). Application of micropropagation methods for blueberries and tamarillos. *Proceedings of the International Plant Propagators' Society, 30*, 144-146.

Cohen, D., & Elliott, D. (1979). Micropropagation methods for blueberries and tamarillos [*Cyphomandra betacea*, tree-tomato]. *Proceedings of the International Plant Propagators' Society, 29*, 177-179.

Cokus, S. J., Feng, S., Zhang, X., Chen, Z., Merriman, B., Haudenschild, C. D., Pradhan, S., Nelson, S. F., Pellegrini, M., & Jacobsen, S. E. (2008). Shotgun bisulphite sequencing of the *Arabidopsis* genome reveals DNA methylation patterning. *Nature, 452*(7184), 215-219. doi:10.1038/nature06745

Compton, M. (1994). Statistical methods suitable for the analysis of plant tissue culture data. *Plant Cell, Tissue and Organ Culture, 37*(3), 217-242. doi:10.1007/BF00042336

Connor, A. M., Luby, J. J., Hancock, J. F., Berkheimer, S., & Hanson, E. J. (2002a). Changes in fruit antioxidant activity among blueberry cultivars during cold-temperature storage. *Journal of Agricultural and Food Chemistry, 50*(4), 893-898. doi:10.1021/jf011212y

Connor, A. M., Luby, J. J., & Tong, C. B. S. (2002b). Variation and heritability estimates for antioxidant activity, total phenolic content, and anthocyanin content in blueberry progenies. *Journal of the American Society for Horticultural Science, 127*(1), 82-88.

Connor, A. M., Luby, J. J., Tong, C. B. S., Finn, C. E., & Hancock, J. F. (2002c). Genotypic and environmental variation in antioxidant activity, total phenolic content, and

anthocyanin content among blueberry cultivars. *Journal of the American Society for Horticultural Science, 127*(1), 89-97.

Correa-Betanzo, J., Allen-Vercoe, E., McDonald, J., Schroeter, K., Corredig, M., & Paliyath, G. (2014). Stability and biological activity of wild blueberry (*Vaccinium angustifolium*) polyphenols during simulated *in vitro* gastrointestinal digestion. *Food Chemistry, 165*, 522-531. doi:10.1016/j.foodchem.2014.05.135

Dahlberg, J. A. (2000). Classification and characterization of sorghum. In C. W. Smith & R. A. Frederiksen (Eds.), *Sorghum: Origin, History, Technology and Production* (pp. 99–130.). New York: John Wiley & Sons.

Dai, L., Wang, B., Zhao, H., & Peng, J. (2013). Transferability of genomic simple sequence repeat and expressed sequence tag-simple sequence repeat markers from sorghum to *Miscanthus sinensis*, a potential biomass crop. *Crop Science, 53*(3), 977-986. doi:10.2135/cropsci2011.12.0671

Dann, A., & Wilson, C. (2011). Comparative assessment of genetic and epigenetic variation among regenerants of potato (*Solanum tuberosum*) derived from long-term nodal tissue-culture and cell selection. *Plant Cell Reports, 30*(4), 631-639. doi:10.1007/s00299-010-0983-9

Davey, M. R., & Anthony, P. (2010). *Plant Cell Culture: Essential Methods*. Chichester, UK: John Wiley & Sons Ltd.

De Klerk, G. J. (2002). Rooting of microcuttings: theory and practice. *In vitro Cellular & Developmental Biology - Plant, 38*(5), 415-422. doi:10.1079/IVP2002335

Debnath, S. C. (2004). *In vitro* culture of lowbush blueberry (*Vaccinium angustifolium* Ait.). *Small Fruits Review, 3*(3/4), 393-408. doi:10.1300/J301v03n03_16

Debnath, S. C. (2005a). Morphological development of lingonberry as affected by *in vitro* and *ex vitro* propagation methods and source propagule. *HortScience, 40*(3), 760-763.

Debnath, S. C. (2005b). Differentiation of *Vaccinium* cultivars and wild clones using RAPD markers. *Journal of Plant Biochemistry and Biotechnology, 14*(2), 173-177. doi:10.1007/bf03355954

Debnath, S. C. (2006). Influence of propagation method and indole-3-butyric acid on growth and development of *in vitro*- and *ex vitro*-derived lingonberry plants. *Canadian Journal of Plant Science, 86*(1), 235-243. doi:10.4141/P04-142

Debnath, S. C. (2007a). Propagation of *Vaccinium in vitro*: a review. *International Journal of Fruit Science, 6*(2), 47-71. doi:10.1300/J492v06n02_04

Debnath, S. C. (2007b). Influence of indole-3-butyric acid and propagation method on growth and development of *in vitro*- and *ex vitro*-derived lowbush blueberry plants. *Plant Growth Regulation, 51*(3), 245-253. doi:10.1007/s10725-006-9164-9

Debnath, S. C. (2007c). Strategies to propagate *Vaccinium* nuclear stocks for the Canadian berry industry. *Canadian Journal of Plant Science, 87*(4), 911-922. doi:10.4141/P06-131

Debnath, S. C. (2008). Molecular analysis for *Vaccinium* germplasm improvement- a review. *Current Topics in Plant Biology, 9*, 1-12.

Debnath, S. C. (2009a). A two-step procedure for adventitious shoot regeneration on excised leaves of lowbush blueberry. *In vitro Cellular and Developmental Biology - Plant, 45*(2), 122-128. doi:10.1007/s11627-008-9186-2

Debnath, S. C. (2009b). A scale-up system for lowbush blueberry micropropagation using a bioreactor. *HortScience, 44*(7), 1962-1966.

Debnath, S. C. (2009c). Characteristics of strawberry plants propagated by *in vitro* bioreactor culture and *ex vitro* propagation method. *Engineering in Life Sciences, 9*(3), 239-246. doi:10.1002/elsc.200800095

Debnath, S. C. (2009d). Development of ISSR markers for genetic diversity studies in *Vaccinium angustifolium*. *Nordic Journal of Botany, 27*(2), 141-148. doi:10.1111/j.1756-1051.2009.00402.x

Debnath, S. C. (2010). Berry bioreactor micropropagation and molecular analysis for genetic fidelity. *Acta Horticulturae, 865*, 43-51. doi:10.17660/ActaHortic.2010.865.4

Debnath, S. C. (2011). Adventitious shoot regeneration in a bioreactor system and EST-PCR based clonal fidelity in lowbush blueberry (*Vaccinium angustifolium* Ait.). *Scientia Horticulturae, 128*(2), 124-130. doi:10.1016/j.scienta.2011.01.012

Debnath, S. C. (2014a). Strategies to propagate *Vaccinium* nuclear stock and use of molecular markers for clonal fidelity. *Acta Horticulturae, 1017*, 93-99. doi:10.17660/ActaHortic.2014.1017.8

Debnath, S. C. (2014b). Structured diversity using EST-PCR and EST-SSR markers in a set of wild blueberry clones and cultivars. *Biochemical Systematics and Ecology, 54*(0), 337-347. doi:10.1016/j.bse.2014.03.018

Debnath, S. C. (2014c). Bioreactor-induced adventitious shoot regeneration affects genotype-dependent morphology but maintains clonal fidelity in red raspberry. *In vitro Cellular & Developmental Biology - Plant, 50*(6), 777-788. doi:10.1007/s11627-014-9632-2

Debnath, S. C. (2016). Genetic diversity and erosion in berries. In M. R. Ahuja & S. M. Jain (Eds.), *Genetic Diversity and Erosion in Plants: Case Histories* (pp. 75-129). Cham: Springer.

Debnath, S. C. (2017). Temporary immersion and stationary bioreactors for mass propagation of true-to-type highbush, half-high, and hybrid blueberries (*Vaccinium* spp.). *Journal of Horticultural Science and Biotechnology, 92*(1), 72-80. doi:10.1080/14620316.2016.1224606

Debnath, S. C., & McRae, K. B. (2001a). An efficient *in vitro* shoot propagation of cranberry (*Vaccinium macrocarpon* Ait.) by axillary bud proliferation. *In vitro Cellular and Developmental Biology - Plant, 37*(2), 243-249. doi:10.1007/s11627-001-0043-9

Debnath, S. C., & McRae, K. B. (2001b). *In vitro* culture of lingonberry (*Vaccinium vitis-idaea* L.): the influence of cytokinins and media types on propagation. *Small Fruits Review, 1*(3), 3-19. doi:10.1300/J301v01n03_02

Debnath, S. C., & McRae, K. B. (2002). An efficient adventitious shoot regeneration system on excised leaves of micropropagated lingonberry (*Vaccinium vitis- idaea* L.). *Journal of Horticultural Science and Biotechnology, 77*(6), 744-752. doi:10.1080/14620316.2002.11511567

Debnath, S. C., & McRae, K. B. (2005). A one-step *in vitro* cloning procedure for cranberry (*Vaccinium macrocarpon* Ait.). *Small Fruits Review, 4*(3), 57-75. doi:10.1300/J301v04n03_05

Debnath, S. C., Siow, Y. L., Petkau, J., An, D., & Bykova, N. V. (2012a). Molecular markers and antioxidant activity in berry crops: genetic diversity analysis. *Canadian Journal of Plant Science, 92*(6), 1121-1133. doi:10.4141/CJPS2011-240

Debnath, S. C., Vyas, P., Goyali, J. C., & Igamberdiev, A. U. (2012b). Morphological and molecular analyses in micropropagated berry plants acclimatized under *ex vitro* condition. *Canadian Journal of Plant Science, 92*(6), 1065-1073. doi:10.1139/CJPS2011-194

Deikman, J. (1995). Induction of anthocyanin accumulation by cytokinins in *Arabidopsis thaliana*. *Plant Physiology, 108*(1), 47-57. doi:10.1104/pp.108.1.47

Deng, R., Donnelly, D. J., & Buszard, D. J. (1993). Field performance of micropropagated and conventionally propagated red raspberry. *Acta Horticulturae, 336*, 185-191. doi:10.17660/ActaHortic.1993.336.23

Dhanaraj, A. L., Slovin, J. P., & Rowland, L. J. (2004). Analysis of gene expression associated with cold acclimation in blueberry floral buds using expressed sequence tags. *Plant Science, 166*(4), 863-872. doi:10.1016/j.plantsci.2003.11.013

Dhar, M., Pethe, V., Gupta, V., & Ranjekar, P. (1990). Predominance and tissue specificity of adenine methylation in rice. *Theoretical and Applied Genetics, 80*(3), 402-408. doi:10.1007/BF00210080

Dias, M. I., Sousa, M. J., Alves, R. C., & Ferreira, I. C. F. R. (2016). Exploring plant tissue culture to improve the production of phenolic compounds: a review. *Industrial Crops and Products, 82*(Supplement C), 9-22. doi:10.1016/j.indcrop.2015.12.016

Díaz-Martínez, M., Nava-Cedillo, A., Guzmán-López, J. A., Escobar-Guzmán, R., & Simpson, J. (2012). Polymorphism and methylation patterns in *Agave tequilana* Weber var. 'Azul' plants propagated asexually by three different methods. *Plant Science, 185-186*, 321-330. doi:10.1016/j.plantsci.2012.01.004

Dixon, R. A., & Paiva, N. L. (1995). Stress-induced phenylpropanoid metabolism. *The Plant Cell, 71*, 1085-1097. doi:10.1105/tpc.7.7.1085

Dweikat, I. M., & Lyrene, P. M. (1988). Adventitious shoot production from leaves of blueberry cultured *in vitro*. *HortScience, 23*(3), 629.

Działo, M., Mierziak, J., Korzun, U., Preisner, M., Szopa, J., & Kulma, A. (2016). The potential of plant phenolics in prevention and therapy of skin disorders. *International Journal of Molecular Sciences, 17*(2), 160. doi:10.3390/ijms17020160

Ehala, S., Vaher, M., & Kaljurand, M. (2005). Characterization of phenolic profiles of Northern European berries by capillary electrophoresis and determination of their antioxidant activity. *Journal of Agricultural and Food Chemistry, 53*(16), 6484-6490. doi:10.1021/jf050397w

Ehlenfeldt, M. K., & Prior, R. L. (2001). Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. *Journal of Agricultural and Food Chemistry, 49*(5), 2222-2227. doi:10.1021/jf0013656

El-Shiekh, A., Wildung, D. K., Luby, J. J., Sargent, K. L., & Read, P. E. (1996). Long-term effects of propagation by tissue culture or softwood single-node cuttings on growth habit, yield, and berry weight of 'Northblue' blueberry. *Journal of the American Society for Horticultural Science, 121*(2), 339-342.

Enríquez-del Valle, J. R., Antonio-Luis, K. H., Rodríguez-Ortiz, G., & Campos-Ángeles, G. V. (2016). Effect of culture medium and incubation on the characteristics of micropropagated agave plants. *Ciencia e Investigacion Agraria, 43*(2), 263-272. doi:10.4067/S0718-16202016000200009

Faria, A., Pestana, D., Teixeira, D., De Freitas, V., Mateus, N., & Calhau, C. (2010). Blueberry anthocyanins and pyruvic acid adducts: anticancer properties in breast cancer cell lines. *Phytotherapy Research, 24*(12), 1862-1869. doi:10.1002/ptr.3213

Feng, S., Cokus, S. J., Zhang, X., Chen, P. Y., Bostick, M., & Goll, M. G. (2010). Conservation and divergence of methylation patterning in plants and animals.

Proceedings of the National Academy of Sciences of the United States of America, 107, 8689-8694. doi:10.1073/pnas.1002720107

Ferlemi, A.-V., Lamari, F. N., & Klimis-Zacas, D. (2016). Berry leaves: an alternative source of bioactive natural products of nutritional and medicinal value. *Antioxidants, 5*(2), 17. doi:10.3390/antiox5020017

Feuser, S., Meler, K., Daquinta, M., Guerra, M. P., & Nodari, R. O. (2003). Genotypic fidelity of micropropagated pineapple (*Ananas comosus*) plantlets assessed by isozyme and RAPD markers. *Plant Cell, Tissue and Organ Culture, 72*(3), 221-227. doi:10.1023/A:1022321405683

Finnegan, E. J., & Kovac, K. A. (2000). Plant DNA methyltransferases. *Plant Molecular Biology, 43*(2), 189-201. doi:10.1023/A:1006427226972

Finnegan, E. J., Peacock, W. J., & Dennis, E. S. (1996). Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. *Proceedings of the National Academy of Sciences of the United States of America, 93*(16), 8449-8454. doi:10.1073/pnas.93.16.8449

Flinn, M. A., & Pringle, J. K. (1983). Heat tolerance of rhizomes of several understory species. *Canadian Journal of Botany, 61*(2), 452-457. doi:10.1139/b83-052

Foley, S. L., & Debnath, S. C. (2007). Influence of *in vitro* and *ex vitro* propagation on anthocyanin content and anti-oxidant activity of lingonberries. *Journal of Horticultural Science and Biotechnology, 82*(1), 114-118. doi:10.1080/14620316.2007.11512207

Forney, C. F., Kalt, W., Jordan, M. A., Vinqvist-Tymchuk, M. R., & Fillmore, S. A. E. (2012). Blueberry and cranberry fruit composition during development. *Journal of Berry Research, 2*(3), 169-177. doi:10.3233/JBR-2012-034

Fraga, M. F., & Esteller, M. (2002). DNA methylation: a profile of methods and applications. *BioTechniques, 33*(3), 632-649.

Fraga, M. F., Rodríguez, R., & Cañal, M. J. (2002). Genomic DNA methylation-demethylation during aging and reinvigoration of *Pinus radiata*. *Tree Physiology, 22*(11), 813-816. doi:10.1093/treephys/22.11.813

Frankel, E. N., & Meyer, A. S. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of Agriculture and Food Chemistry, 48*, 1925-1941. doi: 10.1002/1097-0010(200010)80:13<1925::AID-JSFA714>3.0.CO;2-4

Frett, J. J., & Smagula, J. M. (1983). *In vitro* shoot production of lowbush blueberry. *Canadian Journal of Plant Science, 63*(2), 467-472. doi:10.4141/cjps83-054

Frommer, M., McDonald, L. E., Millar, D. S., Collis, C. M., Watt, F., Grigg, G. W., Molloy, P. L., & Paul, C. L. (1992). A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proceedings of the National Academy of Sciences of the United States of America, 89*(5), 1827-1831.

