In vitro and molecular approaches for propagation and germplasm improvement of blueberries

by

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Abstract

Blueberries are known as a “super-fruit” and have tremendous commercial importance due to their high antioxidant contents. During in vitro culture, by introducing various plant growth regulators (PGRs) in the culture medium redifferentiation is stimulated in regenerating cells. During organogenesis and somatic embryogenesis (SE), a somatic cell goes through the process of dedifferentiation which is predominantly controlled by various epigenetic factors. I have investigated the effect of different PGRs on SE and established a protocol in half-high blueberry plants, for the first time, using thidiazuron (TDZ) on a semi-solid medium (SSM). I compared the antioxidant capacity of the in vitro grown plants with their donor counterparts to see the effect of SE on the biochemical profile of the regenerants. It was seen that not only the SE process but also the different concentrations of TDZ and the physiological age of the explants significantly affects the antioxidant activity. To get a more detailed insight into the effect of in vitro propagation on differential methylation pattern, I have analysed the global methylation pattern of young leaves and regenerated calli of one hybrid blueberry and three lowbush blueberry clones using methylation sensitive amplification polymorphism (MSAP) technique. Methylation assay results showed that calli regenerated in the SSM supplemented with TDZ are significantly hypermethylated relative to the donor plants, and the level of methylation varies with different concentrations of TDZ. Not only that but different plant genotypes showed differential effect on methylation pattern. These findings further confirm the effect of different aspects of plant tissue culture techniques on altered DNA methylation pattern. Finally, to gain further insight into how various in vitro culture

systems affect the global methylation pattern, I performed global methylation analysis on half-highbush blueberry plantlets regenerated from SSM and liquid medium in a temporary immersion bioreactor (TIB) in the presence of TDZ and zeatin. From this experiment I found that significant increase in total methylation percentage and methylation polymorphism were present in plantlets from TIB system in comparison to SSM. Overall, my results indicate that each component of in vitro propagation has strong effects on the epigenetic and biochemical profile of the regenerants.

General Summary

Blueberries have high market value because of their high antioxidant contents. To meet the market demand of blueberries, plant tissue culture techniques have been used for commercial propagation of blueberries. In this study I have developed a protocol to regenerate shoot and root simultaneously using an artificial growth medium. I have compared the antioxidant properties among the plants grown in the laboratory with their greenhouse grown counterparts. It was found that not only the plant tissue culture process, but plant growth regulators (PGRs) used in the media, and age of the plant tissues in the culture media influence antioxidant activities.

DNA Methylation is a type of chemical modification. Complete understanding of altered DNA methylation pattern will help to get an idea how surrounding environment (i.e. laboratory/ in vitro condition) affects the developmental process. To get a more detailed insight into the effect of in vitro propagation on differential methylation pattern, I have analysed the global methylation pattern of young leaves and unorganised cells (callus/plural calli) regenerated in the tissue culture system of four blueberry clones using methylation sensitive amplification polymorphism (MSAP) technique. Methylation assay results showed that calli regenerated in the media are significantly more highly methylated than the donor plants. Moreover different concentrations of PGRs, and genotype also have effects on altered DNA methylation pattern. Finally, to gain further insight into how various in vitro culture systems affect the global methylation pattern, I performed global methylation analysis on half-highbush blueberry plantlets regenerated from semi-solid medium (SSM) and liquid medium (without agar gelled) in a temporary

immersion bioreactor (TIB) in the presence of two PGRs. From this experiment I found that significant increase in total methylation percent and methylation polymorphism was present in plantlets from TIB system in comparison to SSM. Overall, my results indicate that each components of in vitro propagation have the strong effect on the epigenetic and biochemical profile of the regenerants.

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List of Abbreviations, Symbols and Nomenclature

Abbreviation	Meaning
$	dollar
%	percent
M	million
2,4-D	2,4-Dichlorophenoxyacetic acid
4-mC	N4-methylcytosine
5-azaC	5–azacytidine
5-mC	5-methylated cytosine
6-mA	N6-methyladenine
AA	antioxidant activity
AFLP	amplified fragment length polymorphism
AGO	Argonaute
ANOVA	analysis of variance
AP1	*Apetala 1*
BA	benzyladenine
BM	basal medium
BS-seq	Bisulfite sequencing
CE	catechin equivalents
CMT2	chromomethylase 2
CMT3	chromomethylase 3
CNR	*Colourless non-ripening*
CPPU	N-(2-chloro-4-pyridyl)-N′-phenylurea
CRD	completely randomized design
d	days
DCL3	Dicer-like protein 3
DDM1	decrease in DNA methylation 1
DMRT	Duncan Multiple Range Test
DNMT3	DNA methyltransferase 3
DPPH	2,2-diphenyl-1-picrylhydrazyl
DRM2	domains rearranged methyltransferase 2
DRMs	domain rearranged methyltransferases
dsRNA	double stranded RNA
epiRIL	epigenetic recombinant inbred line
FOLT1	*Folate transporter 1*
GA_3	gibberellic acid
GAE	gallic acid equivalents
h	hours
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
IBA	indole–3–butyric acid
JMJ14	*JUMONJI 14*

KYP	*KRYPTONITE*
Lcyc	linaria cycloidea-like gene
lncRNAs	long noncoding RNAs
lw	leaf weight
MeDIP	chromatin immunoprecipitation
MERE1-1	*Medicago retroelement 1-1*
MET1/ DMT1	DNA methyltransferase 1
METS	methyltransferases
MS	Murashige and Skoog
MSAP	methylation sensitive amplification polymorphism
ncRNA	non-coding RNA
OsSPL14	*Squamosa promoter binding protein like – 14*
PGR	plant growth regulator
POL II	polymerase II
POL IV	RNA polymerase IV
PPFD	photosynthetic photon flux density
r	correlation coefficient
r^2	coefficient of determination
RdDM	RNA-directed DNA methylation
RDR2	RNA-dependent RNA polymerase 2
RFLP	randomly amplified length polymorphism
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SE	somatic embryogenesis
siRNA	small interfering RNA
SSM	semi-solid medium
SSR	simple sequence repeat
SUP	*SUPERMAN*
TAC	total anthocyanin content
TBE	Tris-borate-EDTA
TCIV	tissue culture-induced variation
TDZ	Thidiazuron (N-phenyl-N′-1,2,3-thiadiazol-5-ylurea)
TFC	total flavonoid content
TIB	temporary immersion bioreactors
TPAC	total proanthocyanidins content
TPC	total phenolic content
V	volt
WFP	*Wealthy farmer's panicle*
WGBS	whole genome bisulfite sequencing
WUS	*WUSCHEL*
ZEA	zeatin

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Co authorship statement

Chapter 2 is a manuscript published in Molecular Biology Reports (Amrita Ghosh, Abir U. Igamberdiev, and Samir C. Debnath (2021). Tissue culture-induced DNA methylation in crop plants: a review, Molecular Biology Reports, 48, 823–841. I have carried out the outlined, experiments, wrote the manuscript, prepared the tables, and the figures. A.U. Igamberdiev revised and reviewed the manuscript. S.C. Debnath conceptualized, revised, and reviewed the manuscript.

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Chapter – 1 Introduction and overview

1.1. Blueberry - origin, botany, and systematics

Blueberry plants are terrestrial or epiphytic in nature; belonging to *Vaccinium* L. genus (family: *Ericaceae,* Syn. Heath) (Vander Kloet, 1988). There are 400 – 500 species in the genus *Vaccinium* which are widely spread throughout the globe, native to all the continents other than Antarctica (Song & Hancock 2011; Strik 2004; Strik & Yarborough 2005). There are almost 90 species found in China among which 51 are endemic (Luby et al. 1991). Almost 40% of the *Vaccinium* species originated from Southeast Asia including Malayan Peninsula, New Guinea, India, China, and Japan; the rest 35% is native to America (out of which 25% North America, 10% Central and South America), and 25% distributed all over the world (Song & Hancock 2011). Blueberry plants can tolerate an extensive range of climatic conditions and mostly grow on acidic soil (pH 4.0 – 5.0) with an optimum organic matter of between 5 – 10% present in the soil (Yarborough 2012). A study on lowbush blueberry in China showed summer flooding was tremendously damaging for the plantings (Lin et al. 2002). Extreme cold weather can be another limiting factor for blueberry plantations, which may cause winter kill and spring frost thus causing low yield by damaging the flowers (Yarborough 2012).