Fu, C., Li, L., Wu, W., Li, M., Yu, X., & Yu, L. (2012). Assessment of genetic and epigenetic variation during long- term Taxus cell culture. *Plant Cell Reports, 31*(7), 1321-1331. doi:10.1007/s00299-012-1251-y

Fulneček, J., & Kovařík, A. (2014). How to interpret methylation sensitive amplified polymorphism (MSAP) profiles? *BMC Genetics, 15*, 2. doi:10.1186/1471-2156-15-2

Fulneček, J., Matyášek, R., Kovařík, A., & Bezděk, M. (1998). Mapping of 5-methylcytosine residues in *Nicotiana tabacum* 5S rRNA genes by genomic

sequencing. *Molecular and General Genetics, 259*(2), 133-141. doi:10.1007/s004380050798

Gaj, M. (2004). Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. *Plant Growth Regulation, 43*(1), 27-47. doi:10.1023/B:GROW.0000038275.29262.fb

Gajdošová, A., Ostrolucká, M. G., Libiaková, G., Ondrušková, E., & Šimala, D. (2006). Microclonal propagation of *Vaccinium* sp. and *Rubus* sp. and detection of genetic variability in culture *in vitro*. *Journal of Fruit and Ornamental Plant Research, 14*(Supplement 1), 103-119.

Galletta, G. J., & Ballington, J. R. (1996). Blueberries, cranberries, and lingonberries. In J. Janick & J. N. Moore (Eds.), *Fruit Breeding, Vol. II: Vine and Small Fruits* (pp. 1-108). New York: John Wiley & Sons, Inc.

Gao, L., Jing, R., Huo, N., Li, Y., Li, X., Zhou, R., Chang, X., Tang, J., Ma, Z., & Jia, J. (2004). One hundred and one new microsatellite loci derived from ESTs (EST-SSRs) in bread wheat. *Theoretical and Applied Genetics, 108*(7), 1392-1400. doi:10.1007/s00122-003-1554-z

Gao, L., & Mazza, G. (1994). Quantitation and distribution of simple and acylated anthocyanins and other phenolics in blueberries. *Journal of Food Science, 59*(5), 1057-1059. doi:10.1111/j.1365-2621.1994.tb08189.x

Gao, X., Yang, D., Cao, D., Ao, M., Sui, X., Wang, Q., Kimatu, J., & Wang, L. (2010). *In vitro* micropropagation of *Freesia hybrida* and the assessment of genetic and epigenetic stability in regenerated plantlets. *Journal of Plant Growth Regulation, 29*(3), 257-267. doi:10.1007/s00344-009-9133-4

Gao, Y., Ran, L., Kong, Y., Jiang, J., Sokolov, V., & Wang, Y. (2014). Assessment of DNA methylation changes in tissue culture of *Brassica napus*. *Russian Journal of Genetics, 50*(11), 1186-1191. doi:10.1134/S1022795414100032

Gavrilova, V., Kajdžanoska, M., Gjamovski, V., & Stefova, M. (2011). Separation, characterization and quantification of phenolic compounds in blueberries and red and black currants by HPLC−DAD−ESI-MS. *Journal of Agricultural and Food Chemistry, 59*(8), 4009-4018. doi:10.1021/jf104565y

Ge, H., Li, Y., Fu, H., Long, G., Luo, L., Li, R., & Deng, Z. (2015). Production of sweet orange somaclones tolerant to citrus canker disease by *in vitro* mutagenesis with EMS. *Plant Cell, Tissue and Organ Culture, 123*(1), 29-38. doi:10.1007/s11240-015-0810-7

Gehring, M., Bubb, K. L., & Henikoff, S. (2009). Extensive demethylation of repetitive elements during seed development underlies gene imprinting. *Science, 324*(5933), 1447-1451. doi:10.1126/science.1171609

George, E. F. (2008). Plant tissue culture procedure – background. In E. F. George, M. A. Hall & G-J. De Klerk (Eds.), Plant propagation by tissue culture (pp. 1-28). Dordrecht, The Netherlands: Springer.

Georgiev, V., Weber, J., Kneschke, E.-M., Denev, P., Bley, T., & Pavlov, A. (2010). Antioxidant activity and phenolic content of betalain extracts from intact plants and hairy root cultures of the red beetroot *Beta vulgaris* cv. Detroit dark red. *Plant Foods for Human Nutrition, 65*(2), 105-111. doi:10.1007/s11130-010-0156-6

Georgieva, M. (2013). Micropropagation of lowbush blueberry (*Vaccinium angustifolium*). *Banat's Journal of Biotechnology, 4*(8), 42-47. doi:10.7904/2068-4738-IV(8)-42

Georgieva, M., Badjakov, I., Dincheva, I., Yancheva, S., & Kondakova, V. (2016). *In vitro* propagation of wild Bulgarian small berry fruits (bilberry, lingonberry, raspberry and strawberry). *Bulgarian Journal of Agricultural Science, 22*(1), 46-51.

Ghosh, A., Igamberdiev, A. U., & Debnath, S. C. (2017). Detection of DNA methylation pattern in thidiazuron-induced blueberry callus using methylation-sensitive amplification polymorphism. *Biologia Plantarum, 61*(3), 511-519. doi:10.1007/s10535-016-0678-3

Gillaspy, G., Ben-David, H., & Gruissem, W. (1993). Fruits: a developmental perspective. *The Plant Cell, 5*(10), 1439-1451. doi:10.1105/tpc.5.10.1439

Gillis, K., Gielis, J., Peeters, H., Dhooghe, E., & Oprins, J. (2007). Somatic embryogenesis from mature Bambusa balcooa Roxburgh as basis for mass production of elite forestry bamboos. *Plant Cell, Tissue and Organ Culture, 91*(2), 115-123. doi:10.1007/s11240-007-9236-1

Giongo, L., Ieri, F., Vrhovsek, U., Grisenti, M., Mattivi, F., & Eccher, M. (2006). Characterization of *Vaccinium* cultivars: horticultural and antioxidant profile. *Acta Horticulturae, 715*, 147-151. doi:10.17660/ActaHortic.2006.715.21

Giovanelli, G., Brambilla, A., & Sinelli, N. (2013). Effects of osmo-air dehydration treatments on chemical, antioxidant and morphological characteristics of blueberries. *LWT - Food Science and Technology, 54*(2), 577-584. doi:10.1016/j.lwt.2013.06.008

Giovanelli, G., & Buratti, S. (2009). Comparison of polyphenolic composition and antioxidant activity of wild Italian blueberries and some cultivated varieties. *Food Chemistry, 112*(4), 903-908. doi:10.1016/j.foodchem.2008.06.066

Giri, C. C., & Zaheer, M. (2016). Chemical elicitors versus secondary metabolite production *in vitro* using plant cell, tissue and organ cultures: recent trends and a

sky eye view appraisal. *Plant Cell, Tissue and Organ Culture, 126*, 1-8. doi:10.1007/s11240-016-0985-6

Goldy, R. G., & Lyrene, P. M. (1984). Meiotic abnormalities of *Vaccinium ashei* x *Vaccinium darrowi* hybrids. *Canadian Journal of Genetics and Cytology, 26*(2), 146-151. doi:10.1139/g84-025

González, A., Sáiz, A., Acedo, A., Ruiz, M., & Polanco, C. (2013a). Analysis of genomic DNA methylation patterns in regenerated and control plants of rye (*Secale cereale* L.). *Plant Growth Regulation, 70*(3), 227-236. doi:10.1007/s10725-013-9794-7

González, A. I., Sáiz, A., Acedo, A., Ruiz, M. L., & Polanco, C. (2013b). Analysis of genomic DNA methylation patterns in regenerated and control plants of rye (*Secale cereale* L.). *Plant Growth Regulation, 70*(3), 227-236. doi:10.1007/s10725-013-9794-7

Gonzalez, M. V., Lopez, M., Valdes, A. E., & Ordas, R. J. (2000). Micropropagation of three berry fruit species using nodal segments from field-grown plants. *Annals of Applied Biology, 137*(1), 73-78. doi:10.1111/j.1744-7348.2000.tb00059.x

Goyali, J. C., Igamberdiev, A. U., & Debnath, S. C. (2013). Morphology, phenolic content and antioxidant capacity of lowbush blueberry (*Vaccinium angustifolium* Ait.) plants as affected by *in vitro* and *ex vitro* propagation methods. *Canadian Journal of Plant Science, 93*(6), 1001-1008. doi:10.4141/CJPS2012-307

Goyali, J. C., Igamberdiev, A. U., & Debnath, S. C. (2015a). Propagation methods affect fruit morphology and antioxidant properties but maintain clonal fidelity in lowbush blueberry. *HortScience, 50*(6), 888-896.

Goyali, J. C., Igamberdiev, A. U., & Debnath, S. C. (2015b). Micropropagation affects not only the fruit morphology of lowbush blueberry (*Vaccinium angustifolium* Ait.) but

also its medicinal properties. *Acta Horticulturae, 1098*, 137-142. doi:10.17660/ActaHortic.2015.1098.14

Graham, J., Greig, K., & McNicol, R. J. (1996). Transformation of blueberry without antibiotic selection. *Annals of Applied Biology, 128*(3), 557-564. doi:10.1111/j.1744-7348.1996.tb07114.x

Graham, T. L. (1991). Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. *Plant Physiology, 95*(2), 594-603. doi:10.1104/pp.95.2.594

Grant-Downton, R. T., & Dickinson, H. G. (2005). Epigenetics and its implications for plant biology. 1. The epigenetic network in plants. *Annals of Botany, 96*(7), 1143-1164. doi:10.1093/aob/mci273

Grodzicker, T., Williams, J., Sharp, P., & Sambrook, J. (1974). Physical mapping of temperature-sensitive mutations of adenoviruses. *Cold Spring Harbor Symposia on Quantitative Biology, 39*, 439-446. doi:10.1101/SQB.1974.039.01.056

Grout, J. M., Read, P. E., & Wildung, D. K. (1986). Influence of tissue culture and leaf-bud propagation on the growth habit of 'Northblue' blueberry. *Journal of the American Society for Horticultural Science, 111*(3), 372-375.

Gruenbaum, Y., Naveh-many, T., Cedar, H., & Razin, A. (1981). Sequence specificity of methylation in higher plant DNA. *Nature, 292*(5826), 860-862. doi:10.1038/292860a0

Gündüz, K., Serçe, S., & Hancock, J. F. (2015). Variation among highbush and rabbiteye cultivars of blueberry for fruit quality and phytochemical characteristics. *Journal of Food Composition and Analysis, 38*, 69-79. doi:10.1016/j.jfca.2014.09.007

Guo, W., Wu, R., Zhang, Y., Liu, X., Wang, H., Gong, L., Zhang, Z., & Liu, B. (2007). Tissue culture-induced locus-specific alteration in DNA methylation and its correlation with genetic variation in *Codonopsis lanceolata* Benth. et Hook. f. *Plant Cell Reports, 26*(8), 1297-1307. doi:10.1007/s00299-007-0320-0

Gupta, M., Chyi, Y. S., Romero-Severson, J., & Owen, J. (1994). Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats. *Theoretical and Applied Genetics, 89*(7), 998-1006. doi:10.1007/BF00224530

Gupta, R., Modgil, M., & Chakrabarti, S. K. (2009). Assessment of genetic fidelity of micropropagated apple rootstock plants, EMLA 111, using RAPD markers. *Indian Journal of Experimental Biology, 47*(11), 925-928.

Gupta, V., Estrada, A. D., Blakley, I., Reid, R., Patel, K., Meyer, M. D., Andersen, S. U. j., Brown, A. F., Lila, M. A., & Loraine, A. E. (2015). RNA-Seq analysis and annotation of a draft blueberry genome assembly identifies candidate genes involved in fruit ripening, biosynthesis of bioactive compounds, and stage-specific alternative splicing. *GigaScience, 4*, 5. doi:10.1186/s13742-015-0046-9

Gustavsson, B. A., & Stanys, V. (2000). Field performance of 'Sanna' lingonberry derived by micropropagation vs. stem cuttings. *HortScience, 35*(4), 742-744.

Haberlandt, G. (1902). Kulturversuche mit isolierten Pflanzenzellen. Sitzungsber. *Math. Naturwiss. Kl. Kais. Akad. Wiss. Wien., 111*(8), 69-92.

Haddad, P. S., Depot, M., Settaf, A., Chabli, A., & Cherrah, Y. (2003). Comparative study on the medicinal plants most recommended by traditional practitioners in Morocco and Canada. *Journal of Herbs, Spices & Medicinal Plants, 10*(3), 25-45. doi:10.1300/J044v10n03_04

Hahlbrock, K., & Scheel, D. (1989). Physiology and molecular biology of phenylpropanoid metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology, 40*, 347-369. doi:10.1146/annurev.pp.40.060189.002023

Hakkim, F. L., Shankar, C. G., & Girija, S. (2007). Chemical composition and antioxidant property of holy basil (*Ocimum sanctum* L.) leaves, stems, and inflorescence and their *in vitro* callus cultures. *Journal of Agricultural and Food Chemistry, 55*(22), 9109-9117. doi:10.1021/jf071509h

Häkkinen, S., Heinonen, M., Kärenlampi, S., Mykkänen, H., Ruuskanen, J., & Törrönen, R. (1999). Screening of selected flavonoids and phenolic acids in 19 berries. *Food Research International, 32*(5), 345-353. doi:10.1016/S0963-9969(99)00095-2

Häkkinen, S. H., & Törrönen, A. R. (2000). Content of flavonols and selected phenolic acids in strawberries and *Vaccinium* species: influence of cultivar, cultivation site and technique. *Food Research International, 33*(6), 517-524. doi:10.1016/S0963-9969(00)00086-7

Halbwirth, H., Puhl, I., Haas, U., Jezik, K., Treutter, D., & Stich, K. (2006). Two-phase flavonoid formation in developing strawberry (*Fragaria* x *ananassa*) fruit. *Journal of Agricultural and Food Chemistry, 54*(4), 1479-1485. doi:10.1021/jf0524170

Hall, I. V. (1983). Genetic improvement of the lowbush blueberry, *Vaccinium angustifolium*. *Canadian Journal of Plant Science, 63*(4), 1091-1092. doi:10.4141/cjps83-141

Hall, I. V., Jamieson, A. R., & Brydon, A. D. (1988). Cumberland and Fundy lowbush blueberries. *Canadian Journal of Plant Science, 68*(2), 553-555. doi:10.4141/cjps88-068

Hammerschlag, F., Ritchie, D., Werner, D., Hashmil, G., Krusberg, L., Meyer, R., & Huettel, R. (1995). *In vitro* selection of disease resistance in fruit trees. *Acta Horticulturae, 392*, 19-26. doi:10.17660/ActaHortic.1995.392.2

Hanai, L. R., Floh, E. I. S., Fungaro, M. H. P., Santa-Catarina, C., de Paula, F. M., Viana, A. M., & Vieira, M. L. C. (2010). Methylation patterns revealed by MSAP profiling in genetically stable somatic embryogenie cultures of *Ocotea catharinensis* (Lauraceae). *In vitro Cellular and Developmental Biology - Plant, 46*(4), 368-377. doi:10.1007/s11627-010-9291-x

Hancock, J. F., Luby, J. J., & Beaudry, R. (2003). Fruits of the *Ericaceae*. In L. Trugo, P. M. Finglas, & B. Caballero (Eds.), *Encyclopedia of Food Science, Food Technology and Nutrition* (pp. 2762-2768). London, UK: Academic Press.

Hancock, J. F., Lyrene, P., Finn, C. E., Vorsa, N., & Lobos, G. A. (2008). Blueberries and cranberries. In J. F. Hancock (Ed.), *Temperate Fruit Crop Breeding: Germplasm to Genomics* (pp. 115-149). Dordrecht, The Netherlands: Springer.

Hansen, A., Jonasson, S., Michelsen, A., & Julkunen-Tiitto, R. (2006). Long-term experimental warming, shading and nutrient addition affect the concentration of phenolic compounds in arctic-alpine deciduous and evergreen dwarf shrubs. *Oecologia, 147*(1), 1-11. doi:10.1007/s00442-005-0233-y

Harborne, J. B. (1994). *The Flavonoids: Advances in Research since 1986*. New York,USA: Chapman & Hall.

Harborne, J. B., & Williams, C. A. (2000). Advances in flavonoid research since 1992. *Phytochemistry, 55*(6), 481-504. doi:10.1016/S0031-9422(00)00235-1

Harding, K. (1994). The methylation status of DNA derived from potato plants recovered from slow growth. *Plant Cell, Tissue and Organ Culture, 37*(1), 31-38. doi:10.1007/BF00048114

Harris, C. S., Burt, A. J., Saleem, A., Le, P. M., Martineau, L. C., Haddad, P. S., Bennett, S. A. L., & Arnason, J. T. (2007). Single HPLC-PAD-APCI/MS method for the quantitative comparison of phenolic compounds found in leaf, stem, root and fruit extracts of *Vaccinium angustifolium*. *Phytochemical Analysis, 18*(2), 161-169. doi:10.1002/pca.970

Hashmi, G., Huettel, R., Meyer, R., Krusberg, L., & Hammerschlag, F. (1997). RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. *Plant Cell Reports, 16*(9), 624-627. doi:10.1007/BF01275503

Hassimotto, N. M. A., Genovese, M. I., & Lajolo, F. M. (2005). Antioxidant activity of dietary fruits, vegetables, and commercial frozen fruit pulps. *Journal of Agricultural and Food Chemistry, 53*(8), 2928-2935. doi:10.1021/jf047894h

Hatano, T., Kagawa, H., Yasuhara, T., & Okuda, T. (1988). Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. *Chemical and Pharmaceutical Bulletin, 36*(6), 2090-2097. doi:10.1248/cpb.36.2090

Heinonen, M. (2007). Antioxidant activity and antimicrobial effect of berry phenolics – a Finnish perspective. *Molecular Nutrition & Food Research, 51*(6), 684-691. doi:10.1002/mnfr.200700006

Henderson, I. R., Chan, S. R., Cao, X., Johnson, L., & Jacobsen, S. E. (2010). Accurate sodium bisulphite sequencing in plants. *Epigenetics, 5*(1), 47-49. doi:10.4161/epi.5.1.10560

Hicks, B. J. (2011). Pollination of lowbush blueberry (*Vaccinium angustifolium*) in Newfoundland by native and introduced bees. *Journal of the Acadian Entntomological Society, 7*(15), 108-118.

Hokanson, K., & Hancock, J. (1998). Levels of allozymic diversity in diploid and tetraploid *Vaccinium* sect. *Cyanococcus* (blueberries). *Canadian Journal of Plant Science, 78*(2), 327-332. doi:10.4141/P97-081

How-Kit, A., Daunay, A., Mazaleyrat, N., Busato, F., Daviaud, C., Teyssier, E., Deleuze, J. F., Gallusci, P., & Tost, J. (2015). Accurate CpG and non-CpG cytosine methylation analysis by high-throughput locus-specific pyrosequencing in plants. *Plant Molecular Biology, 88*(4-5), 471-485. doi:10.1007/s11103-015-0336-8

Howard, L. R., Clark, J. R., & Brownmiller, C. (2003). Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. *Journal of the Science of Food and Agriculture, 83*(12), 1238-1247. doi:10.1002/jsfa.1532

Howell, A., Kalt, W., Duy, J. C., Forney, C. F., & McDonald, J. E. (2001). Horticultural factors affecting antioxidant capacity of blueberries and other small fruit. *HortTechnology, 11*(4), 523-528.

Hruskoci, J. D., & Read, P. E. (1993). *In vitro* shoot regeneration from internode segments and internode-derived callus of blueberry (*Vaccinium* spp.). *Acta Horticulturae, 346*, 125-130. doi:10.17660/ActaHortic.1993.346.17

Hsieh, C.-L. (1999). *In vivo* activity of murine *de novo* methyltransferases, Dnmt3a and Dnmt3b. *Molecular and Cellular Biology, 19*(12), 8211-8218. doi: 10.1128/MCB.19.12.8211

Huang, H., Han, S., Wang, Y., Zhang, X., & Han, Z. (2012a). Variations in leaf morphology and DNA methylation following *in vitro* culture of *Malus xiaojinensis*. *Plant Cell, Tissue and Organ Culture, 111*(2), 153-161. doi:10.1007/s11240-012-0179-9

Huang, W.-Y., Zhang, H.-C., Liu, W.-X., & Li, C.-Y. (2012b). Survey of antioxidant capacity and phenolic composition of blueberry, blackberry, and strawberry in

Nanjing. *Journal of Zhejiang University Science B, 13*(2), 94-102. doi:10.1631/jzus.B1100137

Hung, C. D., Hong, C. H., Kim, S. K., Lee, K. H., Park, J. Y., Dung, C. D., Nam, M. W., Choi, D. H., & Lee, H. I. (2016a). *In vitro* proliferation and *ex vitro* rooting of microshoots of commercially important rabbiteye blueberry (*Vaccinium ashei* Reade) using spectral lights. *Scientia Horticulturae, 211*, 248-254. doi:10.1016/j.scienta.2016.09.003

Hung, C. D., Hong, C. H., Kim, S. K., Lee, K. H., Park, J. Y., Nam, M. W., Choi, D. H., & Lee, H. I. (2016b). LED light for *in vitro* and *ex vitro* efficient growth of economically important highbush blueberry (*Vaccinium corymbosum* L.). *Acta Physiologiae Plantarum, 38*(6), 152. doi:10.1007/s11738-016-2164-0

Hunter, R. L., & Markert, C. L. (1957). Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. *Science, 125*(3261), 1294-1295. doi:10.1126/science.125.3261.1294-a

Ibarra, C. A., Feng, X., Schoft, V. K., Hsieh, T.-F., Uzawa, R., Rodrigues, J. A., Zemach, A., Chumak, N., Machlicova, A., Nishimura, T., Rojas, D., Fischer, R. L., Tamaru, H., & Zilberman, D. (2012). Active DNA demethylation in plant companion cells reinforces transposon methylation in gametes. *Science, 337*(6100), 1360-1364. doi:10.1126/science.1224839

Ikeuchi, M., Sugimoto, K., & Iwase, A. (2013). Plant callus: mechanisms of induction and repression. *Plant Cell, 25*, 3159-3173. doi:10.1105/tpc.113.116053

Imazio, S., Labra, M., Grassi, F., Winfield, M., Bardini, M., & Scienza, A. (2002). Molecular tools for clone identification: The case of the grapevine cultivar 'Traminer'. *Plant Breeding, 121*(6), 531-535. doi:10.1046/j.1439-0523.2002.00762.x

Jaakola, L., Määttä, K., Pirttilä, A. M., Törrönen, R., Kärenlampi, S., & Hohtola, A. (2002). Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonol levels during bilberry fruit development. *Plant Physiology, 130*(2), 729-739. doi:10.1104/pp.006957

Jain, S. M. (2001). Tissue culture-derived variation in crop improvement. *Euphytica, 118*(2), 153-166. doi:10.1023/A:1004124519479

Jaligot, E., Hooi, W. Y., Debladis, E., Richaud, F., Beulé, T., Collin, M., Agbessi, M. D. T., Sabot, F., Garsmeur, O., D'Hont, A., Alwee, S. S. R. S., Rival, A., & Bendahmane, M. (2014). DNA methylation and expression of the *EgDEF1* gene and neighboring retrotransposons in mantled somaclonal variants of oil palm. *PLoS ONE, 9*(3), e91896. doi:10.1371/journal.pone.0091896

Jaligot, E., Rival, A., Beulé, T., Dussert, S., & Verdeil, J. L. (2000). Somaclonal variation in oil palm (*Elaeis guineensis* Jacq.): the DNA methylation hypothesis. *Plant Cell Reports, 19*(7), 684-690. doi:10.1007/s002999900177

Jamieson, A. R., & Nickerson, N. L. (2003). Field performance of the lowbush blueberry propagated by seed, stem cuttings and micropropagation. *Acta Horticulturae, 626,* 431-436. doi:10.17660/ActaHortic.2003.626.58

Johnston, M., Onwueme, I. C., Dowling, A. J., & Rodoni, B. C. (1997). Comparison of suckering, leaf and corm characteristics of taro grown from tissue culture and conventional planting material. *Australian Journal of Experimental Agriculture, 37*(4), 469-475. doi:10.1071/EA96059

Jones, C. G., & Hartley, S. E. (1999). A protein competition model of phenolic allocation. *Oikos, 86*(1), 27-44. doi:10.2307/3546567

Jones, C. J., Edwards, K. J., Castaglione, S., Winfield, M. O., Sala, F., van de Wiel, C., Bredemeijer, G., Vosman, B., Matthes, M., Daly, A., Brettschneider, R., Bettini, P.,

Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Volckaert, G., Rueda, J., Linacero, R., Vazquez, A., & Karp, A. (1997). Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding, 3*(5), 381-390. doi:10.1023/A:1009612517139

Joyce, S., & Cassells, A. (2002). Variation in potato microplant morphology *in vitro* and DNA methylation. *Plant Cell, Tissue and Organ Culture, 70*(2), 125-137. doi:10.1023/A:1016312303320

Joyce, S., Cassells, A., & Mohan Jain, S. (2003). Stress and aberrant phenotypes *in vitro* culture. *Plant Cell, Tissue and Organ Culture, 74*(2), 103-121. doi:10.1023/A:1023911927116

Kaeppler, S., Kaeppler, H., & Rhee, Y. (2000). Epigenetic aspects of somaclonal variation in plants. *Plant Molecular Biology, 43*(2-3), 179-188. doi:10.1023/A:1006423110134

Kaeppler, S. M., & Phillips, R. L. (1993). Tissue culture-induced DNA methylation variation in maize. *Proceedings of the National Academy of Sciences of the United States of America, 90*(19), 8773-8776.

Kähkönen, M. P., Hopia, A. I., & Heinonen, M. (2001). Berry phenolics and their antioxidant activity. *Journal of Agricultural and Food Chemistry, 49*(8), 4076-4082. doi:10.1021/jf010152t

Kaldmäe, H., Karp, K., Starast, M., & Paal, T. (2006). Effect of donor plant physiological condition on *in vitro* establishment of *Vaccinium angustifolium* shoot explants. *Acta Horticulturae, 715*, 433-438. doi:10.17660/ActaHortic.2006.715.65

Kalt, W., & Dufour, D. (1997). Health functionality of blueberries. *HortTechnology, 7*(3), 216-221.

Kalt, W., Forney, C. F., Martin, A., & Prior, R. L. (1999a). Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. *Journal of Agricultural and Food Chemistry, 47*(11), 4638-4644. doi:10.1021/jf990266t

Kalt, W., Howell, A., Duy, J. C., Forney, C. F., & McDonald, J. E. (2001a). Horticultural factors affecting antioxidant capacity of blueberries and other small fruit. *HortTechnology, 11*(4), 523-528.

Kalt, W., Howell, A. B., Mackinnon, S. L., & Goldman, I. L. (2007). Selected bioactivities of *Vaccinium* berries and other fruit crops in relation to their phenolic contents. *Journal of the Science of Food and Agriculture, 87*(12), 2279-2285. doi:10.1002/jsfa.2985

Kalt, W., Lawand, C., Ryan, D. A. J., McDonald, J. E., Donner, H., & Forney, C. F. (2003). Oxygen radical absorbing capacity, anthocyanin and phenolic content of highbush blueberries (*Vaccinium corymbosum* L.) during ripening and storage. *Journal of the American Society for Horticultural Science, 128*(6), 917-923.

Kalt, W., & McDonald, J. E. (1996). Chemical composition of lowbush blueberry cultivars. *American Society for Horticultural Science, 121*(1), 142-146.

Kalt, W., McDonald, J. E., Ricker, R. D., & Lu, X. (1999b). Anthocyanin content and profile within and among blueberry species. *Canadian Journal of Plant Science, 79*(4), 617-623. doi:10.4141/P99-009

Kalt, W., Ryan, D. A. J., Duy, J. C., Prior, R. L., Ehlenfeldt, M. K., & Vander Kloet, S. P. (2001b). Interspecific variation in anthocyanins, phenolics, and antioxidant capacity among genotypes of highbush and lowbush blueberries (*Vaccinium* Section *cyanococcus* spp.). *Journal of Agricultural and Food Chemistry, 49*(10), 4761-4767. doi:10.1021/jf010653e

Kan, Y. W., & Dozy, A. M. (1978). Polymorphism of DNA sequence adjacent to human beta-globin structural gene: relationship to sickle mutation. *Proceedings of the National Academy of Sciences of the United States of America, 75*(11), 5631-5635.

Kang, B.-H., Racicot, K., Pilkenton, S., Kwon, Y.-I., & Apostolidis, E. (2015). Blueberry extract inhibits carbohydrate-hydrolyzing enzymes and these inhibitory activities are not proanthocyanidin dependent. *Journal of the Korean Society for Applied Biological Chemistry, 58*(1), 127-136. doi:10.1007/s13765-015-0001-6

Kang, B., Racicot, K., Pilkenton, S., & Apostolidis, E. (2016). In-vitro evaluation of bioactive fractions of blueberry extract for phenolic-mediated inhibition of carbohydrate hydrolyzing enzymes. *International Journal of Applied Research in Natural Products, 9*(2), 33-38.

Karp, A. (1995). Somaclonal variation as a tool for crop improvement. *Euphytica, 85*(1), 295-302. doi:10.1007/BF00023959

Karuppusamy, S. (2009). A review on trends in production of secondary metabolites from higher plants by *in vitro* tissue, organ and cell cultures. *Journal of Medicinal Plants Research, 3*(13), 1222-1239.

Kashkush, K., Feldman, M., & Levy, A. A. (2002). Transcriptional activation of retrotransposons alters the expression of adjacent genes in wheat. *Nature Genetics, 33*(1), 102-106. doi:10.1038/ng1063

Kay, C. D., & Holub, B. J. (2002). The effect of wild blueberry (*Vaccinium angustifolium*) consumption on postprandial serum antioxidant status in human subjects. *The British Journal of Nutrition, 88*(4), 389-398. doi:10.1079/BJN2002665

Kesseli, R. V., Paran, I., & Michelmore, R. W. (1994). Analysis of a detailed genetic linkage map of *Lactuca sativa* (lettuce) constructed from RFLP and RAPD markers. *Genetics, 136*(4), 1435-1446.

Khalaf, N. A., Shakya, A. K., Al-Othman, A., El-Agbar, Z., & Farah, H. (2008). Antioxidant activity of some common plants. *Turkish Journal of Biology, 32*(1), 51-55.

Khawale, R., Singh, S., & Vimala, Y. (2006). Assessment of clonal fidelity of micropropagated grape (*Vitis vinifera* L.) plants by RAPD analysis. *Physiology and Molecular Biology of Plants, 12*(2), 189-192.

King, A. M. Y., & Young, G. (1999). Characteristics and occurrence of phenolic phytochemicals. *Journal of the American Dietetic Association, 99*(2), 213-218. doi:10.1016/S0002-8223(99)00051-6

Kliewer, W. M. (1977). Influence of temperature, solar radiation and nitrogen on coloration and composition of Emperor grapes. *American Journal of Enology and Viticulture, 28*(2), 96-103.

Knop, W. (1865). Quantitative untersuchungen uber die ernahrungsprozesse der Pflanzen. *Landwirtsch Vers. Stn., 7*, 93-107.

Koca, I., & Karadeniz, B. (2009). Antioxidant properties of blackberry and blueberry fruits grown in the Black Sea Region of Turkey. *Scientia Horticulturae, 121*(4), 447-450. doi:10.1016/j.scienta.2009.03.015

Kohlenbach, H.-W. (1959). Streckungs und tellungswachstum isolierter mesophyllzellen von *Macleaya cordata* (Willd.) R. Br. *Naturwissenschaften, 46*(3), 116-117. doi:10.1007/BF00638335

Konar, R. N., Thomas, E., & Street, H. E. (1972). Origin and structure of embryoids arising from epidermal cells of the stem of *Ranunculus sceleratus* L. *Journal of Cell Science, 11*(1), 77-93.

Kosar, M., Kafkas, E., Paydas, S., & Baser, K. H. C. (2004). Phenolic composition of strawberry genotypes at different maturation stages. *Journal of Agricultural and Food Chemistry, 52*(6), 1586. doi:10.1021/jf035093t

Kovarˇik, A., Koukalová, B., Bezdeˇk, M. & Opatrn´, Z. (1997b). Hypermethylation of tobacco heterochromatic loci in response to osmotic stress. *Theoretical and Applied Genetics*, 95(1), 301-306. doi:10.1007/s001220050563

Kovarík, A., Matyásek, R., Leitch, A., Gazdová, B., Fulnecek, J., & Bezdek, M. (1997b). Variability in CpNpG methylation in higher plant genomes. *Gene, 204*(1), 25-33. doi: 10.1016/S0378-1119(97)00503-9.

Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M., & Sadh, R. (2016). Somaclonal variations and their applications in horticultural crops improvement. *3 Biotech, 6*(1), 54. doi:10.1007/s13205-016-0389-7

Krizova, K., Fojtova, M., Depicker, A., & Kovarik, A. (2009). Cell culture-induced gradual and frequent epigenetic reprogramming of invertedly repeated tobacco transgene epialleles. *Plant Physiology, 149*(3), 1493-1504. doi:10.1104/pp.108.133165

Krupa, T., & Tomala, K. (2007). Antioxidant capacity, anthocyanin content profile in 'Bluecrop' blueberry fruit. *Vegetable Crops Research Bulletin, 66*, 129-141. doi:10.2478/v10032-007-0015-7

Kubis, S., Castilho, A. M. F., Vershinin, A., & Heslop-Harrison, J. (2003). Retroelements, transposons and methylation status in the genome of oil palm (*Elaeis guineensis*) and the relationship to somaclonal variation. *Plant Molecular Biology, 52*(1), 69-79. doi:10.1023/A:1023942309092

Kuo, K. C., McCune, R. A., Gehrke, C. W., Midgett, R., & Ehrlich, M. (1980). Quantitative reversed-phase high performance liquid chromatographic determination of major

and modified deoxyribonucleosides in DNA. *Nucleic Acids Research, 8*(20), 4763-4776. doi:10.1093/nar/8.20.4763

Lakshmanan, V., Venkataramareddy, S. R., & Neelwarne, B. (2007). Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers. *Electronic Journal of Biotechnology, 10*(1), 106-113. doi:10.2225/vol10-issue5-fulltext-12

Landey, R. B., Cenci, A., Georget, F., Bertrand, B., Camayo, G., Dechamp, E., Herrera, J. C., Santoni, S., Lashermes, P., Simpson, J., & Etienne, H. (2013). High genetic and epigenetic stability in *Coffea arabica* plants derived from embryogenic suspensions and secondary embryogenesis as revealed by AFLP, MSAP and the phenotypic variation rate. *PLoS ONE, 8*(2), e56372. doi:10.1371/journal.pone.0056372

Landey, R. B., Cenci, A., Guyot, R., Bertrand, B., Georget, F., Dechamp, E., Herrera, J.-C., Aribi, J., Lashermes, P., & Etienne, H. (2015). Assessment of genetic and epigenetic changes during cell culture ageing and relations with somaclonal variation in *Coffea arabica*. *Plant Cell, Tissue and Organ Culture, 122*(3), 517-531. doi:10.1007/s11240-015-0772-9

Larkin, P., & Scowcroft, W. (1981). Somaclonal variation – a novel source of variability from cell cultures for plant improvement. *Theoretical and Applied Genetics, 60*(4), 197-214. doi:10.1007/BF02342540

Larson, R. A. (1988). The antioxidants of higher plants. *Phytochemistry, 27*(4), 969-978. doi:10.1016/0031-9422(88)80254-1

Law, R. D., & Suttle, J. C. (2005). Chromatin remodeling in plant cell culture: patterns of DNA methylation and histone H3 and H4 acetylation vary during growth of asynchronous potato cell suspensions. *Plant Physiology and Biochemistry, 43*(6), 527-534. doi:10.1016/j.plaphy.2005.03.014

Leduc, C., Coonishish, J., Haddad, P., & Cuerrier, A. (2006). Plants used by the Cree Nation of Eeyou Istchee (Quebec, Canada) for the treatment of diabetes: a novel approach in quantitative ethnobotany. *Journal of Ethnopharmacology, 105*(1), 55-63. doi:10.1016/j.jep.2005.09.038

Lee, J., Durst, R. W., & Wrolstad, R. E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. *Journal of AOAC International, 88*(5), 1269-1278.

Leljak-Levanić, D., Bauer, N., Mihaljević, S., & Jelaska, S. (2004). Changes in DNA methylation during somatic embryogenesis in *Cucurbita pepo* L. *Plant Cell Reports, 23*(3), 120-127. doi:10.1007/s00299-004-0819-6

Leonardi, M. (1993). Treatment of fibrocystic disease of the breast with *Myrtillus* anthocyanins. Our experience. *Minerva Ginecologica, 45*(12), 617-621.

Lepiniec, L., Debeaujon, I., Routaboul, J.-M., Baudry, A., Pourcel, L., Nesi, N., & Caboche, M. (2006). Genetics and biochemistry of seed flavonoids. *Annual Review of Plant Biology, 57*(1), 405-430. doi:doi:10.1146/annurev.arplant.57.032905.105252

Levi, A., & Rowland, L. J. (1997). Identifying blueberry cultivars and evaluating their genetic relationships using randomly amplified polymorphic DNA (RAPD) and simple sequence repeat- (SSR-) anchored primers. *Journal of the American Society for Horticultural Science, 122*(1), 74-78.

Li, D., Li, B., Ma, Y., Sun, X., Lin, Y., & Meng, X. (2017). Polyphenols, anthocyanins, and flavonoids contents and the antioxidant capacity of various cultivars of highbush and half-high blueberries. *Journal of Food Composition and Analysis, 62*(Supplement C), 84-93. doi:10.1016/j.jfca.2017.03.006

Li, H., Geng, M., Liu, Q., Jin, C., Zhang, Q., Chen, C., Song, W., & Wang, C. (2014). Characteristics of cytosine methylation status and methyltransferase genes in the early development stage of cauliflower (*Brassica oleracea* L. var. Otrytis). *Plant Cell, Tissue and Organ Culture, 117*(2), 187-199. doi:10.1007/s11240-014-0432-5

Li, X., Xu, M., & Korban, S. S. (2002). DNA methylation profiles differ between field- and *in vitro*-grown leaves of apple. *Journal of Plant Physiology, 159*(11), 1229-1234. doi:10.1078/0176-1617-00899

Li, X., Yu, X., Wang, N., Feng, Q., Dong, Z., Liu, L., Shen, J., & Liu, B. (2007). Genetic and epigenetic instabilities induced by tissue culture in wild barley (*Hordeum brevisubulatum* (Trin.) Link). *Plant Cell, Tissue and Organ Culture, 90*(2), 153-168. doi:10.1007/s11240-007-9224-5

Li, Y.-C., Korol, A. B., Fahima, T., & Nevo, E. (2004). Microsatellites within genes: structure, function, and evolution. *Molecular Biology and Evolution, 21*(6), 991-1007. doi:10.1093/molbev/msh073

Li, Y., & Hong, Y. (2009). The current status and future of the blueberry industry in China. *Acta Horticulturae, 810*, 445-456. doi:10.17660/ActaHortic.2009.810.58

Linacero, R., Rueda, J., Esquivel, E., Bellido, A., Domingo, A., & Vázquez, A. M. (2011). Genetic and epigenetic relationship in rye, *Secale cereale* L., somaclonal variation within somatic embryo-derived plants. *In vitro Cellular and Developmental Biology - Plant, 47*(5), 618-628. doi:10.1007/s11627-011-9407-y

Lister, R., O'Malley, R. C., Tonti-Filippini, J., Gregory, B. D., Berry, C. C., Millar, A. H., & Ecker, J. R. (2008). Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. *Cell, 133*(3), 523-536. doi:10.1016/j.cell.2008.03.029

Lister, R., Pelizzola, M., Dowen, R. H., Hawkins, R. D., Hon, G., Tonti-Filippini, J., Nery, J. R., Lee, L., Ye, Z., Ngo, Q.-M., Edsall, L., Antosiewicz-Bourget, J., Stewart, R.,

Ruotti, V., Millar, A. H., Thomson, J. A., Ren, B., & Ecker, J. R. (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature, 462*(7271), 315-322. doi:10.1038/nature08514

Litwińczuk, W. (2013). Micropropagation of *Vaccinium* sp. by *in vitro* axillary shoot proliferation. In M. Lambardi, E. A. Ozudogru, & S. M. Jain (Eds.), *Protocols for Micropropagation of Selected Economically-Important Horticultural Plants* (pp. 63-76). Totowa, NJ: Humana Press.

Litwińczuk, W., Szczerba, G., & Wrona, D. (2005). Field performance of highbush blueberries (*Vaccinium* x *corymbosum* L.) cv. 'Herbert' propagated by cuttings and tissue culture. *Scientia Horticulturae, 106*(2), 162-169. doi:10.1016/j.scienta.2005.02.025

Litwińczuk, W., & Wadas, M. (2008). Auxin-dependent development and habituation of highbush blueberry (*Vaccinium* × *covilleanum* But. et Pl.) 'Herbert' *in vitro* shoot cultures. *Scientia Horticulturae, 119*(1), 41-48. doi:10.1016/j.scienta.2008.07.007

Liu, C., Callow, P., Rowland, L., Hancock, J., & Song, G.-Q. (2010). Adventitious shoot regeneration from leaf explants of southern highbush blueberry cultivars. *Plant Cell, Tissue and Organ Culture, 103*(1), 137-144. doi:10.1007/s11240-010-9755-z

Liu, Y., Tan, D., Tong, C., Zhang, Y., Xu, Y., Liu, X., Gao, Y., & Hou, M. (2015). Blueberry anthocyanins ameliorate radiation-induced lung injury through the protein kinase RNA-activated pathway. *Chemico-Biological Interactions, 242*, 363-371. doi:10.1016/j.cbi.2015.11.001

Lloyd, G., & McCown, B. (1980). Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia,* by use of shoot tip culture. *Proceedings of the International Plant Propagator's Society, 30*, 421-427.

Lopes, T., Pinto, G., Loureiro, J., Costa, A., & Santos, C. (2006). Determination of genetic stability in long-term somatic embryogenic cultures and derived plantlets of cork oak using microsatellite markers. *Tree Physiology, 26*(9), 1145-1152. doi:10.1093/treephys/26.9.1145

LoSchiavo, F., Pitto, L., Giuliano, G., Torti, G., Nuti-Ronchi, V., Marazziti, D., Vergara, R., Orselli, S., & Terzi, M. (1989). DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. *Theoretical and Applied Genetics, 77*(3), 325-331. doi:10.1007/BF00305823

Lu, Y., Rong, T., & Cao, M. (2008). Analysis of DNA methylation in different maize tissues. *Journal of Genetics and Genomics, 35*(1), 41-48. doi:10.1016/S1673-8527(08)60006-5

Luby, J. J., Ballington, J. R., Draper, A. D., Pliszka, K., & Austin, M. E. (1991). Blueberries and cranberries (*Vaccinium*). *Acta Horticulturae, 290*, 393-458. doi:10.17660/ActaHortic.1991.290.9

Luczkiewicz, M., Kokotkiewicz, A., & Glod, D. (2014). Plant growth regulators affect biosynthesis and accumulation profile of isoflavone phytoestrogens in high-productive *in vitro* cultures of *Genista tinctoria*. *Plant Cell Tissue and Organ Culture, 118*(3), 419-429. doi:10.1007/s11240-014-0494-4

Lugato, D., Simão, M., Garcia, R., Mansur, E., & Pacheco, G. (2014). Determination of antioxidant activity and phenolic content of extracts from *in vivo* plants and *in vitro* materials of *Passiflora alata* Curtis. *Plant Cell, Tissue and Organ Culture, 118*(2), 339-346. doi:10.1007/s11240-014-0486-4

Lyrene, P. M. (1980). Micropropagation of rabbiteye blueberries [Shoot culture]. *HortScience, 15*(1), 80-81.

Lyrene, P. M., & Perry, J. (1983). Production and selection of blueberry polyploids *in vitro*. *Journal of Heredity, 73*(5), 377-378. doi:10.1093/oxfordjournals.jhered.a109674

Lyrene, P. M. (1981). Juvenility and production of fast- rooting cuttings from blueberry shoot cultures. *Journal of the American Society for Horticultural Science, 106*(3), 396-398.

Maas, J. L., Wang, S. Y., & Galletta, G. J. (1991). Evaluation of strawberry cultivars for ellagic acid content. *HortScience, 26*(1), 66-68.

Machczyńska, J., Orłowska, R., Mańkowski, D., Zimny, J., & Bednarek, P. (2014). DNA methylation changes in triticale due to *in vitro* culture plant regeneration and consecutive reproduction. *Plant Cell, Tissue and Organ Culture, 119*(2), 289-299. doi:10.1007/s11240-014-0533-1

Macheix, J. J., Fleuriet, A., & Billot, J. (1990). *Fruit phenolics*. Boca Raton, FL, USA: CRC Press.

Madlung, A., & Comai, L. (2004). The effect of stress on genome regulation and structure. *Annals of Botany, 94*(4), 481-495. doi:10.1093/aob/mch172

Mahmood, T., Anwar, F., Abbas, M., & Saari, N. (2012). Effect of maturity on phenolics (phenolic acids and flavonoids) profile of strawberry cultivars and mulberry species from pakistan. *International Journal of Molecular Sciences, 13*(4), 4591-4607. doi:10.3390/ijms13044591

Mainland, C. M., & Tucker, J. W. (2002). Blueberry health information - some new mostly review. *Acta Horticulturae*(574), 39-43. doi:10.17660/ActaHortic.2002.574.3

Mallón, R., Rodríguez-Oubiña, J., & González, M. (2010). *In vitro* propagation of the endangered plant *Centaurea ultreiae*: assessment of genetic stability by cytological

studies, flow cytometry and RAPD analysis. *Plant Cell, Tissue and Organ Culture, 101*(1), 31-39. doi:10.1007/s11240-009-9659-y

Marino, S. R., Williamson, J. G., Olmstead, J. W., & Harmon, P. F. (2014). Vegetative growth of three Southern highbush blueberry cultivars obtained from micropropagation and softwood cuttings in two Florida locations. *HortScience, 49*(5), 556-561.

Martelli, G., Greco, I., Mezzetti, B., & Rosati, P. (1993). Isozymic analysis of somaclonal variation among regenerants from apple rootstock leaf tissue. *Acta Horticulturae*, 336, 381-388. doi:10.17660/ActaHortic.1993.336.50

Martineau, L. C., Couture, A., Spoor, D., Benhaddou-Andaloussi, A., Harris, C., Meddah, B., Leduc, C., Burt, A., Vuong, T., Mai Le, P., Prentki, M., Bennett, S. A., Arnason, J. T., & Haddad, P. S. (2006). Anti-diabetic properties of the Canadian lowbush blueberry *Vaccinium angustifolium* Ait. *Phytomedicine, 13*(9-10), 612. doi:10.1016/j.phymed.2006.08.005

Mastan, S., Rathore, M., Bhatt, V., Chikara, J., & Ghosh, A. (2014). DNA methylation and methylation polymorphism in ecotypes of *Jatropha curcas* L. using methylation-sensitive AFLP markers. *Molecular Biology Reports, 41*(12), 8261-8271. doi:10.1007/s11033-014-3734-0

Matchett, M. D., MacKinnon, S. L., Sweeney, M. I., Gottschall-Pass, K. T., & Hurta, R. A. R. (2005). Blueberry flavonoids inhibit matrix metalloproteinase activity in DU145 human prostate cancer cells. *Biochemistry and Cell Biology, 83*(5), 637-643. doi:10.1139/o05-063

Matkowski, A. (2008). Plant *in vitro* culture for the production of antioxidants - review. *Biotechnology Advances, 26*(6), 548-560. doi:http://dx.doi.org/10.1016/j.biotechadv.2008.07.001

McClelland, M., Nelson, M., & Raschke, E. (1994). Effect of site-specific modification on restriction endonucleases and DNA modification methyltransferases. *Nucleic Acids Research, 22*(17), 3640-3659. doi:10.1093/nar/22.17.3640

McClintock, B. (1984). The significance of responses of the genome to challenge. *Science, 226*(4676), 792-801. doi:10.1126/science.15739260

Meiners, J., Schwab, M., & Szankowski, I. (2007). Efficient *in vitro* regeneration systems for *Vaccinium* species. *Plant Cell, Tissue and Organ Culture, 89*(2-3), 169-176. doi:10.1007/s11240-007-9230-7

Messeguer, R., Ganal, M., Steffens, J., & Tanksley, S. (1991). Characterization of the level, target sites and inheritance of cytosine methylation in tomato nuclear DNA. *Plant Molecular Biology, 16*(5), 753-770. doi:10.1007/BF00015069

Miguel, C., & Marum, L. (2011). An epigenetic view of plant cells cultured *in vitro*: somaclonal variation and beyond. *Journal of Experimental Botany, 62*(11), 3713-3725. doi:10.1093/jxb/err155

Modgil, M., Mahajan, K., Chakrabarti, S. K., Sharma, D. R., & Sobti, R. C. (2005). Molecular analysis of genetic stability in micropropagated apple rootstock MM106. *Scientia Horticulturae, 104*(2), 151-160. doi:10.1016/j.scienta.2004.07.009

Mohr, N. A., & Kevan, P. G. (1987). Pollinators and pollination requirements of lowbush blueberry (*Vaccinium angustifolium* Ait. and *V. myrtiloides* Michx.) and cranberry (*V. macrocarpon* Ait.) in Ontario with notes on highbush blueberry (*V. corymbosum* L.) and lignonberry (*V. vitis-ideae* L.). *Proceedings of the Entomological Society of Ontario, 118,* 149-154.