In the genus *Vaccinium* L. economically important species are found in the sections *Cyanococcus, Oxycoccus, Vitis-idaea, Myritillus,* and *Vaccinium*. (Song & Hancock 2011). Blueberry belongs to the section *Cyanococcus*. Although representation of *Cyanococcus* has been difficult due to complex polyploidy, in spite of that Camp (1945)

characterized them, where nine species falls into diploid (2n = 2x = 24), twelve of them in tetraploid (2n = 4x = 48), and there of them categorized as hexaploid (2n = 6x = 72) species. Later Vander Kloet (1980) compressed them to diploid (six), tetraploid (five), and hexaploid (one) taxa. Depending on their stature blueberries have been differentiated in five classes such as i) highbush (*Vaccinium corymbosum* L., tetraploid), ii) lowbush (*V. angustifolium* Ait., tetraploid; *V. myrtilloides*, diploid; *V. borale*, diploid), iii) half-high (*V. corymbosum* L. × *V. angustifolium* Ait., tetraploid), iv) rabbiteye (*V. ashei* Reade, hexaploid), and v) southern highbush (*V. corymbosum* L., tetraploid) (Debnath 2007b). Highbush blueberry plants (1.8 – 2.5 m tall) are generally found in wetlands and dry upland slopes of Nova Scotia to Wisconsin, and also in South Georgia to Alabama (Song & Hancock 2011). Lowbush plants (0.3 – 0.6 m tall) including *V. angustifolium* grow from the Arctic to Minnesota, hills of New York and New Hampshire. Velvety-leaf *V. myrtilloids* are found in the wilds of New England and west (Song & Hancock 2011). The commercial production of lowbush blueberries are mostly confined to Maine, Quebec, New Brunswick and Nova Scotia (Strik & Yarborough 2005). Half-high blueberry plants are of intermediate height between high and lowbush blueberries (Galletta & Ballington, 1996) and at maturity they grow up to 0.6 – 1.25 m (Ratnaparkhe 2007). They are mainly cultivated in the upper Midwest of the USA (Song & Hancock 2011). Southern USA is the natural habitat of rabbiteye blueberry plants (up to 0.6 m tall), especially Southern Georgia and the Northern part Florida (Galletta & Ballington 1996; Vander Kloet 1980). Southern highbush blueberries are mainly *V. corymbosum* germplasm, including the hybrids which contain genetic material from at least two up to four *Vaccinium* species (Ratnaparkhe 2007).

Before 1916, all blueberries were harvested from the wild, from many wild North American species until the first shipment of F. V. Coville's hybrid blueberry fruits from Elizabeth White's Farm at Whitesbog, New Jersey, in 1916 (Coville 1921). In 1920, introduction of a few cultivars such as 'Pioneer', 'Cabot', and 'Katherine' from Coville's breeding program escalated the growth of new agricultural industry (Coville 1937). With passing time this industry started expanding with the development of improved and better cultivars from three domesticated classes of blueberries. However, most production comes from the *Cyanococcus* inclusive of *V. corymbosum* L., *V. angustifolium* Ait., and *V. ashei* Reade blueberries which are fairly important in the southeast of North America (Song & Hancock 2011). Hybrids of highbush and lowbush blueberries are known as half-highs; although they do not significantly contribute to the blueberry fruit market they have been used substantially as ornamental crops for landscaping (Song & Hancock 2011).

1.2. Commercial importance of blueberries

Blueberries are an economically high-value crop; deciduous woody dwarf small-fruit-bearing shrubs, they can be grown in acidic soil which is of lesser agriculture value (Rowland et al. 2003). Although blueberries have been cultivated and produced around the globe, North America is the principal producer of blueberries (Rowland et al. 2012). They are commercially important in Atlantic Canadian provinces, Quebec and Maine where they can be found in wild stands which are a collection of genetically diversified

heterogeneous clones (Debnath 2004; Hoefs & Shay 1981). However, cultivation of blueberries is not a new horticultural practise; in earlier times, indigenous people used to burn their wild blueberry stands for better yield and production purposes (Strik 2004). Most of the lowbush blueberry commercial fields have been established from abandoned woodlands and farmlands (Nestby et al. 2011). Lowbush blueberries have been developed in Maine; however, Quebec and the Atlantic Canadian provinces are responsible for developing genetically diversified clones (Aalders et al. 1979; Hall 1979).

Although domestication of wild blueberries started in the nineteenth century, the recent improvement in the crop management practices increased the yield four-fold over the past 20 years (Yarborough 2004). There is a recent increase in blueberry consumption due to their health-promoting benefits. Blueberry fruits contain a high level of anthocyanin (Kalt et al. 1999), which has been found to improve night vision, helps to prevent macular degeneration and heart diseases, and also has anticancer, antitumor, and anti-inflammatory activities (Cho et al. 2004; Kalt et al. 1999; Wang et al. 1999). Rimando et al. (2004) found the compound resveratrol in blueberries which has been linked to reduce the risk of heart disease and cancer, and also found pterostilbene which has been found to reduce high cholesterol levels. In addition, lowbush blueberries have also been used as one of the anti-diabetic plants alongside fenugreek by traditional practitioners in Quebec (Haddad et al. 2003). There are several literatures published highlighting the antioxidant properties of *Vaccinium* species (Böttcher et al. 2011; Kalt et al. 1999; Kalt et al. 2001; Prior et al. 1998; Prior et al. 2001; Yarborough 2012). Considering the fact that the wild blueberries are high in health-promoting

phytochemicals and high nutritional value, they have been popularly known as a “super-fruit” (Prior et al. 1998). Due to this, blueberry production has increased tremendously in the past two decades and since then the blueberry industry has expanded significantly (Nestby et al. 2011).

According to Statistics Canada (2019), Canada is the second-largest producer of blueberries yielding up to 178,745 tons behind only the United States of America (269,257 tons); however, Canada has the highest acreage 54.5 (hectares) than the USA (37.5 hectares). Among all the fresh produced fruits in Canada, blueberries (including low and highbush blueberries) have the highest farm gate value of 262M Canadian dollars ($) followed by apple (223M $) and grapes (151M $), which include table grapes and wine grapes) (Statistics Canada, 2016). Among all the small fruit crops produced in Canada in 2019, blueberry has the largest production, followed by cranberries and grapes (Table 1.1).

Table 1.1: Production of small fruit crops in Canada[s]

Common name	Marketed production (tons)				
	2015	**2016**	**2017**	**2018**	**2019**
Blueberries	193,498	232,798	173,641	169,503	194,171
Cranberries	159,991	198,727	138,415	195,007	187,749
Grapes	88,769	116,627	131,112	119,488	126,034
Strawberries	27,201	29,265	31,275	29,809	29,131
Sweet Cherries	19,351	18,395	26,797	27,857	24,318
Sour Cherries	4,471	6,772	5,096	4,202	5,058

[s]Statistics Canada (2019)

1.3. Blueberry and health benefits

Cultivated blueberries have economic importance due of their potential health-promoting properties. Blueberries are highly acknowledged among the fruits for their anthocyanin and flavonoid contents. Flavonoids are divided into six subclasses i.e., flavones, flavanols, flavonols, flavanones, isoflavones, and anthocyanins depending on the oxidation state of their central carbon ring (Dai & Mumper 2010). Anthocyanins have

wide ranging health benefits and are particularly accountable for red and blue colours in various fruits and vegetables (Zhou et al. 2020). The natural anthocyanin pigments in plants are mostly comprised of anthocyanidins such as delphinidin, peonidin, petunidin, cyanidin, malvidin, and pelargonidin which are linked via glycosidic bonds to various sugar moieties (Yousef et al. 2013). In addition to anthocyanins, blueberries also contains high amounts of hydroxybenzoic acid, flavones and flavonols (Prior et al., 1998; Skrovankova et al. 2015). Quercetin, one of the flavonoid compound found in red wines functions as a cardioprotective compound (Formica & Regelson 1995). This particular type of dietary flavonol has also been found to be effective fighting against specific type of tumors (Verma et al. 1988). Lowbush blueberries contain condensed tannins mainly composed of proanthocyanidins (Smith et al. 2000). Various studies have shown that proanthocyanidin extracted from lowbush blueberries functions as an active agent to suppress the early stage of chemically induced carcinoma (Bomser et al. 1996). Proanthocyanidin also arrests the spread of bacteria causing urinary tract infection by reducing their adherence ability (Howell et al. 1998).

Blueberry fruits have high ascorbic acid (Kalt and Dufour 1997), although the ascorbic acid content is not the main source of their antioxidant properties (Prior et al., 1998). In blueberries antioxidant activity is positively correlated with their polyphenol contents (Zhou et al., 2020). Phenolic acids are divided in two groups i.e. gallic acid-derivatives of benzoic acid; and coumaric, caffeic, and ferulic acid-derivatives of cinnamic acid (Dai & Mumper 2010). Caffeic acid is the most frequently found phenolic acid in vegetable and fruits, whereas ferulic acid majorly found in cereals (Archivio et al.

2007; Dai & Mumper 2010). Polyphenols present in blueberries are related to anti-inflammatory, anti-proliferative, anti-obesity, and neuroprotective functions (Auzanneau et al. 2018; Diaconeasa et al. 2015). Blueberries contains catechins like gallocatechin, epigallocatechin, egallic acid, and gallic acid, which have the tremendous capability of free radical (e.g. superoxide, peroxide, hydroxyl, and dioxidene) scavenging (Kalt et al. 1999). This may lead to anti-cancerous activity and antimicrobial activity in colon and esophagus, urinary tract infection (Kalt et al. 1999). European and American *Vaccinium* species are known for their anti-diabetic properties (Chambers & Camire 2003) and have been used in traditional medicines to treat secondary complications of diabetes (Jellin, Batz, & Hitchens 2004). Moreover, European blueberries and their relatives treat age-related macular degeneration and improve vision (Trevithick & Mitton 1999). It was also found that leaf extracts of wild blueberries (*V. angustifolium* Ait.) and bilberry (*V. myrtillus*) can be used to reduce the blood glucose level in animal models (Murray 1997).

1.4 In vitro propagation of blueberry

The theoretical concept of plant tissue culture was explained by Gottlieb Haberlandt in his lecture at German Academy of Sciences based on his experiments on single cell culture (Haberlandt 1902). Haberlandt experimented with leaf and other actively differentiating cells and failed to give rise to artificial embryos. He further added that cultivating vegetative cells under optimal hormonal influence can be established as a new approach of plant propagation (Thorpe 2007). Although he did not succeeded in his

experiments, his practice of regeneration of artificial embryos from vegetative cells supported the concept of totipotency (Thorpe 2007). Haberlandt (1902) was successful attuning the survival of in vitro-grown tissue, cell division in in vitro condition was first observed by Hannig (1904) and regeneration from callus tissue was first documented by Simon (1908).

Regeneration of plants via tissue culture is dependent on two primary concepts which are totipotency and developmental plasticity. According to Skoog and Miller (1957), totipotency can be explained as the ability of a plant cell to differentiate, proliferate, and eventually grow into a plantlet under optimal culture conditions under hormonal influence. In general, cells from young tissues and meristems are totipotent in nature, however, differentiated cells also occasionally exhibit totipotency (Debnath 2007a). On the other hand, plasticity is the capability of the plant tissues to adjust their metabolism, growth pattern, and development in order to survive under various environmental conditions. Plant tissue culture which is also known as in vitro cell culture, is an important area of basic and applied science studies (Thorpe 1990). Due to the availability of several in vitro propagation techniques, starting around mid 1960s to 1980s these techniques gained a huge popularity to solve various biological, agricultural, horticultural and forestry problems (Thorpe 1990).

During in vitro culture process an individual cell, tissue or organ of a plant can be used as an explant. Explants are then cultured in an artificial medium containing macro and micronutrients, a carbohydrate source, vitamins, plant growth regulators (PGRs) and a chelating agent depending on media type. Under the optimum hormonal stimuli and

appropriate environment explants develop into an identical copy of the source plant under aseptic condition which is called a clone (Altman & Loberant 2003). Clones can be regenerated during in vitro propagation via organogenesis by either forming shoot or root meristem, and/or somatic embryogenesis (SE) where shoot and root meristems form simultaneously (Steward et al. 1970). Organogenesis follows the pathway of shoot proliferation from a pre-existing bud followed by adventitious shoot regeneration (Steward et al. 1970). In a tissue culture system, plants produce a hundreds of identical copies within a short period. The commercial use of micropropagation techniques includes maintenance of pathogen-free germplasm, production of nuclear stock, and yearlong production of clones of hybrid and parental lines (Debnath 2013). In recent years for large scale commercial micropropagation of elite varieties, industries have implemented micropropagation techniques. Consequently, many tissue culture laboratories were set up around the globe especially in developing countries due to cheaper labour cost (Kumar & Reddy 2011). However first report on use of micropropagation techniques for commercial purposes were reported in the 1970s by Boxus (1974) in strawberry and Anderson (1975) in rhododendron.

Some other major discoveries were chemical and hormonal regulation of plant regeneration (Skoog & Miller 1957), application of in vitro propagation techniques in basic and applied science (Komamine et al. 1992; Reinert 1959), regeneration of virus free plantlets, haploid culture (Guha & Maheshwari 1964; Nitsch & Nitsch 1969), plantlet formation from protoplast culture (Cocking 1960), secondary metabolite production (Kaul & Staba 1965), and cell culture in liquid medium in a bioreactor (Noguchi et al. 1977).

Skoog and Miller (1957) hypothesized the effect of auxin-cytokinin on plant morphogenesis. They concluded from his experiment on tobacco pith culture that the auxin-cytokinin ratio is the deciding element for shoot and root formation. Skoog and Miller (1957) hypothesized that the culture the medium supplemented with a higher concentration of cytokinin gives rise to shoot, increased concentration of auxin induces root formation, while a balanced ratio of auxin and cytokinin leads to formation of a callus.

Micropropagation in *Vaccinium* spp. is being used for year-round propagation of virus-free (indexed) clones which can be used as a first step of a nuclear stock crop production system (Debnath 2018). Softwood or rhizome cuttings of desirable clones can be used to establish new blueberry stands as they are comparatively easy to root; however, their flowering ability makes the establishment process very slow for plantings (Debnath 2004). With the intervention of micropropagation techniques, this problem can be largely avoided (Smagula & Lyrene 1984). Many of the small fruit crops have been traditionally produced via plant tissue culture techniques to attain rapid fruit-bearing condition and to maintain genetic fidelity (Debnath 2018). Especially in the case of lowbush blueberry, micropropagated plants develop similar spreading behaviour as seedlings. In addition to that, they exhibit consistent productivity behaviours similar to the rooted cuttings (Frett & Smagula 1983). Furthermore, in vitro propagation methods match the traditional way of introducing new desirable characters into the progeny and multiplying them in a short period of time (Meiners et al. 2007; Rowland & Ogden 1992).

In vitro propagation of blueberry was first reported in early 1970s using rhizome explants without the help of any PGR (Barker & Collins 1963) in White's medium (White 1943). With the onset of micropropagation of blueberry, Smagula and Lyrene (1984) proposed the use of micropropagation techniques to overcome the problem of establishment of blueberry plantings using softwood or rhizome cuttings. It was previously reported that micropropagated and seedlings of lowbush blueberries are easy to establish and spread than softwood or rhizome cuttings (Debnath 2007a). Since then lot of researches has been done on various aspects of blueberry tissue culture and investigated effects of different culture media, culture condition, type of explants, basal media, and PGRs (Table 1.2). Many researchers have documented blueberry micropropagation on semi-solid gelled media via either organogenesis or SE such as, highbush (Cao et al. 2003; Fan et al. 2017; Hung et al. 2016; Lisa & Elizabeth 1992; Rowland & Ogden 1992; Tetsumura et al. 2008; Zhao et al. 2011), lowbush (Debnath 2004, 2007a, 2009c; Frett & Smagula 1983; Nickerson 1978), half-high (Ghosh et al. 2018; Zhao et al. 2011), and rabbiteye (Fan et al. 2017; Lyrene 1980).

Micropropagation of blueberries using liquid media in a bioreactor system is one of the most updated techniques of mass propagation. Over time, automated bioreactors have become an important tool for the success of large-scale commercial production of tissue culture plants (Debnath, 2013). Haberlandt (1902) first used sucrose supplemented in Knop's liquid medium for propagating individual bract cells of *Lamium purpureum* (Preil 2005). Kohlenbach (1959) used mesophyll cells of *Macleaya* as an explant to develop cell forming organs, somatic embryos and cell clusters sixty years after

Haberlandt's experiment. Liquid media have been used in stationary and temporary immersion bioreactors (TIBs) to induce organogenesis and SE (Ziv 2005). Paek et al. (2005) described bioreactors as a sterilized, independent unit that works on the principal of in and outflow of the liquid medium. Automated bioreactors are capable of easily managing the microenvironment such as aeration, agitation, dissolved oxygen level etc. during the intensive culture process (Paek et al. 2005). The environment of the culture room is also responsible for determining the microenvironment inside the bioreactor (Morini & Melai 2003). Bioreactors also provide a better control of the gaseous exchange of plants, pH of the medium, and temperature (Levin & Tanny 2004). Keeping above mentioned factors in mind two types of bioreactors have been developed a) agitated and b) non-agitated bioreactors (Debnath 2011b).

Levin and Vasil (1989) introduced bioreactor systems for mass propagation of various horticultural crops: ever since agitated and non-agitated bioreactors have been used successfully for various ornamental and vegetative crops such as oriental lily (Lian et al. 2003) and potato (Piao et al. 2003). Micropropagation of berry plants using liquid medium in bioreactors has been investigated by many researchers (Arencibia-Rodríguez et al. 2018; Arencibia et al. 2013; Arigundam et al. 2020; Debnath 2008, 2009b, 2010, 2014a, 2014b, 2017). Strawberry shoot culture was obtained in commercially available TIBs using leaves of five cultivars as explants in Murashige and Skoog (MS) medium supplemented with 9 µM thidiazuron (TDZ) and 2.5 µM indole–3–butyric acid (IBA) (Hanhineva et al. 2005). Debnath (2008) optimized a shoot regeneration and proliferation protocol in strawberry cv. 'Bounty' using liquid medium in a TIB system coupled with

semi-solid medium (SSM) with 2 – 4 µM TDZ. Similarly, micropropagation of two lowbush blueberry genotypes and one cultivar were established using TIB in combination in combination with SSM 1, 2 or 4 µM zeatin (ZEA) (Debnath 2009b). Adventitious shoots of three lowbush blueberry genotypes 'PB1', 'QB1', and 'QB2' were regenerated in a liquid medium supplemented with 1.2 – 2.3 µM thidiazuron (TDZ) in a bioreactor (Debnath 2011a). Arencibia et al. (2013) reported micropropagation of three raspberry cultivars 'Meeker', 'Amity', and 'Heritage' using shoot tips cultures (~ 5 cm) in liquid media in a TIB supplemented with various sucrose concentrations. In vitro multiplication of raspberry cultivars 'Festival', 'Heritage', and 'Latham' has been reported by Debnath (2010) in TDZ supplemented media. Adventitious shoot regeneration of wild lingonberry clones were obtained using stationary bioreactors and TIBs with liquid media supplemented with 9.1 µM TDZ and 1.8 µM ZEA (Arigundam et al. 2020). Organogenesis in half-high blueberry cv. 'Patriot' is shown in Fig. 1.1 using a semi-solid and a liquid medium in a TIB (Ghosh et al. unpublished results).