Morgante, M., & Olivieri, A. M. (1993). PCR-amplified microsatellites as markers in plant genetics. *Plant Journal, 3*(1), 175-182. doi:10.1111/j.1365-313X.1993.tb00020.x

Morrison, S. E., & Smagula, J. M. (1986). Morphology, growth, and rhizome development of lowbush blueberry tissue culture plants, seedlings, and rooted softwood cuttings. *HortScience, 21*, 734-741.

Morrison, S. E., Smagula, J. M., & Litten, W. (2000). Morphology, growth, and rhizome development of *Vaccinium angustifolium* Ait. seedlings, rooted softwood cuttings, and micropropagated plantlets. *HortScience, 35*(4), 738-741.

Moyer, R. A., Hummer, K. E., Finn, C. E., Frei, B., & Wrolstad, R. E. (2002). Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: *Vaccinium*, *Rubus*, and *Ribes*. *Journal of Agricultural and Food Chemistry, 50*(3), 519-525. doi:10.1021/jf011062r

Mullis, K. B., & Faloona, F. A. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. *Methods in Enzymology, 155*, 335-350. doi:10.1016/0076-6879(87)55023-6

Muralitharan, M. S., Chandler, S. F., & Steveninck, R. F. M. v. (1992). Effects of Na_2SO_4, K_2SO_4 and KCl on growth and ion uptake of callus cultures of *Vaccinium corymbosum* L. cv. Bluecrop. *Annals of Botany, 69*(5), 459-462. doi:10.1093/oxfordjournals.aob.a088368

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum, 15*(3), 473-497. doi:10.1111/j.1399-3054.1962.tb08052.x

Naczk, M., Amarowicz, R., Zadernowski, R., Pegg, R. B., & Shahidi, F. (2003). Antioxidant activity of crude phenolic extracts from wild blueberry leaves. *Polish Journal of Food and Nutrition Sciences, 12*(1), 166-169.

Nag, D., Suri, M., & Stenson, E. (2004). Both CAG repeats and inverted DNA repeats stimulate spontaneous unequal sister-chromatid exchange in *Saccharomyces cerevisiae*. *Nucleic Acids Research, 32*(19), 5677-5684. doi:10.1093/nar/gkh901

Neelakandan, A., & Wang, K. (2012). Recent progress in the understanding of tissue culture-induced genome level changes in plants and potential applications. *Plant Cell Reports, 31*(4), 597-620. doi:10.1007/s00299-011-1202-z

Neto, C. C. (2007). Cranberry and blueberry: evidence for protective effects against cancer and vascular diseases. *Molecular Nutrition & Food Research, 51*(6), 652-664. doi:10.1002/mnfr.200600279

Ng, H.-H., & Adrian, B. (1999). DNA methylation and chromatin modification. *Current Opinion in Genetics and Development, 9*(2), 158-163. doi:http://dx.doi.org/10.1016/S0959-437X(99)80024-0

Nichenametla, S., Taruscio, T., Barney, D., & Exon, J. (2006). A review of the effects and mechanisms of polyphenolics in cancer. *Critical Reviews in Food Science and Nutrition, 46*(2), 161-183. doi:10.1080/10408390591000541

Nickerson, N. L. (1978a). *In vitro* shoot formation in lowbush blueberry seedling explants. *HortScience, 13*(6), 698.

Nickerson, N. L. (1978b). Callus formation in lowbush blueberry fruit explants cultured *in vitro*. *Horticultural Research, 18*(2), 85-91.

Nickerson, N. L., & Hall, I. V. (1976). Callus formation in stem internode sections of lowbush blueberry (*Vaccinium angustifolium* Ait.) cultured on a medium containing plant growth regulators. *Horticultural Research, 16*(1), 29-35.

Nimmakayala, P., Vajja, G., Gist, R., Tomason, Y., Levi, A., & Reddy, U. (2011). Effect of DNA methylation on molecular diversity of watermelon heirlooms and stability

of methylation specific polymorphisms across the genealogies. *Euphytica, 177*(1), 79-89. doi:10.1007/s10681-010-0259-z

Nookaraju, A., & Agrawal, D. C. (2011). Genetic homogeneity of *in vitro* raised plants of grapevine cv. Crimson Seedless revealed by ISSR and microsatellite markers. *South African Journal of Botany, 78*, 302-306. doi:10.1016/j.sajb.2011.08.009

Nordstrom, A., Tarkowski, P., Tarkowska, D., Norbaek, R., Astot, C., Dolezal, K., & Sandberg, G. (2004). Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: a factor of potential importance for auxin-cytokinin-regulated development. *Proceedings of the National Academy of Sciences of the United States of America, 101*(21), 8039-8044. doi:10.1073/pnas.0402504101

Norton, C., Kalea, A. Z., Harris, P. D., & Klimis-Zacas, D. J. (2005). Wild blueberry-rich diets affect the contractile machinery of the vascular smooth muscle in the sprague–dawley rat. *Journal of Medicinal Food, 8*(1), 8-13. doi:10.1089/jmf.2005.8.8

Nybom, H. (2004). Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. *Molecular Ecology, 13*(5), 1143-1155. doi:10.1111/j.1365-294X.2004.02141.x

Okie, W. R. (2002). Register of new fruit and nut varieties list 41. *HortScience, 37*(2), 251-272.

Olsson, M. E., Ekvall, J., Gustavsson, K. E., Nilsson, J., Pillai, D., Sjoholm, I., Svensson, U., Akesson, B., & Nyman, M. G. L. (2004). Antioxidants, low molecular weight carbohydrates, and total antioxidant capacity in strawberries (*Fragaria* x *ananassa*): effects of cultivar, ripening, and storage. *Journal of Agricultural and Food Chemistry, 52*(9), 2490-2498. doi:10.1021/jf030461e

Orlando, R., Magro, P., & Rugini, E. (1997). Pectic enzymes as a selective pressure tool for *in vitro* recovery of strawberry plants with fungal disease resistance. *Plant Cell Reports, 16*(5), 272-276. doi:10.1007/BF01088279

Orłowska, R., Machczyńska, J., Oleszczuk, S., Zimny, J., & Bednarek, P. T. (2016). DNA methylation changes and TE activity induced in tissue cultures of barley (*Hordeum vulgare* L.). *Journal Of Biological Research-Thessaloniki, 23*(1), 19. doi:10.1186/s40709-016-0056-5

Ozden, M., & Karaaslan, M. (2011). Effects of cytokinin on callus proliferation associated with physiological and biochemical changes in *Vitis vinifera* L. *Acta Physiologiae Plantarum, 33*(4), 1451-1459. doi:10.1007/s11738-010-0681-9

Paek, K. Y., Chakrabarty, D., & Hahn, E. J. (2005). Application of bioreactor systems for large scale production of horticultural and medicinal plants. *Plant Cell, Tissue and Organ Culture, 81*(3), 287-300. doi:10.1007/s11240-004-6648-z

Papandreou, M. A., Dimakopoulou, A., Linardaki, Z. I., Cordopatis, P., Klimis-Zacas, D., Margarity, M., & Lamari, F. N. (2009). Effect of a polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholinesterase activity. *Behavioural Brain Research, 198*(2), 352-358. doi:10.1016/j.bbr.2008.11.013

Pardue, M. L. (1991). Dynamic instability of chromosomes and genomes. *Cell, 66*(3), 427-431. doi:10.1016/0092-8674(81)90007-6

Park, S., Murthy, H., Chakrabarthy, D., & Paek, K. (2009). Detection of epigenetic variation in tissue-culture-derived plants of *Doritaenopsis* by methylation-sensitive amplification polymorphism (MSAP) analysis. *In vitro Cellular and Developmental Biology - Plant, 45*(1), 104-108. doi:10.1007/s11627-008-9166-6

Pathak, H., & Dhawan, V. (2012). ISSR assay for ascertaining genetic fidelity of micropropagated plants of apple rootstock Merton 793. *In vitro Cellular and Developmental Biology - Plant, 48*(1), 137-143. doi:10.1007/s11627-011-9385-0

Pathirana, R., Wiedow, C., Pathirana, S., Hedderley, D., Morgan, E., Scalzo, J., Frew, T., & Timmerman-Vaughan, G. (2015). Ovule culture and embryo rescue facilitate interspecific hybridisation in blueberry (*Vaccinium* spp.). *Acta Horticulturae, 1083*, 123-132. doi:10.17660/ActaHortic.2015.1083.13

Pavlica, M., Papeš, D., & Nagy, B. (1991). 2,4-Dichlorophenoxyacetic acid causes chromatin and chromosome abnormalities in plant cells and mutation in cultured mammalian cells. *Mutation Research Letters, 263*(2), 77-81. doi:10.1016/0165-7992(91)90063-A

Peraza-Echeverria, S., Herrera-Valencia, V., & Kay, A.-J. (2001). Detection of DNA methylation changes in micropropagated banana plants using methylation-sensitive amplification polymorphism (MSAP). *Plant Science, 161*(2), 359-367. doi:10.1016/S0168-9452(01)00421-6

Percival, D., & MacKenzie, J. L. (2007). Use of plant growth regulators to increase polyphenolic compounds in the wild blueberry. *Canadian Journal of Plant Science, 87*(2), 333-336. doi:10.4141/P06-120

Peredo, E. L., Arroyo-García, R., & Revilla, M. Á. (2009). Epigenetic changes detected in micropropagated hop plants. *Journal of Plant Physiology, 166*(10), 1101-1111. doi:10.1016/j.jplph.2008.12.015

Perry, J., & Lyrene, P. (1984). In vitro induction of tetraploidy in *Vaccinium darrowi*, *V. elliottii*, and *V. darrowi* × *V. elliottii* with colchicine treatment. *Journal of the American Society for Horticulture Science,* 109(1), 4-6.

Phillips, R. L., Kaeppler, S. M., & Olhoft, P. (1994). Genetic instability of plant tissue cultures: breakdown of normal controls. *Proceedings of the National Academy of Sciences of the United States of America, 91*(12), 5222-5226.

Pierik, R. (1991). Commercial aspects of micropropagation. In J. Prokash & R. L. M. Pierik (Eds.), *Horticulture - New Technologies and Applications* (pp. 141-153). Dordrecht, The Netherlands: Kluwer Academic Publishers.

Piljac-Zegarac, J., Belscak, A., & Piljac, A. (2009). Antioxidant capacity and polyphenolic content of blueberry (*Vaccinium corymbosum* L.) leaf infusions. *Journal of Medicinal Food, 12*(3), 608. doi:10.1089/jmf.2008.0081

Piola, F., Rohr, R., & Heizmann, P. (1999). Rapid detection of genetic variation within and among *in vitro* propagated cedar (*Cedrus libani* Loudon) clones. *Plant Science, 141*(2), 159-163. doi:dx.doi.org/10.1016/S0168-9452(98)00229-5

Polashock, J. J., & Vorsa, N. (1997). Evaluation of fingerprinting techniques for differentiation of cranberry and blueberry varieties. *Acta horticulturae*(446), 239-242. doi:10.17660/ActaHortic.1997.446.35

Popescu, A. N., Isac, V. S., Coman, M. S., & Radulescu, M. S. (1997). Somaclonal variation in plants regenerated by organogenesis from callus culture of strawberry (*Fragaria* x *ananassa*). *Acta Horticulturae, 439*, 89-96. doi:10.17660/ActaHortic.1997.439.8

Portis, E., Acquadro, A., Comino, C., & Lanteri, S. (2004). Analysis of DNA methylation during germination of pepper (*Capsicum annuum* L.) seeds using methylation-sensitive amplification polymorphism (MSAP). *Plant Science, 166*(1), 169-178. doi:10.1016/j.plantsci.2003.09.004

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., & Rafalski, A. (1996). The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers

for germplasm analysis. *Molecular Breeding, 2*(3), 225-238. doi:10.1007/BF00564200

Pradhan, S., Urwin, N. A. R., Jenkins, G. I., & Adams, R. L. P. (1999). Effect of CWG methylation on expression of plant genes. *Biochemical Journal*, *341*(3), 473-476. doi:10.1042/0264-6021:3410473

Prakash, A. P., Kush, A., Lakshmanan, P., & Kumar, P. P. (2003). Cytosine methylation occurs in a CDC48 homologue and a MADS-box gene during adventitious shoot induction in Petunia leaf explants. *Journal of Experimental Botany, 54*(386), 1361-1371. doi:10.1093/jxb/erg155

Predieri, S. (2001). Mutation induction and tissue culture in improving fruits. *Plant Cell, Tissue and Organ Culture, 64*(2), 185-210. doi:10.1023/A:1010623203554

Preil, W. (2005). General introduction: a personal reflection on the use of liquid media for *in vitro* culture. In A. K. Hvoslef-Eide & W. Preil (Eds.), *Liquid Culture Systems for in vitro Plant Propagation* (pp. 1-18). Dordrecht, The Netherlands: Springer

Price, M. L., Van Scoyoc, S., & Butler, L. G. (1978). A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *Journal of Agricultural and Food Chemistry, 26*(5), 1214-1218. doi:10.1021/jf60219a031

Prior, R. L., Cao, G., Martin, A., Sofic, E., McEwen, J., O'Brien, C., Lischner, N., Ehlenfeldt, M., Kalt, W., Krewer, G., & Mainland, C. M. (1998). Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. *Journal of Agricultural and Food Chemistry, 46*(7), 2686-2693. doi:10.1021/jf980145d

Prior, R. L., Hammerstone, J. F., Lazarus, S. A., Cao, G., & Muccitelli, H. (2001). Identification of procyanidins and anthocyanins in blueberries and cranberries (*Vaccinium* spp.) using high-performance liquid chromatography/mass

spectrometry. *Journal of Agricultural and Food Chemistry, 49*(3), 1270-1276. doi:10.1021/jf001211q

Qian, W., Ge, S., & Hong, D. Y. (2001). Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by RAPD and ISSR markers. *Theoretical and Applied Genetics, 102*(2), 440-449. doi:10.1007/s001220051665

Qu, L., Polashock, J., & Vorsa, N. (2000). A highly efficient *in vitro* cranberry regeneration system using leaf explants. *HortScience, 35*(5), 948-952.

Qureshi, J., Hucl, P., & Kartha, K. (1992). Is somaclonal variation a reliable tool for spring wheat improvement? *Euphytica, 60*(3), 221-228. doi:10.1007/BF00039402

Qureshi, S. N., Saha, S., Kantety, R. V., & Jenkins, J. N. (2004). EST-SSR: a new class of genetic markers in cotton. *Journal of Cotton Science, 8*(2), 112-123.

Radhakrishnan, R., & Ranjitha Kumari, B. D. (2008). Morphological and agronomic evaluation of tissue culture derived Indian soybean plants. *Acta Agriculturae Slovenica, 91*(2), 391-396. doi:10.2478/v10014-008-0019-0

Rahman, M. H., & Rajora, O. P. (2001). Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*). *Plant Cell Reports, 20*(6), 531-536. doi:10.1007/s002990100365

Randhir, R., Lin, Y.-T., & Shetty, K. (2004). Phenolics, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. *Asia Pacific Journal of Clinical Nutrition, 13*(3), 295-307.

Rangwala, S. H., & Richards, E. J. (2004). The value-added genome: building and maintaining genomic cytosine methylation landscapes. *Current Opinion in Genetics and Development, 14*(6), 686-691. doi:10.1016/j.gde.2004.09.009

Rani, V., & Raina, S. (2000). Genetic fidelity of organized meristem-derived micropropagated plants: a critical reappraisal. *In vitro Cellular and Developmental Biology - Plant, 36*(5), 319-330. doi:10.1007/s11627-000-0059-6

Ratel, D., Ravanat, J.-L., Berger, F., & Wion, D. (2006). N6-methyladenine: The other methylated base of DNA. *BioEssays, 28*(3), 309-315. doi:10.1002/bies.20342

Rathore, M. S., Chikara, J., Mastan, S. G., Rahman, H., Anand, K. G., & Shekhawat, N. S. (2011). Assessment of genetic stability and instability of tissue culture-propagated plantlets of *Aloe vera* L. by RAPD and ISSR Markers. *Applied Biochemistry and Biotechnology, 165*(5), 1356-1365. doi:10.1007/s12010-011-9352-6

Rathore, M. S., & Jha, B. (2016). DNA methylation and methylation polymorphism in genetically stable *in vitro* regenerates of *Jatropha curcas* L. using methylation-sensitive AFLP Markers. *Applied Biochemistry and Biotechnology, 178*(5), 1002-1014. doi:10.1007/s12010-015-1924-4

Rathore, M. S., Mastan, S. G., & Agarwal, P. K. (2015). Evaluation of DNA methylation using methylation-sensitive amplification polymorphism in plant tissues grown *in vivo* and *in vitro*. *Plant Growth Regulation, 75*(1), 11-19. doi:10.1007/s10725-014-9926-8

Ratnaparkhe, M. B. (2007). Blueberry. In C. Kole (Ed.), *Genome Mapping and Molecular Breeding in Plants: Fruits and Nuts* (Vol. 4, pp. 217-227). Heidelberg, Germany: Springer.