1.4.1. Axillary shoot proliferation

Shoot proliferation from axillary buds is one of the most common pathways of organogenesis. It is also most dependable technique to attain true-to-type clones from the donor plants due to least chances of occurrence of genetic alterations (Debnath 2018). Axillary shoot proliferation does not involve cell differentiation; rather, it follows the natural pathway of plantlet formation from pre-existing meristems (Novikova & Zaytseva

2018). Meristem tissues are comparatively less prone to mutations or genetic alteration than unorganized tissues such as calluses; however chance of epigenetic changes occurring still persists (Baránek et al. 2015). Explants such as shoot tips or nodal explants containing axillary buds can be sterilized and cultured on SSM or liquid medium in a bioreactor. These axillary buds remain dormant until higher level of cytokinins is not induced in the medium in composition with lower level of auxins (Debnath 2018). Higher concentration of cytokinins are usually used in the culture medium to give rise to shoot and breaking of apical dominance (Debnath 2003). In the presence of the optimal amount of cytokinin, explants overcome apical dominance; cytokinin also helps in branching via lateral bud formation (Debnath 2018). Usually in some woody species shoot explants demonstrate the natural monopodial growth habit without branching in the presence of aminopurine cytokinin growth supplements (Huetteman & Preece 1993). However, culture medium supplemented with TDZ breaks the lateral bud dormancy (Wang et al. 1986) leading to axillary shoot proliferation in a comparatively greater frequency than in the presence of commonly used cytokinins (Thomas & Katterman 1986).

Micropropagation via axillary shoot has been practiced in blueberries for years by many researchers like Cao et al. 2003; Debnath 2004, 2007a, 2017; Fan et al. 2017; Jiang et al. 2009.

1.4.2. Adventitious shoot regeneration

De novo shoot regeneration can occur two ways: directly without any involvement of callus or indirectly via callus formation. However, direct shoot regeneration is the more preferable technique for mass propagation as the chances of occurrences of somaclonal variance are scarce (Debnath 2018; Novikova & Zaytseva 2018). Nevertheless indirect of shoot regeneration is the principal pathway to attain genetic improvement of woody plant species which are usually difficult to propagate (Novikova & Zaytseva 2018). During indirect regeneration process shoot originates from the surface of the callus tissue thus have better chance to be genetically transformed (José et al. 2014). According to Ammirato (1985) adventitious shoot regeneration can initiate from unipolar organs either with shoot or root meristem (organogenesis) or from bipolar organs, simultaneously containing shoot and root meristem (SE). The shoot organogenesis process includes several steps such as a) adventitious bud formation, b) elongation of shoots c) root formation from the elongated shoots (Qu et al. 2000). Although totipotency theory states that each cell has the capacity to regenerate into a whole new plant, the regeneration capability of a plant cell depends on explant types and cultivars (Ganeshan et al. 2002). In addition, there are various other biotic factors involved in successful shoot regeneration; for instance, genotype of the source plant, culture medium, type and concentration of PGRs, explant's stage of development etc. (Liu et al. 2010). The adventitious shoot regeneration technique has been successfully employed for mass propagation of blueberries for many years. Shoot regeneration from

adventitious buds originating from leaf explants was seen in Southern highbush blueberry hybrid cultivars ‘Legacy’ and ‘Ozarkblue’ (Meiners et al. 2007). A shoot regeneration protocol was developed from the internodal segments of blueberry plants in Zimmerman’s Z-2 medium with 25 µM ZEA or TDZ (Hruskoci & Read 1993). Many other examples of adventitious shoot regeneration are available on highbush (Brevis et al. 2008; Cao et al. 2003; Meiners et al. 2007; Ostrolucká et al. 2004), lowbush (Debnath 2009c, 2011a; Nickerson 1978), half-high (Zhao et al. 2011), and rabbiteye blueberry (Schuchovski et al. 2020).

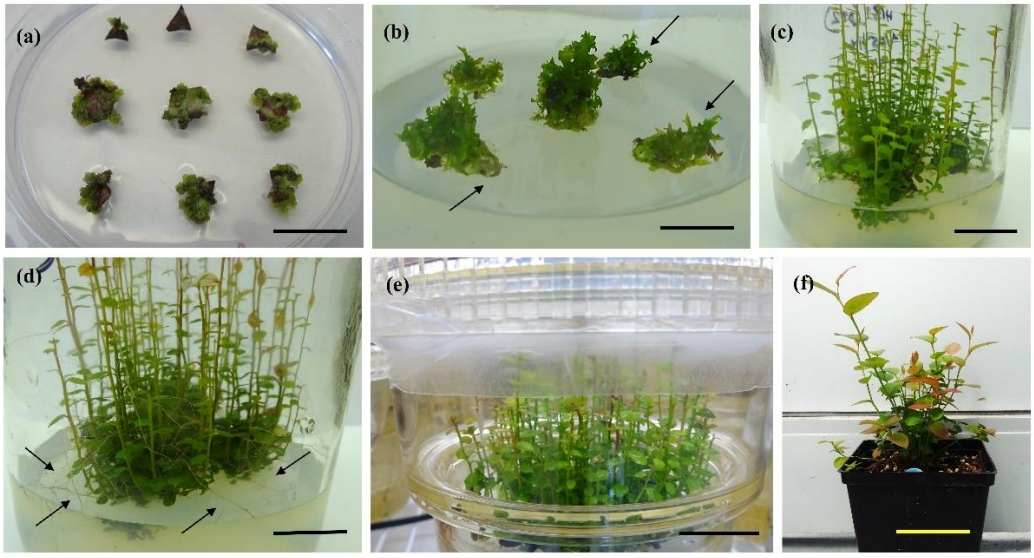

Figure 1.1: In vitro organogenesis in half-high blueberry cv. 'Patriot'. (a) Callus formation after 4 weeks of culture on a semi-solid medium (SSM) with 2.3 µM zeatin (ZEA) (bar = 2 cm), (b) bud initiation from leaf explant (arrows) after 8 weeks of culture on SSM with 9.2 µM ZEA (bar = 2 cm), (c) shoot regeneration after 12 weeks of culture on SSM with 9.2 µM ZEA (bar = 2 cm), (d) shoot elongation and root formation (arrows) after 16 weeks of culture on SSM with 9.2 µM ZEA (bar = 2 cm), (e) shoot elongation after 16 weeks of culture in a temporary immersion bioreactor (TIB) containing liquid medium with 9.2 µM ZEA (bar = 3 cm), (f) one year old hardened-off plants in a greenhouse (bar = 8 cm) (Ghosh et al.unpublished results).

1.4.3. Somatic embryogenesis (SE)

Somatic embryogenesis is explained as the process of formation of embryos from differentiating somatic cells. Somatic embryos and zygotic embryos are remarkably similar both temporally and morphologically, although they develop independently than the physical constrains of the maternal tissues (Zimmerman 1993). Somatic embryos are bipolar and contain radicals, hypocotyl, cotyledons, and usual embryonic organs (von Arnold et al. 2002). Similar to zygotic, somatic embryos also develop globular-shaped, heart-shaped, torpedo-shaped embryos followed by formation of shoot and root meristem simultaneously (Zimmerman 1993). Like organogenesis, SE can be direct from an explant or indirect, involving callus formation (Williams & Maheswaran 1986). Microspores, ovules, embryos (zygotic and somatic), and seedlings are a few examples of explants which are commonly used in direct embryogenesis (von Arnold et al. 2002). The morphological differences between direct and indirect SE are still not clear. However, according to an older hypothesis, direct SE starts from pre-destined embryonic cells while indirect SE is induced from undetermined unorganized callus tissues. However, calluses developed during the indirect SE process can either be embryogenic or non-embryogenic (von Arnold et al. 2002).

The initial description of SE was reported from carrot callus culture (Steward et al., 1958) and since then carrot has been used as the principal model system to study the early regulatory and morphogenic processes occurring during SE (Zimmerman 1993). SE is a unique developmental pathway which is well recognized for plantlet regeneration pathway from cell culture systems (Zimmerman 1993). Unlike organogenesis pathway SE

is not commonly employed for mass multiplication of blueberry species. The first report of development of embryogenic callus tissues from blueberry leaf explant in TDZ supplemented SSM was reported by Ghosh et al. (2017). Later Ghosh et al. (2018) established a protocol for direct SE from young leaf explants of four half-high blueberry cultivars in TDZ supplemented medium. The greatest percentage of embryo formation was observed at 9 µM TDZ, while embryo germination was highest at a lower concentration of TDZ (2.3 µM). These are the only available detailed reports on SE in blueberries. However, there are literatures available on SE in grapes and other small fruit crops (Dhekney et al. 2016; Nakajima & Matsuta 2003; Vidal et al. 2009) and strawberry (Biswas et al. 2009; Husaini et al. 2008; Husaini et al. 2011). SE in half-high blueberry cv. 'Northblue' is shown in Fig. 1.2.

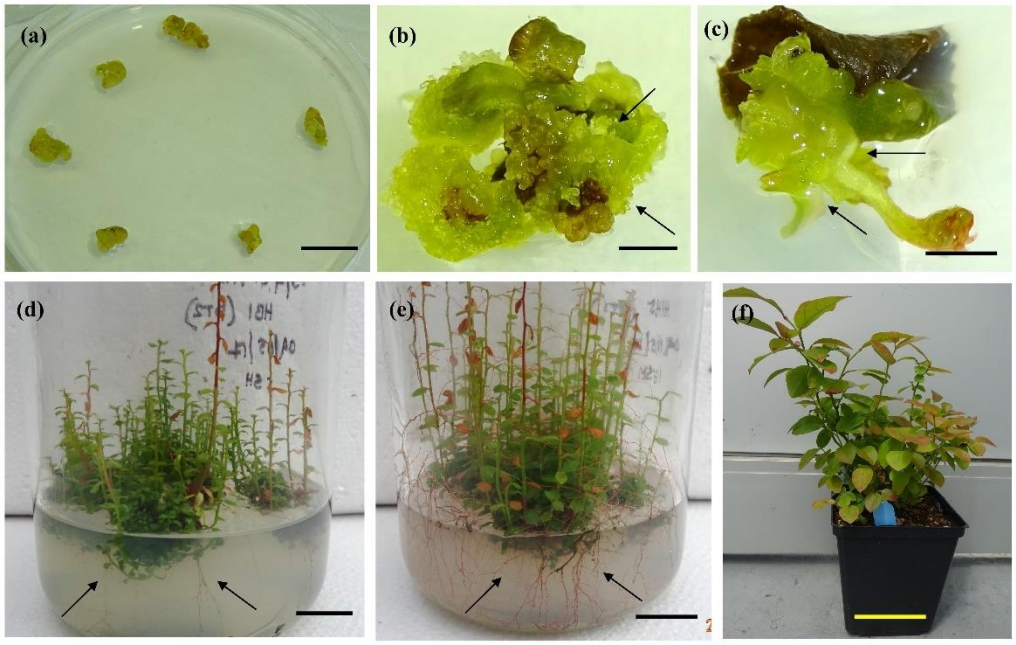

Figure 1.2: In vitro propagation via somatic embryogenesis in half-high blueberry cv. 'Northblue'. (a) young leaf cultured on a medium with 9 µM of thidiazuron (TDZ), (b) development of globular embryos (arrows) after 4 weeks of culture in 9 µM TDZ supplemented medium, (c) shoot and root apex development from the embryos (arrow) after 10 weeks of culture with 9 µM of TDZ. (Bar = 0.5 cm), (d) root elongation (arrows) in a nutrient medium containing 2.3 µM of TDZ, after 4 weeks of culture in a glass jar. (Bar = 2 cm), (e) rooting system development (arrows) in a nutrient medium with 2.3 µM of TDZ after 6 weeks of culture (Bar = 2 cm), and (f) one year old hardened-off plants in a greenhouse (Bar = 5 cm) (Ghosh et al.unpublished results).

1.4.4. Advantages and disadvantages of in vitro propagation

Rapid clonal propagation is one of the most exploited plant biotechnology techniques for clonal propagation of many plant species. It has many advantages over conventional breeding process such as: a) small amount of space needed from high volume of production, b) specific techniques (meristem culture) are available to create regenerants free from pathogens, c) easy multiplication of plants which are difficult to propagate vegetatively, d) all year-round production independent of seasonal changes, e) propagation techniques can be automated, because plant material does not need much attention in between subcultures, f) increased chances of survival of plant materials in the in vitro system and high chances of survival upon transferring to the field, and g) plant tissue culture techniques can be used to eliminate morphological and physiological abnormalities (George et al. 2007; Kozai & Kubota 2005).

Besides having all these advantages, in vitro culture techniques have some limitations as well. The main disadvantage of micropropagation techniques is that they require specialized training and knowledge to maintain in vitro conditions. Other disadvantages are a) specific methods and expensive production facilities are needed to maintain the plant materials, b) sometimes high relative humidity inside the culture vessel causes hyperhydricity among the regenerated plantlets, and c) micropropagation techniques often imposes the somaclonal variation among the regenerants (Debnath 2013; George et al. 2007; Kozai & Kubota 2005). The occurrence of somaclonal variation can be disadvantageous from commercial propagation point of view where maintaining clonal fidelity is a must.

Table 1.2: In vitro propagation of blueberry using various media types, explant types, and micropropagation techniques

Species	Media types	Explant types	Micropropagation technique	References
Vaccinium corymbosum L.	semi-solid	leaf	shoot proliferation	(Cao et al. 2003)
V. corymbosum L.	semi-solid	nodal segments	shoot regeneration, shoot proliferation	(Cappelletti et al. 2016)
V. angustifolium Ait	semi-solid	stem segments, axillary buds	shoot proliferation	(Debnath, 2004)
V. angustifolium Ait.	semi-solid	stem segments	shoot proliferation	(Debnath 2007a)
V. angustifolium Ait.	semi-solid	shoot tip	shoot proliferation	(Debnath 2009b)

V. angustifolium Ait.	semi-solid, liquid	leaf segments	shoot regeneration	(Debnath 2009c)
V. angustifolium Ait.	semi-solid, liquid	leaf segments	shoot regeneration	(Debnath 2011a)
V. angustifolium Ait., *V. corymbosum* L., *V. corymbosum* L. × *V. angustifolium* Ait.	semi-solid, liquid	shoot-tip	shoot proliferation	(Debnath 2017)
V. corymbosum L.	semi-solid	young stem	shoot proliferation	(Fan et al. 2017)
V. angustifolium Ait.	semi-solid	shoot segment	shoot proliferation	(Frett & Smagula 1983)
V. corymbosum L. × *V. angustifolium* Ait.	semi-solid	leaf section	somatic embryogenesis	(Ghosh et al. 2018)
V. angustifolium Ait.	semi-solid	internodes	shoot regeneration	(Hruskoci & Read 1993)

V. corymbosum L.	semi-solid	nodal segment	shoot proliferation	(Hung et al. 2016)
V. corymbosum L.	semi-solid	shoot-tip	shoot regeneration, shoot proliferation	(Litwińczuk et al. 2005)
V. corymbosum L.	semi-solid	leaf section	shoot regeneration	(Rowland & Ogden 1992)
V. corymbosum L.	semi-solid	nodal segments	shoot proliferation	(Tetsumura et al. 2008)
V. corymbosum L.	semi-solid	leaf section	shoot regeneration	(Xing et al. 2009)

1.5. Antioxidant activities (AA) and in vitro propagation

Blueberries (*Vaccinium* spp.) are popularly known as “super-fruits” because of their high level of antioxidant properties due to the ample presence of polyphenolic compounds (Kalt et al. 2020). However, the antioxidant capacity of these phenolic compounds is not directly accessible under in situ condition due to their low bioavailability (Williamson & Clifford 2010). In blueberries, antioxidant capacity depends on the presence of phenolic and flavonoid compounds, redox potential and their chemical structures (Prior et al. 1998). It was previously demonstrated that there is a positive and steady correlation between total phenolic (TPC) and anthocyanin content (TAC) and antioxidant activities (AA) in blueberries (Giovanelli et al. 2013; Moyer et al. 2002). However, the relationship between TPC and AA is much stronger than TAC and AA (Moyer et al. 2002). Moreover Ghosh et al. (2018) found that in half-high blueberry plants, TPC and AA are strongly related in comparison to total flavonoid content (TFC) and AA. Nonetheless, quantification of total AA in a plant is a complex process as it is influenced by linkage of different phytochemicals among themselves and working synergistically or antagonistically in the presence of various environmental factors (Hassimotto et al. 2005).

Micropropagation of blueberry plants has been an area of interest for plant scientists due to possibility to improve phytochemical properties. It has been used in various medicinal plant species to elevate the level of phenolic components with AA to satisfy industrial pharmaceutical needs (Dias et al. 2016; Giri & Zaheer 2016). It has been found earlier that micropropagated berries have higher levels of antioxidant in

comparison to the conventionally propagated blueberries (Debnath & Goyali 2020). The reason behind increasing phenolic content during plant tissue culture can be due to the use of PGR in the growth media which may be involved in up and down-regulation of genes engaged in secondary metabolite production pathways (Sakakibara, Takei, & Hirose 2006; Zifkin et al. 2012). During direct shoot regeneration of aloe species, it was found that presence of cytokinin in the media alone or in combination with auxin increased the amount of TPC, TFC, and condensed tannin to the plants regenerated from the media without any PGR (Amoo et al. 2012). It was also proven that the type of cytokinin and concentration in the culture media affects the biosynthesis pathways of secondary metabolites (Debnath & Goyali 2020). Phenolic compounds are the most commonly found secondary metabolite in plats and are derivatives of phenylalanine. Cytokinin concentration is positively correlated with the level of transcription of genes encoding enzymes such as ammonia lyase, chalcone synthase, chalcone isomerase, and dihydroflavonol reductase which are associated with the flavonoid biosynthesis pathway thus indirectly correlated to the TAC in Arabidopsis (Deikman & Hammer 1995). Not only that, but various environmental factors like low concentrations of nutrients and light increase phenylalanine ammonia lyase activity which is an important managing factor of plant metabolic pathways (Taiz & Zeiger 2006). It was found that lowbush blueberry genotype ‘QB9C’ and cultivar ‘Fundy’ were affected by micropropagation techniques and ‘QB9C’ fruits showed higher content of secondary metabolites and AA than ‘Fundy’ regenerants (Goyali et al. 2013). Later Goyali et al. (2015) reported increased levels of TPC, TAC, TFC, total proanthocyanidins (TPAC), and AA in the tissue culture regenerants of lowbush blueberry clone ‘QB9C’ and cultivar ‘Fundy’ in comparison to

the stem cutting counter parts. There are several examples available on in vitro propagation techniques affecting AA in various berry plants such as lingonberry (Georgieva et al. 2016; Vyas et al. 2013), bilberry (Georgieva et al. 2016), strawberry (Debnath 2009a; Georgieva et al. 2016), and raspberry (Georgieva et al. 2016).