Read, P. E., Hartley, C. A., Sandahl, J. G., & Wildung, D. K. (1988). Field performance of *in vitro* propagated blueberries. *Proceedings of the International Plant Propagator's Society, 37*, 450-452.

Read, P. E., Wildung, D. K., & Hanky, C. A. (1989). Field performance of *in vitro*-propagated 'Northblue' blueberries. *Acta Horticulturae, 241*, 191-194. doi:10.17660/ActaHortic.1989.241.30

Reddy, P. M., Sarla, N., & Siddiq, E. A. (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica, 128*(1), 9-17. doi:10.1023/A:1020691618797

Reed, B. M., & Abdelnouresquivel, A. (1991). The use of zeatin to initiate *in vitro* cultures of *Vaccinium* species and cultivars. *HortScience, 26*(10), 1320-1322.

Regalado, J., Carmona Martín, E., Castro, P., Moreno, R., Gil, J., & Encina, C. (2015). Study of the somaclonal variation produced by different methods of polyploidization in *Asparagus officinalis* L. *Plant Cell, Tissue and Organ Culture, 122*(1), 31-44. doi:10.1007/s11240-015-0747-x

Renau-Morata, B., Nebauer, S. G., Arrillaga, I., & Segura, J. (2005). Assessments of somaclonal variation in micropropagated shoots of Cedrus: consequences of axillary bud breaking. *Tree Genetics and Genomes, 1*(1), 3-10. doi:10.1007/s11295-004-0001-x

Retamales, J. B., & Hancock, J. F. (2012). *Blueberries*. Cambridge, MA, USA: CABI.

Reyes-Díaz, M., Inostroza-Blancheteau, C., Millaleo, R., Cruces, E., Wulff-Zottele, C., Alberdi, M., & Mora, M. d. l. L. (2010). Long-term aluminum exposure effects on physiological and biochemical features of highbush blueberry cultivars. *Journal of the American Society for Horticultural Science, 135*(3), 212-222.

Reyna-López, G. E., Simpson, J., & Ruiz-Herrera, J. (1997). Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. *Molecular and general genetics, 253*(6), 703-710. doi:10.1007/s004380050374

Ribera, A. E., Reyes-Díaz, M., Alberdi, M., Zuñiga, G. E., & Mora, M. L. (2010). Antioxidant compounds in skin and pulp of fruits change among genotypes and maturity stages in highbush blueberry (*Vaccinium corymbosum* L.) grown in Southern Chile. *Journal of Soil Science and Plant Nutrition, 10*(4), 509-536. doi:10.4067/S0718-95162010000200010

Richards, E. J. (2006). Inherited epigenetic variation - revisiting soft inheritance. *Nature Reviews Genetics, 7*(5), 395-401. doi:10.1038/nrg1834

Riihinen, K., Jaakola, L., Kärenlampi, S., & Hohtola, A. (2008). Organ-specific distribution of phenolic compounds in bilberry (*Vaccinium myrtillus*) and 'Northblue' blueberry (*Vaccinium corymbosum* x *V. angustifolium*). *Food Chemistry, 110*(1), 156-160. doi:10.1016/j.foodchem.2008.01.057

Rival, A., Ilbert, P., Labeyrie, A., Torres, E., Doulbeau, S., Personne, A., Dussert, S., Beulé, T., Durand-Gasselin, T., Tregear, J., & Jaligot, E. (2013). Variations in genomic DNA methylation during the long-term *in vitro* proliferation of oil palm embryogenic suspension cultures. *Plant Cell Reports, 32*(3), 359-368. doi:10.1007/s00299-012-1369-y

Robards, K., Prenzler, P. D., Tucker, G., Swatsitang, P., & Glover, W. (1999). Phenolic compounds and their role in oxidative processes in fruits. *Food Chemistry, 66*(4), 401-436. doi:10.1016/S0308-8146(99)00093-X

Rodríguez-López, C. M., Wetten, A. C., & Wilkinson, M. J. (2010). Progressive erosion of genetic and epigenetic variation in callus-derived cocoa (*Theobroma cacao*) plants. *New Phytologist, 186*(4), 856-868. doi:10.1111/j.1469-8137.2010.03242.x

Rowland, L. J., & Hammerschlag, F. A. (2005). *Vaccinium* spp. blueberry. In R. E. Litz (Ed.), Biotechnology of fruit and nut crops (Vol. Biotechnology in Agriculture Series pp. 222-246). Wallingford, UK: CABI Publishing.

Rowland, L. J., Hancock, J. F., & Bassil, N. V. (2011). Blueberry. In K. M. Folta & C. Kole (Eds.), *Genetics, Genomics and Breeding of Berries* (pp. 1-39). Boca Raton, FL, USA: CRC Press.

Rowland, L. J., Mehra, S., Dhanaraj, A. L., Ogden, E. L., Slovin, J. P., & Ehlenfeldt, M. K. (2003). Development of EST-PCR markers for DNA fingerprinting and genetic relationship studies in blueberry (*Vaccinium*, section *Cyanococcus*). *Journal of the American Society for Horticultural Science, 128*(5), 682-690.

Rowland, L. J., & Ogden, E. L. (1992). Use of a cytokinin conjugate for efficient shoot regeneration from leaf sections of highbush blueberry. *HortScience, 27*(10), 1127-1129.

Rungis, D., Bérubé, Y., Zhang, J., Ralph, S., Ritland, C., Ellis, B., Douglas, C., Bohlmann, J., & Ritland, K. (2004). Robust simple sequence repeat markers for spruce (*Picea* spp.) from expressed sequence tags. *Theoretical and Applied Genetics, 109*(6), 1283-1294. doi:10.1007/s00122-004-1742-5

Russell, J., Booth, A., Fuller, J., Harrower, B., Hedley, P., Machray, G., & Powell, W. (2004). A comparison of sequence-based polymorphism and haplotype content in transcribed and anonymous regions of the barley genome. *Genome, 47*(2), 389-398. doi:10.1139/g03-125

Russo, V. E. A., Martienssen, R. A., & Riggs, A. D. (1996). *Epigenetic Mechanisms of Gene Regulation*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Ružić, D., Vujović, T., Libiakova, G., Cerović, R., & Gajdošova, A. (2012). Micropropagation *in vitro* of highbush blueberry (*Vaccinium corymbosum* L.). *Journal of Berry Research, 2*(2), 97-103. doi:10.3233/JBR-2012-030

Ryan, D., & Robards, K. (1998). Phenolic compounds in olives. *Analyst, 123*(5), 31R-44R.

Ryu, T. H., Yi, S. I., Kwon, Y. S. E. m. y. s. g. k., & Kim, B. D. (2007). Microsatellite DNA somaclonal variation of regenerated plants via cotyledon culture of hot pepper (*Capsicum annuum* L.). *Korean Journal of Genetics, 29*(4), 459-464. doi:10.1007/s00299-009-0797-9

Sakakibara, H., Takei, K., & Hirose, N. (2006). Interactions between nitrogen and cytokinin in the regulation of metabolism and development. *Trends in Plant Science, 11*(9), 440-448. doi:10.1016/j.tplants.2006.07.004

Salvi, N., George, L., & Eapen, S. (2001). Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. *Plant Cell, Tissue and Organ Culture, 66*(2), 113-119. doi:10.1023/A:1010638209377

SAS Institute. (2002). SAS (Statistical Analysis Systems)/STAT user's guide. Version 9.1. Cary, NC, USA: SAS Institute, Inc.

Sato, M., Kawabe, T., Hosokawa, M., Tatsuzawa, F., & Doi, M. (2011). Tissue culture-induced flower-color changes in *Saintpaulia* caused by excision of the transposon inserted in the *flavonoid 3', 5' hydroxylase* (*F3'5'H*) promoter. *Plant Cell Reports, 30*(5), 929-939. doi:10.1007/s00299-011-1016-z

Scalzo, J., Stevenson, D., & Hedderley, D. (2013). Blueberry estimated harvest from seven new cultivars: fruit and anthocyanins. *Food Chemistry, 139*(1-4), 44-50. doi:10.1016/j.foodchem.2013.01.091

Schaffer, W. I. (1990). Terminology associated with cell, tissue and organ culture, molecular biology and molecular genetics. *In vitro Cellular and Developmental Biology - Plant, 26*(1), 97-101. doi:10.1007/BF02624162

Schellenbaum, P., Mohler, V., Wenzel, G., & Walter, B. (2008). Variation in DNA methylation patterns of grapevine somaclones (*Vitis vinifera* L.). *BMC Plant Biology, 8*(1), 78. doi:10.1186/1471-2229-8-78

Schmidt, B. M., Erdman Jr, J. W., & Lila, M. A. (2006). Differential effects of blueberry proanthocyanidins on androgen sensitive and insensitive human prostate cancer cell lines. *Cancer Letters, 231*(2), 240-246. doi:10.1016/j.canlet.2005.02.003

Schmidt, B. M., Howell, A. B., McEniry, B., Knight, C. T., Seigler, D., Erdman Jr, J. W., & Lila, M. A. (2004). Effective separation of potent antiproliferation and antiadhesion components from wild blueberry (*Vaccinium angustifolium* Ait.) fruits. *Journal of Agricultural and Food Chemistry, 52*(21), 6433-6442. doi:10.1021/jf049238n

Scognamiglio, M., D'Abrosca, B., Esposito, A., Pacifico, S., Monaco, P., & Fiorentino, A. (2013). Plant growth inhibitors: allelopathic role or phytotoxic effects? Focus on Mediterranean biomes. *Phytochemistry Reviews, 12*(4), 803-830. doi:10.1007/s11101-013-9281-9

Seeram, N. P., & Nair, M. G. (2002). Inhibition of lipid peroxidation and structure-activity-related studies of the dietary constituents anthocyanins, anthocyanidins, and catechins. *Journal of Agricultural and Food Chemistry, 50*(19), 5308-5312.

Sellappan, S., Akoh, C. C., & Krewer, G. (2002). Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *Journal of Agricultural and Food Chemistry, 50*(8), 2432-2438. doi:10.1021/jf011097r

Senthilvel, S., Jayashree, B., Mahalakshmi, V., Kumar, P. S., Nakka, S., Nepolean, T., & Hash, C. T. (2008). Development and mapping of simple sequence repeat markers for pearl millet from data mining of expressed sequence tags. *BMC Plant Biology, 8*, 119. doi:10.1186/1471-2229-8-119

Sharma, S. K., Bryan, G. J., Winfield, M. O., & Millam, S. (2007). Stability of potato (*Solanum tuberosum* L.) plants regenerated via somatic embryos, axillary bud proliferated shoots, microtubers and true potato seeds: a comparative phenotypic, cytogenetic and molecular assessment. *Planta, 226*(6), 1449-1458. doi:10.1007/s00425-007-0583-2

Shaughnessy, K. S., Boswall, I. A., Scanlan, A. P., Gottschall-Pass, K. T., & Sweeney, M. I. (2009). Diets containing blueberry extract lower blood pressure in spontaneously hypertensive stroke-prone rats. *Nutrition Research, 29*(2), 130-138. doi:10.1016/j.nutres.2009.01.001

Shen, C.-L., Von Bergen, V., Chyu, M.-C., Jenkins, M. R., Mo, H., Chen, C.-H., & Kwun, I.-S. (2012). Fruits and dietary phytochemicals in bone protection. *Nutrition Research, 32*(12), 897-910. doi:10.1016/j.nutres.2012.09.018

Shukitt-Hale, B. (2012). Blueberries and neuronal aging. *Gerontology, 58*(6), 518-523. doi:10.1159/000341101

Sianipar, N. F., Wattimena, G. A., Soehartono, M. T., Aswidinnoor, H., Toruan-Mathius, N., & Ginting, G. (2008). DNA methylation detection of oil palm (*Elaeis guineensis* Jacq) somatic embryo by randomly amplified DNA finger-printing with methylation–sensitive (RAF-SM) and RP-HPLC. *Journal of Biotechnology Research in Tropical Region, 1*, 1-6.

Simon, I., Racz, E., & Zatyko, J. M. (1987). Preliminary notes on somaclonal variations of strawberry. *Fruit Science Reports, 14*(4), 151-154.

Singh, S. R., Dalal, S., Singh, R., Dhawan, A. K., & Kalia, R. K. (2013). Ascertaining clonal fidelity of micropropagated plants of *Dendrocalamus hamiltonii* Nees et Arn. ex Munro using molecular markers. *In vitro Cellular and Developmental Biology - Plant, 49*(5), 572-583. doi:10.1007/s11627-013-9520-1

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*(3), 144-158.

Skirvin, R. M., Norton, M., & McPheeters, K. D. (1993). Somaclonal variation: has it proved useful for plant improvement? *Acta Horticulturae, 336,* 333-340. doi:10.17660/ActaHortic.1993.336.44

Skoog, F., & Miller, C. O. (1957). Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symposia of the Society for Experimental Biology, 11*, 118-131.

Skrovankova, S., Sumczynski, D., Mlcek, J., Jurikova, T., & Sochor, J. (2015). Bioactive compounds and antioxidant activity in different types of berries. *International Journal of Molecular Sciences, 16*(10), 24673-24706. doi:10.3390/ijms161024673

Skupień, K., Oszmiański, J., Kostrzewa-Nowak, D., & Tarasiuk, J. (2006). *In vitro* antileukaemic activity of extracts from berry plant leaves against sensitive and multidrug resistant HL60 cells. *Cancer Letters, 236*(2), 282-291. doi:10.1016/j.canlet.2005.05.018

Smith, M. A. L., Marley, K. A., Seigler, D., Singletary, K. W., & Meline, B. (2000). Bioactive properties of wild blueberry fruits. *Journal of Food Science, 65*(2), 352-356. doi:10.1111/j.1365-2621.2000.tb16006.x

Smith, M. A. L., & Spomer, L. A. (1995). Vessels, gels, liquid media, and support systems. In J. Aitken-Christie, T. Kozai, & M. A. L. Smith (Eds.), *Automation and Environmental Control in Plant Tissue Culture* (pp. 371-404). Dordrecht, The Netherlands: Springer.

Smulders, M., & Klerk, G. (2011). Epigenetics in plant tissue culture. *Plant Growth Regulation, 63*(2), 137-146. doi:10.1007/s10725-010-9531-4

Solecka, D. (1997). Role of phenylpropanoid compounds in plant responses to different stress factors. *Acta Physiologiae Plantarum, 19*(3), 257-268. doi:10.1007/s11738-997-0001-1

Soneji, J., Rao, P., & Mhatre, M. (2002). Suitability of RAPD for analyzing spined and spineless variant regenerants of pineapple (*Ananas comosus* L., Merr.). *Plant Molecular Biology Reporter, 20*(3), 307. doi:10.1007/BF02782469

Song, G. Q., & Hancock, J. F. (2012). Recent advances in blueberry transformation. *International Journal of Fruit Science, 12*(1-3), 316-332. doi:10.1080/15538362.2011.623083

Song, G. Q., Roggers, R. A., Sink, K. C., Particka, M., & Zandstra, B. (2007). Production of herbicide-resistant highbush blueberry 'Legacy' by *Agrobacterium*-mediated transformation of the *bar* gene. *Acta Horticulturae, 738*, 397-407. doi:10.17660/ActaHortic.2007.738.48

Song, X., Yang, J., Gong, Y., Zhao, L., Huang, D., & Liu, L. (2008). DNA methylation pattern in the process of floral bud differentiation in radish (*Raphanus sativus* L.) with MSAP analysis. *Acta Horticulturae, 774*, 409-416. doi:10.17660/ActaHortic.2008.774.56

Statistics Canada (2017). *Fruit and Vegetable Production*. (0022-4898). Retrieved from http://www5.statcan.gc.ca/cansim/a26?lang=eng&id=0010009&p2=17.

Steward, F. C., Ammirato, P. V., & Mapes, M. O. (1970). Growth and development of totipotent cells: some problems, procedures, and perspectives. *Annals of Botany, 34*(137), 761-787.

Strack, D. (1997). Phenolic metabolism. In P. M. Dey & J. B. Harborne (Eds.), *Plant Biochemistry* (pp. 387–416.). London, UK: Academic Press.

Strik, B. (2005). Blueberry: an expanding world berry crop. *Chronica Horticulturae, 45*(1), 7-12.