1.6. DNA methylation

Plant tissue culture techniques have flourished and improved over time for numerous plant species. Many researchers have incorporated micropropagation techniques into many basic and applied aspects of plant science for years (Lee & Phillips 1987). According to Cassells and Curry (2001) there are a few well described physiological, genetic and epigenetic problems associated with plant cultured in vitro such as recalcitrance, hyperhydricity, and somaclonal variation. Variation in tissue culture-regenerated plants has been termed as somaclonal variation (Larkin & Scowcroft 1981). Due to its origin somaclonal variation is also referred to as tissue culture-induced variation (TCIV). Mutations are referred to as heritable genetic variations that emerged without any intervention of genetic recombination or segregation (van Harten, 1998). Mutations can be induced either physically, chemically or by tissue culture (Predieri 2001; Roux 2004). However, mutagenesis due to tissue culture is different than mutation caused by physical and chemical agents because it was unclear for a long time that this was a possible cause of mutagenesis (Larkin 1998). In vitro propagation techniques such as callus induction, embryo formation, and regeneration can be exceptionally stressful to

the plant cells (Lörz et al. 1988). TCIV can occur due to epigenetic (developmental) or genetic (heritable) changes (Lörz et al. 1988; Skirvin et al. 1994). Epigenetic variations are more often transient and inheritable even though the plant material is asexually propagated (Duncan 1996). However, these variations can lasts many generations and phenotypic variants involves changes in expression of specific genes (Hartman & Kester 1983). In multicellular organisms genetic and epigenetic mechanisms are involved in coordinated gene expression. DNA methylation, chromatin modification, and non-coding RNA biosynthesis are the main three epigenetic marks which mediate epigenetic regulation (Bond & Finnegan 2007).

DNA methylation is one of the most researched epigenetic variation. It is a chromatin modification which does not alter genetic sequence but regulates gene expression by supressing transcription factor and DNA association (Smith & Meissner 2013). DNA methylation can be present in various forms depending on the targeted nucleotide at the modification process (Ratel et al. 2006). It is a post-replicative mechanism and generates several methylated bases such as 5-methylated cytosine (5-mC), N6-methyladenine (6-mA), and N4-methylcytosine (4-mC) (Wion & Casadesús 2006). Among these 5-mC is present mostly in all higher plants and mammals unlike 6-mA and 4-mC which are predominant in bacteria, protists, and lower eukaryotes (Wion & Casadesús 2006). Various DNA cytosine methylation enzymes such as methyltransferases (METS) and domain rearranged methyltransferases (DRMs) mediate DNA methylation process (Rival et al. 2008). Rejuvenation describes as the restoration of juvenile traits in an adult plant is an epigenetic phenomenon (Huang et al. 2012). Many woody perennial

plant species lose the ability to rejuvenate with maturity which can impose a major problem in regards to vegetative propagation (Welander 1983). However, according to Huang et al. (2012), plants' capability to rejuvenate can be regained during tissue culture processes. During tissue culture process plant cells go through callus formation (dedifferentiation) and plantlet regeneration (redifferentiation) which creates a highly stressful condition for the plant material, thus normal cellular regulation gets disrupted (Guo et al. 2007). It has been already studied that degree of altered DNA methylation is related to differentiation, and during dedifferentiation and redifferentiation DNA methylation levels change drastically (Huang et al. 2012). There are not many studies available on DNA methylation during plant tissue culture process. Goyali et al. (2015) observed that tissue culture regenerated lowbush blueberry plants showed increased level of antioxidant level than the softwood cutting counterparts. Later molecular analysis with simple sequence repeat (SSR) markers confirmed that there were no genetic changes in the regenerants which further led the authors to consider the involvement of epigenetic factors. Afterwards, a higher percentage of total DNA methylation was detected in callus tissues than leaf tissues of three lowbush blueberry clones and a hybrid genotype by methylation sensitive amplification polymorphism (MSAP) technique. It was also found that methylation events in callus were more polymorphic than leaf samples (Ghosh et al. 2017). Global methylation analysis by MSAP technique detected altered DNA methylation patterns in tissue cultured plantlets than softwood cutting plants of lowbush blueberry (Goyali et al. 2018).

DNA methylation studies can be approached from various standpoints as there are different methods available to detect tissue culture-induced DNA methylation, which is the localization of methylated cytosine in a particular region of the genome. Among the most popular methods are detection by MSAP, high-performance liquid chromatography (HPLC), high-performance capillary electrophoresis (HPCE), and whole genome bisulfite sequencing (WGBS). MSAP is based on the sensitivity of two restriction enzymes (*MspI* and *HpaII*) to identify methylated cytosine bases (Cedar et al. 1979). This technique is a modified version (Reyna-Lopez et al. 1997) of the amplified fragment length polymorphism (AFLP) technique developed by Vos et al. (1995) which was developed to detect DNA methylation in dimorphic fungi. Subsequently, the MSAP technique has been used in many tissue culture regenerants to detect global methylation including blueberry (Ghosh et al. 2017; Goyali et al. 2018), grapes (Baránek et al. 2009; Schellenbaum et al. 2008), ginseng (Chakrabarty et al. 2003), apple (Hao & Deng, 2003), freesia (Gao et al. 2010), rice (Stroud et al. 2013; Wang et al. 2013), and pea (Baránek et al. 2009; Smýkal et al. 2007) etc. At the genomic DNA level, methylated cytosine can be detected by enzymatic means, although it is not as sensitive as the HPLC method as its resolution is restricted to the cleavage sites of the endonucleases. HPLC can quantify DNA methylation via fractionation of the four bases (A, T, G, C) (Fraga & Esteller 2002). When options are available, HPCE has been demontrated to be more beneficial than HPLC as it is faster, cheaper and comparatively more sensitive (Fraga et al. 2000). This methods have not been used in blueberry so far, but used in various other crops to detect cytosine methylation in tissue culture regenerants such as, oil palm (Jaligot et al. 1999), *Acca sellowiana* (Fraga et al. 2012), triticale (Machczyńska et al. 2014), and apple (Li et

al. 2002). Enzymatic methods are commonly used as they do not involve complex equipment and skilled labour. Enzymatic isoschizomer-based methods do not provide any details on how methylcytosine affects cell and molecular biology. However, these questions can be answered with the use of bisulfite modification of methylated DNA. There are various methods available based on the treatment of sodium bisulphite to detect the methylated cytosine located in a specific location of the DNA (Fraga et al. 2000). Sodium bisulphite converts unmethylated cytosine to uracil while methylated cytosine stays the same (Furiuchi et al. 1970). WGBS provides genome-wide methylation profiling without any interference but not much information is available on minimum required coverage and other factors such as, susceptibility, precision, and cost of the assay (Ziller et al. 2014). WGBS has been used in many tissue culture-regenerated plants for example apple (Gulyás et al. 2019), pineapple (Lin et al. 2019), tomato (Yang et al. 2019), rice (Hu et al., 2020), Chinese cabbage (Lee et al. 2021), and blueberry (Ghosh et al. unpublished results). Various aspects of tissue culture-induced DNA methylation are discussed in next chapter (Chapter 2).

1.7. Research goals

Micropropagation of blueberries has been done for years via various regeneration pathways (Aalders et al. 1979; Debnath 2004, 2007a, 2009b, 2009c, 2011a; Fan et al. 2017; Frett & Smagula 1983). However, various aspects of TCIV, including the epigenetic reason behind them, and also their detection, have not previously been studied

in detail. Here, I have discussed TCIV in relation to DNA methylation in elaboration (Chapter 2).

Although different techniques of blueberry in vitro propagation have been done before, the SE technique has not ever been exploited in blueberries so far. This led to the initial hypothesis for this thesis research that SE can be used to develop a cost-effective technique for blueberry micropropagation (Chapter 3). In general blueberries display high AA due to which they are commercially important and are in high demand (Kalt et al., 2020; Kalt & Dufour, 1997; Kalt et al., 1999; Prior et al., 1998). However, there are limited data available showing the effects of micropropagation on TPC and AA of blueberries. In addition, the AA of various phytochemicals available in blueberries have not been studied in SE regenerated blueberry plants. To gather insight on how SE affects the AA of half-high blueberries, I have compared the TPC, TFC, and AA in SE-regenerated blueberry leaves in relation to their donor plants maintained in greenhouse (Chapter 3).

Micropropagation techniques have been used in blueberries to obtain true-to-type plants, but this may not always ocuur. Continuous subculturing to maintain the plant material may give rise to somaclonal variation or TCIV (Larkin & Scowcroft 1981). TCIV could be either beneficial or deleterious for commercial propagation of perennial fruit crops. TCIV in regenerants can be either genetic and epigenetic or both in nature (Biswas et al. 2009). I was able to utilize previously acquired data (Goyali et al. 2015) to predict that changes occurring are epigenetic in nature and DNA methylation is playing an important role behind it. I performed a comparative analysis on global DNA

methylation patterns via the MSAP technique on globular callus regenerated from young lowbush and hybrid blueberry leaves compared to their donor plants grown in the greenhouse to see the differential methylation patterns generated due to the in vitro propagation process (Chapter 4).

I was able to use the data obtained in Chapter 4 to predict the effect of in vitro culture techniques on global DNA methylation status of regenerants. This idea further interested me to determine the effects of SSM and liquid medium on DNA methylation pattern of regenerants. Lastly, I compared the altered DNA methylation patterns of half-high blueberry plants regenerated from two different media types (Chapter 5).

The results of the work presented in this thesis further elucidate the effect of micropropagation on AA and altered DNA methylation pattern of various blueberry genotypes. It also provides starting points for further work on the direction of WGBS among SE regenerated and cutting-generated plants to figure out the genes involved, their differential methylation status, and their level of expression in each scenario.

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Chapter 2 - Tissue culture-induced DNA methylation in crop plants: a review

Abstract

Plant tissue culture techniques have been extensively employed in commercial micropropagation to provide year-round production. Tissue culture regenerants are not always genotypically and phenotypically similar to their parents. Due to the changes in the tissue culture microenvironment, plant cells are exposed to additional stress which induces genetic and epigenetic instabilities in the regenerants. These changes lead to tissue culture-induced variations (TCIV) which are also known as somaclonal variations to categorically specify the inducing environment. TCIV includes molecular and phenotypic changes caused in the in vitro culture due to continuous sub-culturing and tissue culture-derived stress. Epigenetic variations such as altered DNA methylation patterns are induced due to the above-mentioned factors. Reportedly, alteration in DNA methylation patterns are much more frequent in the plant genome during the tissue culture process. DNA methylation plays an important role in gene expression and regulation of plant development. Variants originating in tissue culture process due to heritable methylation changes, can contribute to intra-species phenotypic variation. Several molecular techniques are available to detect DNA methylation at different stages of in vitro culture. Here, we review the aspects of TCIV with respect to DNA methylation and its effect on crop improvement programs. It is anticipated that a precise and comprehensive knowledge of the molecular basis of in vitro-derived DNA methylation

will help to design strategies to overcome the bottlenecks of micropropagation systems and maintain the clonal fidelity of the regenerants.

2.1. Introduction

The advent of plant tissue culture techniques has become one of the most important tools in modern plant science research, and adoption of these techniques in crop production may provide the answer for adequate food manufacturing for the community. These techniques are not only used in the crop breeding programs but also have commercial importance, including micropropagation at a large-scale, recombination DNA technology, germplasm conservation, and natural plant metabolite production (Neelakandan & Wang 2012). Micropropagation techniques have great potential for crop improvement as it allows the production of pathogen-free genetically and physiologically similar plants in large numbers (Debnath 2018). Although tissue culture regenerants are planned to be alike; this is not always the case.

Due to fluctuations in the in vitro microenvironment, plant cells go through additional stresses, which induce genetic and epigenetic variabilities in the regenerants leading to TCIV. Somaclonal variation is a widely accepted term representing TCIV; it was first proposed in plants by Larkin and Scowcroft (1981). Somaclonal variation occurs universally in all cell and tissue cultures regardless of the micropropagation system (Larkin & Scowcroft 1981). The occurrence of TCIV in the micropropagation system, is the reason behind the clones are not true-to-type to donor plants. This type of variations

causes serious problems for the researchers and plant propagators who require fidelity in their clones. Despite of that, variability associated with the in vitro system provides a pool of natural variants upon which selection pressure can be appointed to isolate the desirable regenerants in the form of clones (Jain 2001). TCIV can be demonstrated as a mitotically or meiotically stable phenomenon (Springer 2013), which may occasionally lead to the production of plant variants, which is useful in crop improvement programs (Jain 1998; Peredo et al. 2006). Mitotically stable variants create phenotypes which are physiologically different only among the primary regenerants and that are rarely transmitted to subsequent generations. This technique has been successfully used in case of ornamental plants and tree species where primary regenerants are the end products (Kaeppler et al. 2000). However, in a plant's lifecycle, environmental and genetic changes induce DNA methylation, which could eventually create epigenetic variations and affect up to several generations (Lukens & Zhan 2007). Occationally, epigenetic variations induce heritable changes in gene expression without altering the DNA sequences (Kaeppler & Phillips 1993).

DNA methylation involves the addition of methyl group in the DNA at the cytosine residues (Bird 2002). DNA methylation is allied with various molecular mechanisms such as regulation of genes, chromatin inactivation, genomic imprinting, and cell differentiation in plants (Park et al. 2009). Available evidence shows that altered cytosine methylation pattern is much more frequent in the plant genome in the in vitro system and leads to discrete phenotypic changes (Finnegan et al. 1996; Kaeppler & Phillips 1993). Tissue culture-induced mutations such as, activation of transposable

elements, chromosome breakage, changes in DNA sequence are hypothesized to occur as a reason of DNA methylation, which eventually leads to high-rate occurrence of phenotypic variation (Kaeppler et al. 2000; Park et al. 2009). DNA methylation is the most frequently found type of covalent base modification across the various taxa (Springer & Schmitz 2017). Specific DNA methylases generate several methylated bases during the state of post-replicative DNA modification, among which 5-methylcytosine (5-mC) is prevalent in higher plants and in mammals (Springer & Schmitz 2017).

Various methods are available to detect changes in the genome-wide methylation pattern in tissue culture plants. Most of these methods are based on comprehensive knowledge of an organism's genome sequence (Fulneček & Kovařík 2014), such as bisulfite modification (Cokus et al. 2008; Li et al. 2020) and chromatin immunoprecipitation (ChIP) (Kaufmann et al. 2010; Wang et al. 2010). Methylation-sensitive amplification polymorphism (MSAP) is a modified amplified fragment length polymorphism (AFLP) technique (Vos et al. 1995) that does not require genome sequencing. High-performance separation techniques such as high-performance capillary electrophoresis (HPCE) (Smýkal et al. 2007) and high-performance liquid chromatography (HPLC) (Renau-Morata et al. 2005) are similarly used in detection of cytosine methylation. The review uncovers the studies showing alteration of DNA methylation patterns that are linked with the variation in the characters at single gene or genome level, by which a large number of high quality breeding material or improved agronomic traits can be attainable (Karim et al. 2016). On these grounds, cytosine methylation can be potentially used as an important source of variation for crop

improvement. This review highlights the recent progress on TCIV and its implications on a crop improvement program and fills in any existing gaps which have not yet been clarified.

2.2. Plant tissue culture and micropropagation systems

A few possible pathways are available in plant tissue culture and regenerants can be obtained either by axillary bud proliferation, adventitious shoot regeneration or via formation of somatic embryos (Steward et al. 1970). Organogenesis, i.e. formation of either shoot or root and somatic embryogenesis (SE) – the formation of a bipolar structure containing the root and shoot meristem at the same time from a somatic cell (Steward et al. 1970). This technique is used for clonal propagation of various economically important plants (Us-Camas et al. 2014), including grape (Vidal et al. 2009), pineapple (Sripaoraya et al. 2003), chili (Santana-Buzzy et al. 2005), strawberry (Biswas et al. 2009), Siberian ginseng (Chakrabarty et al. 2003), *Cymbidium* orchids (Ghosh et al. 2014), raspberry (Debnath 2014), and blueberry (Ghosh et al. 2018). Similar to organogenesis, somatic embryos can be obtained directly from the explants or indirectly with the interference of the callus formation at any stage of development (Us-Camas et al. 2014). Although direct SE has been reported from microspores, ovules, embryos , and seedlings (Ghosh et al. 2014; Vidal et al. 2009), leaf explants are also found to be responsive to SE (Chakrabarty et al. 2003; Ghosh et al. 2018). However, there is a great variability on leaves´ response to SE induction across plant taxa.