Strik, B. C., & Yarborough, D. (2005). Blueberry production trends in North America, 1992 to 2003, and predictions for growth. *HortTechnology, 15*(2), 391-398.

Sulkowska, M. K. (2012). Isoenzyme analyses tools used long time in forest science. In K. Ghowsi (Ed.), *Electrophoresis* (pp. 157-172). Rijeka, Croatia: InTech.

Suzuki, M. M., & Bird, A. (2008). DNA methylation landscapes: provocative insights from epigenomics. *Nature Reviews Genetics, 9*(6), 465-476. doi:10.1038/nrg2341

Swartz, H. J., Galletta, G. J., & Zimmerman, R. H. (1981). Field performance and phenotypic stability of tissue culture-propagated strawberries. *Journal American Society for Horticultural Science, 106*(5), 667-673.

Swartz, H. J., Galletta, G. J., & Zimmerman, R. H. (1983). Field performance and phenotypic stability of tissue culture-propagated thornless blackberries. *Journal American Society for Horticultural Science, 108*, 285-290.

Sweeney, M. I., Kalt, W., Mackinnon, S. L., Ashby, J., & Gottschall-Pass, K. T. (2002). Feeding rats diets enriched in lowbush blueberries for six weeks decreases ischemia-induced brain damage. *Nutritional Neuroscience, 5*(6), 427-431. doi:10.1080/1028415021000055970

Tailor, S., Bykova, N. V., Igamberdiev, A. U., & Debnath, S. C. (2017). Structural pattern and genetic diversity in blueberry (*Vaccinium*) clones and cultivars using EST-PCR and microsatellite markers. *Genetic Resources and Crop Evolution, 64*, 1-12. doi:10.1007/s10722-017-0497-1

Taiz, L., & Zeiger, E. (2006). *Plant Phyisiology*. Sunderland, MA, USA: Sinauer Associated Inc.

Takeshita, M., Ishida, Y. I., Akamatsu, E., Ohmori, Y., Sudoh, M., Oto, H., Tsubouchi, H., & Kataoka, H. (2009). Proanthocyanidin from blueberry leaves suppresses expression of subgenomic hepatitis C virus RNA. *Journal of Biological Chemistry, 284*(32), 21165-21176. doi:10.1074/jbc.M109.004945

Tanaka, M., Takei, K., Kojima, M., Sakakibara, H., & Mori, H. (2006). Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. *Plant Journal, 45*(6), 1028-1036. doi:10.1111/j.1365-313X.2006.02656.x

Tanurdzic, M., Vaughn, M. W., Hongmei, J., Tae-Jin, L., Slotkin, R. K., Sosinski, B., Thompson, W. F., Doerge, R. W., & Martienssen, R. A. (2008). Epigenomic consequences of immortalized plant cell suspension culture. *PLoS Biology, 6*(12), e302. doi:10.1371/journal.pbio.0060302

Tariq, M., & Paszkowski, J. (2004). DNA and histone methylation in plants. *Trends in Genetics, 20*(6), 244-251. doi:10.1016/j.tig.2004.04.005

Taskin, K., Özbilen, A., Sezer, F., Çördük, N., & Erden, D. (2015). Determination of the expression levels of DNA methyltransferase genes during a highly efficient regeneration system via shoot organogenesis in the diploid apomict *Boechera divaricarpa*. *Plant Cell, Tissue and Organ Culture, 121*(2), 335-343. doi:10.1007/s11240-014-0704-0

Taulavuori, E., Tahkokorpi, M., Taulavuori, K., & Laine, K. (2004). Anthocyanins and glutathione S-transferase activities in response to low temperature and frost hardening in *Vaccinium myrtillus* (L.). *Journal of Plant Physiology, 161*(8), 903-911. doi://10.1016/j.jplph.2003.12.001

Tautz, D., & Renz, M. (1984). Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Research, 12*(10), 4127-4138. doi:10.1093/nar/12.10.4127

Taveira, M., Pereira, D. M., Sousa, C., Ferreres, F., Andrade, P. B., Martins, A., Pereira, J. A., & Valentão, P. (2009). *In vitro* cultures of *Brassica oleracea* L. var. Costata DC: potential plant bioreactor for antioxidant phenolic compounds. *Journal of Agricultural and Food Chemistry, 57*(4), 1247-1252. doi:10.1021/jf803496x

Teleszko, M., & Wojdyło, A. (2015). Comparison of phenolic compounds and antioxidant potential between selected edible fruits and their leaves. *Journal of Functional Foods, 14*, 736-746. doi:10.1016/j.jff.2015.02.041

Tetsumura, T., Kajiwara, Y., Honsho, C., Sato-Yamauchi, M., Sugimoto, Y., & Kunitake, H. (2012). Effective micropropagation of rabbiteye blueberries for leaf tea production. *Environmental Control in Biology, 50*(3), 289-296. doi:10.2525/ecb.50.289

Tetsumura, T., Matsumoto, Y., Sato, M., Honsho, C., Yamashita, K., Komatsu, H., Sugimoto, Y., & Kunitake, H. (2008). Evaluation of basal media for micropropagation of four highbush blueberry cultivars. *Scientia Horticulturae, 119*(1), 72-74. doi:10.1016/j.scienta.2008.06.028

Thomas, A. D., Vardhman, K. R., Daniel, J. T., Flicek, P., Li, H., Kulesha, E., Stefan, G., Johnson, N., Herrero, J., Eleni, M. T., Natalie, P. T., Liselotte, B., Herberth, M., Kevin, L. H., David, K. J., Marcos, M. M., John, C. M., Birney, E., Tim, J. P. H., Durbin, R., Simon, T., & Beck, S. (2008). A Bayesian deconvolution strategy for immunoprecipitation-based DNA methylome analysis. *Nature Biotechnology, 26*(7), 779-785. doi:10.1038/nbt1414

Thorpe, T. A. (1993). *In vitro* organogenesis and somatic embryogenesis: physiological and biochemical aspects. In K. A. Roubelakis-Angelakis & K. T. Van Thanh (Eds.), *Morphogenesis in Plants: Molecular Approaches* (pp. 19-38). Boston, MA, USA: Springer.

Torres-Morán, M., Escoto-Delgadillo, M., Molina-Moret, S., Rivera-Rodríguez, D., Velasco-Ramírez, A., Infante, D., & Portillo, L. (2010). Assessment of genetic fidelity among *Agave tequilana* plants propagated asexually via rhizomes versus *in vitro* culture. *Plant Cell, Tissue and Organ Culture, 103*(3), 403-409. doi:10.1007/s11240-010-9777-6

Toyoda, H., Horikoshi, K., Yamano, Y., & Ouchi, S. (1991). Selection for *Fusarium* wilt disease resistance from regenerants derived from leaf callus of strawberry. *Plant Cell Reports, 10*(4), 167-170. doi:10.1007/BF00234287

Tsuda, H., Kunitake, H., Aoki, Y., Oyama, A., Tetsumura, T., Komatsu, H., & Yoshioka, K. (2014). Efficient *in vitro* screening for higher soil pH adaptability of intersectional hybrids in blueberry. *HortScience, 49*(2), 141-144.

Uysal, U., Seremet, S., Lamping, J. W., Adams, J. M., Liu, D. Y., Swerdlow, R. H., & Aires, D. J. (2013). Consumption of polyphenol plants may slow aging and associated diseases. *Current Pharmaceutical Design, 19*(34), 6094-6111. doi:10.2174/1381612811319340004

Valledor, L., Hasbún, R., Meijón, M., Rodríguez, J., Santamaría, E., Viejo, M., Berdasco, M., Feito, I., Fraga, M., Cañal, M., & Rodríguez, R. (2007). Involvement of DNA methylation in tree development and micropropagation. *Plant Cell, Tissue and Organ Culture, 91*(2), 75-86. doi:10.1007/s11240-007-9262-z

Valledor, L., Meijon, M., Hasbun, R., Canal, M., & Rodriguez, R. (2010). Variations in DNA methylation, acetylated histone H4, and methylated histone H3 during *Pinus radiata* needle maturation in relation to the loss of *in vitro* organogenic capability. *Journal of Plant Physiology, 167*(5), 351-357. doi:10.1016/j.jplph.2009.09.018

Vander Kloet, S. P. (1980). The taxonomy of the highbush blueberry, *Vaccinium corymbosum*. *Canadian Journal of Botany, 58*(10), 1187-1201. doi:10.1139/b80-148

Vander Kloet, S. P. (1977). The taxonomic status of *Vaccinium boreale*. *Canadian Journal of Botany, 55*(3), 281-288. doi:10.1139/b77-038

Vander Kloet, S. P. (1988). The genus *Vaccinium* in North America. *Research Branch Agriculture Canada Publication* (Vol. 1828).

Vanyushin, B. F., & Ashapkin, V. V. (2011). DNA methylation in higher plants: past, present and future. *Biochimica et Biophysica Acta, 1809*(8), 360-368. doi:10.1016/j.bbagrm.2011.04.006

Varshney, R. K., Graner, A., & Sorrells, M. E. (2005). Genic microsatellite markers in plants: features and applications. *Trends in Biotechnology, 23*(1), 48-55. doi:10.1016/j.tibtech.2004.11.005

Vendrame, S., Del Bo', C., Ciappellano, S., Riso, P., Klimis-Zacas, D., & Battino, M. (2016). Berry fruit consumption and metabolic syndrome. *Antioxidants, 5*(4), 34. doi:10.3390/antiox5040034

Vescan, L. A., Pamfil, D., Clapa, D., Fira, A., Sisea, C. R., Pop, I. F., Petricele, I. V., Ciuzan, O., & Pop, R. (2012). Efficient micropropagation protocol for highbush blueberry (*Vaccinium corymbosum* L.) cv. 'Elliot'. *Romanian Biotechnological Letters, 17*(1), 6893-6902.

Vidal, J. R., Rama, J., Taboada, L., Martin, C., Ibañez, M., Segura, A., & González-Benito, M. E. (2008). Improved somatic embryogenesis of grapevine (*Vitis vinifera*) with focus on induction parameters and efficient plant regeneration. *Plant Cell, Tissue and Organ Culture, 96*(1), 85-94. doi:10.1007/s11240-008-9464-z

Vlasova, T. I., Demidenko, Z. N., Kirnos, M. D., & Vanyushin, B. F. (1995). *In vitro* DNA methylation by wheat nuclear cytosine DNA methyltransferase: effect of phytohormones. *Gene, 157*(1), 279-281. doi:10.1016/0378-1119(94)00784-P

Vooková, B., & Gajdošová, A. (1992). Induction of adventitious buds on the cotyledons of *Abies concolor* × *Abies grandis* hybrid seedlings. *Biologia Plantarum, 34*(1), 23-29. doi:10.1007/BF02925785

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T. V. D., Hornes, M., Friters, A., Pot, J., Paleman, J., Kuiper, M., & Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research, 23*(21), 4407-4414. doi:10.1093/nar/23.21.4407

Vvedenskaya, I. O., & Vorsa, N. (2004). Flavonoid composition over fruit development and maturation in American cranberry, *Vaccinium macrocarpon* Ait. *Plant Science, 167*(5), 1043-1054. doi:10.1016/j.plantsci.2004.06.001

Vyas, P., Debnath, S. C., & Igamberdiev, A. U. (2013a). Metabolism of glutathione and ascorbate in lingonberry cultivars during *in vitro* and *ex vitro* propagation. *Biologia Plantarum, 57*(4), 603-612. doi:10.1007/s10535-013-0339-8

Vyas, P., Kalidindi, S., Chibrikova, L., Igamberdiev, A. U., & Weber, J. T. (2013b). Chemical analysis and effect of blueberry and lingonberry fruits and leaves against glutamate-mediated excitotoxicity. *Journal of Agricultural and Food Chemistry, 61*(32), 7769-7776. doi:10.1021/jf401158a

Wallace, G., & Fry, S. C. (1994). Phenolic components of the plant cell wall. *International Review of Cytology, 151*, 229-267. doi:10.1016/S0074-7696(08)62634-0

Wang, H., Cao, G., & Prior, R. L. (1996). Total antioxidant capacity of fruits. *Journal of Agricultural and Food Chemistry, 44*(3), 701-705. doi:10.1021/jf950579y

Wang, L.-J., Wu, J., Wang, H.-X., Li, S.-S., Zheng, X.-C., Du, H., Xu, Y.-J., & Wang, L.-S. (2015). Composition of phenolic compounds and antioxidant activity in the leaves of blueberry cultivars. *Journal of Functional Foods, 16*, 295-304. doi:10.1016/j.jff.2015.04.027

Wang, L., Bao, X.-M., Liu, Y.-H., & Hao, S. (1994a). Origin of direct somatic embryos from cultured inflorescence axis segments of *Freesia refracta*. *International Journal of Plant Sciences, 155*(6), 672-676. doi:10.1086/297206

Wang, M. L., Barkley, N. A., Yu, J. K., Dean, R. E., Newman, M. L., Sorrells, M. E., & Pederson, G. A. (2005). Transfer of simple sequence repeat (SSR) markers from major cereal crops to minor grass species for germplasm characterization and evaluation. *Plant Genetic Resources, 3*(1), 45-57. doi:10.1079/PGR200461

Wang, S. Y. (2007). Antioxidant capacity and phenolic content of berry fruits as affected by genotype, preharvest conditions, maturity, and postharvest handling. In Y. Zhao (Ed.), *Berry Fruit: Value-Added Products for Health Promotion* (1st ed., pp. 148-178). Boca Raton,FL, USA: CRC Press.

Wang, S. Y., Chen, H., Camp, M. J., & Ehlenfeldt, M. K. (2012). Genotype and growing season influence blueberry antioxidant capacity and other quality attributes. *International Journal of Food Science and Technology, 47*(7), 1540-1549. doi:10.1111/j.1365-2621.2012.03004.x

Wang, S. Y., & Lin, H.-S. (2000). Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *Journal of Agricultural and Food Chemistry, 48*(2), 140-146. doi:10.1021/jf9908345

Wang, S. Y., & Zheng, W. (2001). Effect of plant growth temperature on antioxidant capacity in strawberry. *Journal of Agricultural and Food Chemistry, 49*(10), 4977-4982. doi:10.1021/jf0106244

Wang, S. Y., & Zheng, W. (2005). Preharvest application of methyl jasmonate increases fruit quality and antioxidant capacity in raspberries. *International Journal of Food Science and Technology, 40*(2), 187-195. doi:10.1111/j.1365-2621.2004.00930.x

Wang, Z., Weber, J., Zhong, G., & Tanksley, S. (1994b). Survey of plant short tandem DNA repeats. *Theoretical and Applied Genetics, 88*(1), 1-6. doi:10.1007/BF00222386

Weber, J. L., & May, P. E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *American Journal of Human Genetics, 44*(3), 388-396.

Weidner, S., Amarowicz, R., Karamać, M., & Frączek, E. (2000). Changes in endogenous phenolic acids during development of *Secale cereale* caryopses and after dehydration treatment of unripe rye grains. *Plant Physiology and Biochemistry, 38*(7), 595-602. doi:10.1016/S0981-9428(00)00774-9

Weising, K., Nybom, H., Wolff, K., & Meyer, W. (1995). *DNA Fingerprinting in Plants and Fungi*. Boca Raton, FL, USA: CRC Press.

Welsh, J., & McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research, 18*(24), 7213-7218. doi:10.1093/nar/18.24.7213

Wetzel, S., Duchesne, L. C., & Laporte, M. F. (2006). *Bioproducts from Canada's Forests: New Partnerships in the Bioeconomy*. Dordrecht, The Netherlands: Springer.

White, P. R. (1943). *A Handbook of Plant Tissue Culture* (Vol. 56). Lancaster, Pennsylvania: The Jacques Cattell Press.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research, 18*(22), 6531-6535. doi:10.1093/nar/18.22.6531

Williams, L. L. (2003). Chromatin reorganization accompanying cellular dedifferentiation is associated with modifications of histone H3, redistribution of HP1, and activation of E2F-target genes. *Developmental Dynamics, 228*(1), 113-120. doi:10.1002/dvdy.10348;pmid:

Wolfe, K. L., & Liu, R. H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry, 55*(22), 8896-8907. doi:10.1021/jf0715166

Wolpert, L. (2002). *Principles of Development* (2nd ed.). Toronto, Canada: Oxford University Press.

Wood, G. W. (1968). Self-fertility in the lowbush blueberry. *Canadian Journal of Plant Science, 48*(4), 431-433. doi:10.4141/cjps68-085

Wu, K. S., Jones, R., Danneberger, L., & Scolnik, P. A. (1994). Detection of microsatellite polymorphisms without cloning. *Nucleic Acids Research, 22*(15), 3257-3258.

Wu, X., Kang, J., Xie, C., Burris, R., Ferguson, M., Badger, T., & Nagarajan, S. (2010). Dietary blueberries attenuate atherosclerosis in apolipoprotein E-deficient mice by upregulating antioxidant enzyme expression. *The Journal of Nutrition, 140*(9), 1628-1632. doi:10.3945/jn.110.123927

Xiao, W., Brown, R. C., Lemmon, B. E., Harada, J. J., Goldberg, R. B., & Fischer, R. L. (2006). Regulation of seed size by hypomethylation of maternal and paternal genomes. *Plant Physiology, 142*(3), 1160-1168. doi:10.1104/pp.106.088849

Xu, B. J., & Chang, S. K. C. (2007). A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. *Journal of Food Science, 72*(2), S159-S166. doi: 10.1111/j.1750-3841.2006.00260.x

Xu, M., Li, X., & Korban, S. S. (2004). DNA-methylation alterations and exchanges during *in vitro* cellular differentiation in rose (*Rosa hybrida* L.). *Theoretical and Applied Genetics, 109*(5), 899-910. doi:10.1007/s00122-004-1717-6

Yadong, L., Shuying, L., Zhidong, Z., & Lin, W. (2003). The induction and regeneration of rabbiteye blueberry buds. *Acta Horticulturae, 626*, 259-264. doi:10.17660/ActaHortic.2003.626.35

Yarborough, D. E. (2012). Establishment and management of the cultivated lowbush blueberry (*Vaccinium angustifolium*). *International Journal of Fruit Science, 12*(1-3), 14-22. doi:10.1080/15538362.2011.619130

Yi, W., Akoh, C. C., Fischer, J., & Krewer, G. (2006). Effects of phenolic compounds in blueberries and muscadine grapes on HepG2 cell viability and apoptosis. *Food Research International, 39*(5), 628-638. doi:10.1016/j.foodres.2006.01.001

Yi, W., Fischer, J., Krewer, G., & Akoh, C. C. (2005). Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. *Journal of Agricultural and Food Chemistry, 53*(18), 7320-7329. doi:10.1021/jf051333o

Yildirim, A. B., & Turker, A. U. (2014). Effects of regeneration enhancers on micropropagation of *Fragaria vesca* L. and phenolic content comparison of field-grown and *in vitro*-grown plant materials by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS). *Scientia Horticulturae, 169*, 169-178. doi:10.1016/j.scienta.2014.01.038

Yuan, W., Zhou, L., Deng, G., Wang, P., Creech, D., & Li, S. (2011). Anthocyanins, phenolics, and antioxidant capacity of *Vaccinium* L. in Texas, USA. *Pharmaceutical Crops, 2*(1), 11-23. doi:10.2174/2210290601102010011

Zadernowski, R., Naczk, M., & Nesterowicz, J. (2005). Phenolic acid profiles in some small berries. *Journal of Agricultural and Food Chemistry, 53*(6), 2118-2124. doi:10.1021/jf040411p

Zhang, M., Wang, H., Dong, Z., Qi, B., Xu, K., & Liu, B. (2010). Tissue culture-induced variation at simple sequence repeats in sorghum (*Sorghum bicolor* L.) is genotype-dependent and associated with down-regulated expression of a mismatch repair gene, MLH3. *Plant Cell Reports, 29*(1), 51-59. doi:10.1007/s00299-009-0797-9

Zhang, M., Xu, C., von Wettstein, D., & Liu, B. (2011). Tissue-specific differences in cytosine methylation and their association with differential gene expression in sorghum. *Plant Physiology, 156*(4), 1955-1966. doi:10.1104/pp.111.176842

Zhao, X., Carey, E. E., Wang, W., & Rajashekar, C. B. (2006). Does organic production enhance phytochemical content of vegetables and fruit? Current knowledge and prospects for research. *HortTechnology, 16(3)*, 449-456.

Zhao, X., Zhan, L., & Zou, X. (2011). *In vitro* high-frequency regeneration of half-highbush 'Northland' blueberry. *New Zealand Journal of Crop and Horticultural Science, 39*(1), 51-59. doi:10.1080/01140671.2010.524188

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry, 64*(4), 555-559. doi:http://dx.doi.org/10.1016/S0308-8146(98)00102-2

Zhong, Y., Wang, Y., Guo, J., Chu, H., Gao, Y., & Pang, L. (2015). Blueberry improves the therapeutic effect of etanercept on patients with juvenile idiopathic arthritis: phase III study. *Tohoku Journal of Experimental Medicine, 237*(3), 183-191. doi:10.1620/tjem.237.183

Zietkiewicz, E., Rafalski, A., & Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics, 20*(2), 176-183. doi:10.1006/geno.1994.1151

Zifkin, M., Jin, A., Ozga, J. A., Zaharia, L. I., Schernthaner, J. P., Gesell, A., Abrams, S. R., Kennedy, J. A., & Constabel, C. P. (2012). Gene expression and metabolite profiling of developing highbush blueberry fruit indicates transcriptional regulation of flavonoid metabolism and activation of abscisic acid metabolism. *Plant Physiology, 158*(1), 200-224. doi:10.1104/pp.111.180950

Zimmerman, R. H. (1988). Micropropagation of woody plants: post tissue culture aspects. *Acta Horticulturae, 227*, 489-499. doi:10.17660/ActaHortic.1988.227.102

Zimmerman, R. H., & Broome, O. C. (1980). Blueberry micropropagation. *Proceedings of Nursery Production of Fruit Plants through Tissue Culture - Applications and Feasibility* (pp. 44–47). USDA–SEA Agriculture Research Results ARR-NE-11.

Ziv, M. (1991). Vitrification: morphological and physiological disorders of *in vitro* plants. In P. C. Debergh & R. H. Zimmerman (Eds.), *Micropropagation: Technology and Application* (pp. 45-69). Dordrecht, The Netherlands: Springer.

Zoratti, L., Jaakola, L., Häggman, H., & Giongo, L. (2015). Modification of sunlight radiation through colored photo-selective nets affects anthocyanin profile in *Vaccinium* spp. berries. *PLoS ONE, 10*(8), e0135935. doi:10.1371/journal.pone.0135935

APPENDIX 1

PUBLICATIONS ORIGINATED FROM THE STUDY[3]

1. Debnath, S. C., Vyas, P., **Goyali, J. C.,** & Igamberdiev, A. U. (2012). Morphological and molecular analyses in micropropagated berry plants acclimatized under *ex vitro* condition. *Canadian Journal of Plant Science*, *92*(6), 1065-1073. doi:10.1139/CJPS2011-194
2. **Goyali, J. C.,** Igamberdiev, A. U., & Debnath, S. C. (2013). Morphology, phenolic content and antioxidant capacity of lowbush blueberry (*Vaccinium angustifolium* Alt.) plants as affected by *in vitro* and *ex vitro* propagation methods. *Canadian Journal of Plant Science, 93*(6), 1001-1008. doi:10.4141/CJPS2012-307

[3] **Contributions of author and co-authors:** Manuscript 1 is a review article and prepared by Samir C. Debnath who is the corresponding authors. For the manuscript 2 - 5, J. C. Goyali along with S. C. Debnath and A. U. Igamberdiev formulated the hypothesis, performed literature search and designed the experiments. He carried out experiments set in greenhouse, performed laboratory and statistical analyses and prepared manuscripts. Dr. Debnath provided the plant materials for this research, performed statistical analysis and supervised the research. A. U. Igamberdiev provided critical comments and reviews on the manuscripts and co-supervised the research.

3. **Goyali, J. C.,** Igamberdiev, A. U., & Debnath, S. C. (2015a). Propagation methods affect fruit morphology and antioxidant properties but maintain clonal fidelity in lowbush blueberry. *HortScience, 50*(6), 888-896.
4. **Goyali, J. C.,** Igamberdiev, A. U., & Debnath, S. C. (2015b). Micropropagation affects not only the fruit morphology of lowbush blueberry (*Vaccinium angustifolium* Ait.) but also its medicinal properties. *Acta Horticulturae, 1098,* 137-142. doi:10.17660/ActaHortic.2015.1098.14
5. **Goyali, J. C**., Igamberdiev, A. U. & Debnath, S. C. (2017). DNA methylation in lowbush blueberry (*Vaccinium angustifolium* Ait.) propagated by softwood cutting and tissue culture. *Canadian Journal of Plant Science,* manuscript # CJPS-2017-0297 (submitted).

APPENDIX 2

Table A.1 Pearson's correlation coefficients for number of stems per plant (NSP), number of branches per plant (NBrP); number of branches per stem (NBrS), plant height (PH; cm), stem diameter (SD; mm), leaf length (LL; mm), leaf width (LW; mm), leaf area (LA; mm^2), plant vigour (PV), number of flowers per plant (NFP), number of flower clusters per plant (NCP), number of flowers per cluster (NFC), fruit setting percentage (FSP), number of berries per plant (NBP), berry diameter (BD; mm), individual berry weight (IBW; g) and berry weight per plant (BWP; g) in blueberries in 2011

Characters	NBrP	NBrS	PH	SD	LL	LW	LA	PV	NFP	NCP	NFC	FSP	NBP	BD	IBW	BWP
NSP	0.9**	-0.8**	0.1	-0.7**	0.2	0.1	0.2	-0.3	-0.6*	-0.4	-0.7**	-0.6**	-0.7**	-0.1	0.1	-0.8**
NBrP		-0.6*	0.1	-0.8**	0.1	-0.2	-0.2	-0.5	-0.3	-0.1	-0.6**	-0.7**	-0.5	-0.4	-0.3	-0.7**
NBrS			-0.3	0.3	-0.5*	-0.5	-0.6*	0.1	0.9**	0.8**	0.7**	0.2	0.9**	-0.2	-0.3	0.8**
PH				0.0	0.7**	0.2	0.4	0.2	-0.4	-0.4	-0.3	0.1	-0.4	0.0	0.0	-0.3
SD					-0.1	0.3	0.2	0.3	0.1	0.0	0.4	0.8**	0.3	0.3	0.4	0.5*

* and ** Correlarion is significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table A.1 cont'd

Characters	NBrP	NBrS	PH	SD	LL	LW	LA	PV	NFP	NCP	NFC	FSP	NBP	BD	IBW	BWP
LL						0.1	0.4	0.1	-0.7**	-0.6*	-0.7**	0.1	-0.7**	-0.1	-0.1	-0.7**
LW							0.9**	0.3	-0.6*	-0.8**	0.1	0.5*	-0.5	0.8**	0.9**	0.0
LA								0.3	-0.7**	-0.9**	-0.1	0.5*	-0.6**	0.7**	0.8**	-0.1
PV									-0.1	-0.2	0.2	0.4	0.0	0.3	0.2	0.2
NFP										0.9**	0.7**	-0.1	0.9**	-0.3	-0.3	0.7**
NCP											0.4	-0.3	0.9**	-0.5	-0.6*	0.5*
NFC												0.4	0.8**	0.3	0.3	0.9**
FSP													0.1	0.4	0.6**	0.5*
NBP														-0.2	-0.2	0.8**
BD															0.8**	0.3
IBW																0.3

* and ** Correlarion is significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table A.2 Pearson's correlation coefficients for number of stems per plant (NSP), number of branches per plant (NBrP); number of branches per stem (NBrS), plant height (PH; cm), stem diameter (SD; mm), leaf length (LL; mm), leaf width (LW; mm), leaf area (LA; mm^2), plant vigour (PV), number of flowers per plant (NFP), number of flower clusters per plant (NCP), number of flowers per cluster (NFC), fruit setting percentage (FSP), number of berries per plant (NBP), berry diameter (BD; mm), individual berry weight (IBW; g) and berry weight per plant (BWP; g) in blueberries in 2012

Characters	NBrP	NBrS	PH	SD	LL	LW	LA	PV	NFP	NCP	NFC	FSP	NBP	BD	IBW	BWP
NSP	0.9**	-0.9**	0.1	-0.7**	0.1	0.2	0.3	0.6*	-0.6*	-0.5	-0.6*	-0.7**	-0.7**	0.1	0.1	-0.7**
NBrP		-0.7**	0.2	-0.8**	0.5	-0.2	0.3	0.4	-0.4	-0.2	-0.6**	-0.8**	-0.5*	-0.4	-0.3	-0.8**
NBrS			-0.1	0.6*	-0.1	-0.5	-0.5	-0.4	0.8**	0.7**	0.8**	0.4	0.9**	-0.2	-0.3	0.8**
PH				0.1	0.3	0.1	0.3	-0.1	-0.4	-0.4	-0.4	0.1	-0.3	-0.1	-0.1	-0.3
SD					-0.3	0.2	-0.1	-0.3	0.2	0.1	0.4	0.8**	0.4	0.3	0.2	0.6*
LL						-0.3	0.5*	-0.3	-0.1	0.1	-0.6*	-0.4	-0.2	-0.8**	-0.8**	-0.6*
LW							0.6*	0.1	-0.7**	-0.7**	-0.3	0.5*	-0.6*	0.8**	0.8**	-0.2

* and ** Correlarion is significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table A.2 cont'd

Characters	NBrP	NBrS	PH	SD	LL	LW	LA	PV	NFP	NCP	NFC	FSP	NBP	BD	IBW	BWP
LA								-0.3	-0.7**	-0.6**	-0.7**	0.1	-0.7**	0.1	0.1	-0.7**
PV									-0.1	0.1	0.1	-0.4	-0.1	0.1	0.2	-0.1
NFP										0.9**	0.8**	-0.2	0.9**	-0.3	-0.4	0.7**
NCP											0.7**	-0.3	0.8**	-0.4	-0.4	0.6*
NFC												0.2	0.9**	0.2	0.2	0.9**
FSP													0.1	0.6*	0.5*	0.4
NBP														-0.2	-0.3	0.9**
BD															0.9**	0.3
IBW																0.2

* and ** Correlarion is significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table A.3 Pearson's correlation coefficients for number of stems per plant (NSP), number of branches per plant (NBrP); number of branches per stem (NBrS), plant height (PH; cm), stem diameter (SD; mm), leaf length (LL; mm), leaf width (LW; mm), leaf area (LA; mm^2), plant vigour (PV), number of flowers per plant (NFP), number of flower clusters per plant (NCP), number of flowers per cluster (NFC), fruit setting percentage (FSP), number of berries per plant (NBP), berry diameter (BD; mm), individual berry weight (IBW; g) and berry weight per plant (BWP; g) in blueberries in 2013

Characters	NBrP	NBrS	PH	SD	LL	LW	LA	PV	NFP	NCP	NFC	FSP	NBP	BD	IBW	BWP
NSP	0.8**	-0.9**	0.2	-0.9**	0.1	0.3	0.3	0.5	-0.7**	-0.7**	-0.6*	-0.7**	-0.8**	0.1	0.1	-0.9**
NBrP		-0.6*	0.2	-0.7**	0.4	0.0	0.2	0.4	-0.5*	-0.6*	-0.6**	-0.4	-0.6*	-0.3	-0.3	-0.8**
NBrS			-0.3	0.8**	-0.1	-0.5*	-0.5*	-0.3	0.8**	0.8**	0.7**	0.6*	0.9**	-0.2	-0.2	0.8**
PH				-0.2	0.1	0.3	0.4	0.3	-0.5*	-0.5*	-0.5*	0.1	-0.5	0.1	0.2	-0.3
SD					-0.1	-0.1	-0.1	-0.5	0.5*	0.6*	0.5*	0.8**	0.7**	0.1	0.1	0.9**
LL						-0.2	0.5*	-0.4	-0.3	-0.3	-0.5*	0.3	-0.2	-0.8**	-0.7**	-0.4
LW							0.7**	0.2	-0.7**	-0.7**	-0.4	-0.1	-0.6**	0.7**	0.7**	-0.2

* and ** Correlarion is significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table A.3

Characters	NBrP	NBrS	PH	SD	LL	LW	LA	PV	NFP	NCP	NFC	FSP	NBP	BD	IBW	BWP
LA								-0.1	-0.8**	-0.8**	-0.7**	0.2	-0.7**	0.1	0.1	-0.5
PV									-0.3	-0.3	-0.2	-0.6*	-0.4	0.3	0.3	-0.4
NFP										0.9**	0.9**	0.2	0.9**	-0.2	-0.2	0.8**
NCP											0.9**	0.2	0.9**	-0.1	-0.2	0.8**
NFC												0.2	0.8**	0.1	0.1	0.8**
FSP													0.5*	-0.2	-0.2	0.6*
NBP														-0.2	-0.3	0.8**
BD															0.9**	0.2
IBW																0.2

* and ** Correlarion is significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.