2.3. In vitro-induced variation – history and origin

The term TCIV or somaclonal variation has been widely used to denote the variations originated during the tissue culture process (Larkin & Scowcroft 1981). Many other names are also used to describe this variation such as gametoclonal, protoclonal, and meridional variation, depending on their tissue of origin (Karp 1994). Ideally, clonal propagation process involves only mitotic division to produce plantlets asexually. Thus, all the regenerants should be phenotypically and genotypically identical (Bairu et al. 2010). However, with the first observation of TCIV (Braun 1959), it imposed a major problem in clonal propagation system (Bairu et al. 2010). The unmanageable occurrence of variations might be the reason for not including somaclonal variation regularly in crop improvement programs, as this could disturb the already present desirable traits in the crop plants. Despite that, several tissue culture-induced variants have been released as commercial varieties and cultivars (Katiyar & Chopra 1995). Unlike spontaneous mutations, TCIV arise frequently in explants and the occurrence of spontaneous mutations is much more frequent during in vitro culture than in in vivo culture (Ahloowalia 1986). In tissue culture system, the genetic material is unprotected. Thus chances of exposure to chemicals present in the medium is higher due to which survival of the variants in non-inclusive environment increases with the rate of occurrence of mutation in tissue culture system than in field grown plants (Ahloowalia 1986). Even though, when the mutation rate is same in the somaclones and conventionally grown plants, detection of physiological variants is quite difficult in a crop grown in field because of larger production area (Ahloowalia 1986). The variations in

somaclones emerge due to spontaneously induced major uncontrolled changes in cells, tissues, or organs which may occur genetically or epigenetically (Karp 1994) (Fig. 2.1). For instance, in twenty-five regenerants from callus cultures of wild barley, no phenotypic differences were identified, although there were differences in the their rDNA spacer length and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) profile of hordein (Breiman et al. 1987). However, often somaclones are morphologically different from the donor plants and are permanently different when the variations are genetic and heritable (Kaeppler et al. 2000). On the other hand, temporary changes occur due to the epigenetic or physiological factors which always do not inherit to the next generation (Kaeppler et al. 2000; Bairu et al. 2010). Chromosomal aberrations, sequence variations, point mutations, transposable element activation, and most importantly alterations in DNA methylation patterns are examples of some genetic and epigenetic variations which contribute to somaclonal variation (Jain 2001).

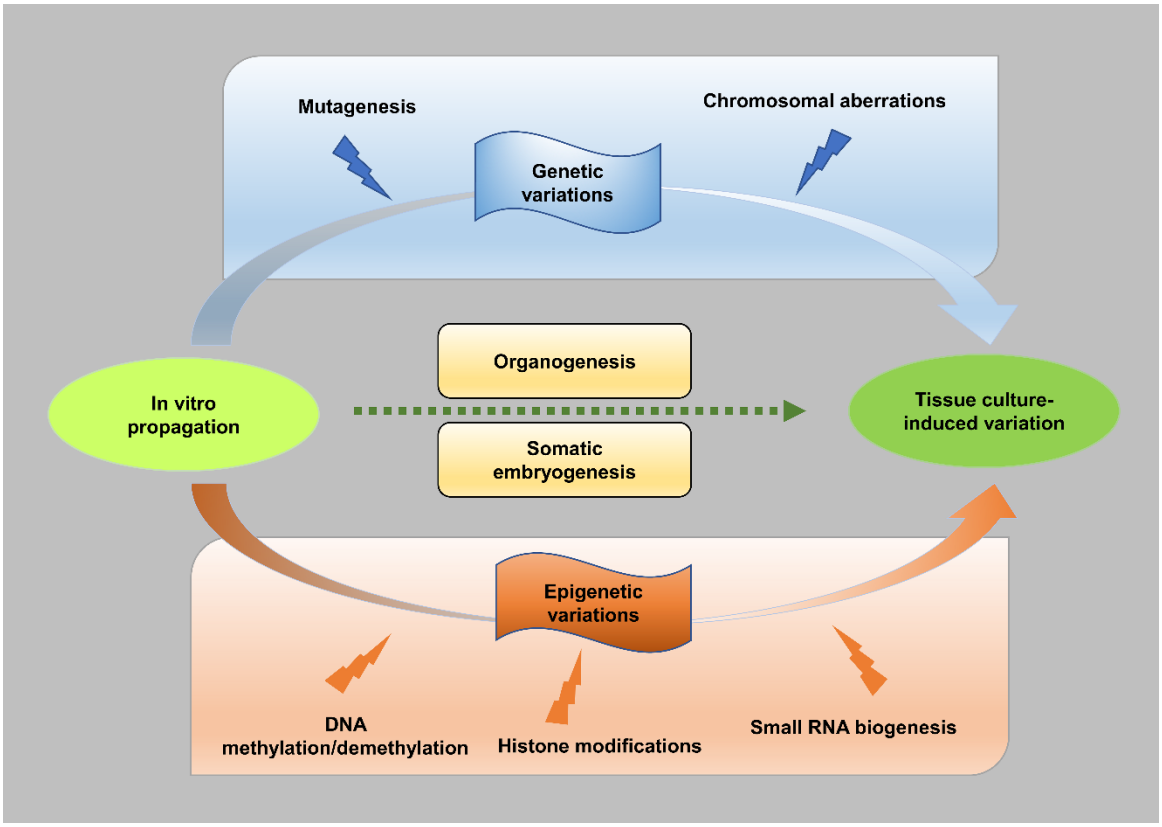

Figure 2.1: Schematic diagram of tissue culture-induced variation

2.4. DNA methylation - a chromatin modification

In plants and mammals, DNA methylation is conserved and specific patterns of genomic DNA methylation are extremely important for development (Zhang et al. 2018). It is well accepted that DNA retains information in a bigger context than the DNA sequence alone, while DNA sequences harbor genetic codes, and in some cases imprint these codes in a epigenetic fashion into various phenotypic traits (Springer & Schmitz 2017). DNA methylation is an evolutionarily ancient covalent modification (cytosine

converts to 5-mC), often associated with gene silencing in eukaryotes (Law & Jacobsen 2010). Among all the heritable and major epigenetic changes DNA methylation is one of the best studied process (Karim et al. 2016). DNA methylation level varies from 0 – 3% in insects, 2 – 7% in vertebrates, up to 10% in fish and amphibians, while plant genome shows a high rate of DNA cytosine methylation of over 30% (Cokus et al. 2008; Law & Jacobsen 2010). In plants, DNA methylation usually occurs in CpG islands in all symmetric (CG or CHG, where H = A, T or G), and asymmetric (CHH) contexts unlike animal where CG methylation prevails (Karim et al. 2016). Among these, three types of cytosine methylation occurs in plants, frequency of CG sequences getting methylated is highest followed by CHG and CHH sequences (Karim et al. 2016). For instance, in Arabidopsis genome-wide cytosine methylation 24% was reported from CG context, while 6.7 and 1.7% were from CHG and CHH contexts (Cokus et al. 2008). Another study on whole genome bisulphite sequencing (WGBS) on mulberry under drought stress tolerance deciphered that methylated cytosines were most prevalent in CG sites (44.28%) followed by CHG (28.50%) and CHH (27.22%) sites (Li et al. 2020).

To ensure if DNA methylation is inherited correctly to the next generation, several molecular mechanisms are involved (Springer & Schmitz 2017), and many of these mechanisms have been studied in detail in Arabidopsis (Law & Jacobsen 2010). In Arabidopsis mutations in the fragment of methylation and demethylation pathways are not always lethal, yet it appears to be crucial during development and environmental stress responses in plants with complex genomes (Zhang et al. 2018). It was long established that in plants DNA methylation is not an active process rather disturbed

stability of DNA methyltransferases may cause decreased level of DNA methylation (Bednarek & Orłowska 2020). In plants, domains rearranged methyltransferase 2 (DRM2) catalyses the *de novo* DNA methylation (Zhang et al. 2018). DRM2 is a homologue of DNA methyltransferase 3 (DNMT3) (Zhang et al. 2018), which is a family of *de novo* methyltransferases in mammals (Law & Jacobsen 2010). Unlike mammals, for *de novo* methylation in plants an RNA-directed DNA methylation (RdDM) pathway is of high importance (Zhang et al. 2018). In the canonical RdDM pathway as studied in Arabidopsis (Zhang & Zhu 2011), RNA polymerase IV (POL IV) synthesizes short single stranded RNAs then RNA-dependent RNA polymerase 2 (RDR2) convert them to double stranded RNAs (dsRNAs) (Zhang et al. 2018; Gallego-Bartolomé 2020). Dicer-like protein 3 (DCL3) cleaves the dsRNAs into small interfering RNAs (siRNAs) and incorporated onto Argonaute (AGO) proteins, especially AGO4 and AGO6 (Gallego-Bartolomé 2020). The second stage of canonical RdDM pathway is established on RNA polymerase V (POL V) dependent transcription of non-coding RNAs (ncRNAs) (Gallego-Bartolomé 2020). The ncRNAs are connected through the sequence complementarity of AGO4 and AGO6 proteins loaded with siRNAs (Zhang et al. 2018). Right after the AGO, siRNA, ncRNA and POL V ribonucleoprotein complex is assembled, DRM2 comes into play to target DNA methylation (Zhang et al. 2018). Canonical RdDM pathway is moderated by POL IV-dependent 24-nt siRNAs, small RNAs from various origins such as polymerase II (POL II) transcripts and viruses and moderate non-canonical RdDM pathways (Cuerda-Gil & Slotkin 2016). Initially, the species of RNA triggering RdDM was unknown, later studies found out involvement of siRNAs and long noncoding RNAs (lncRNAs) in this mechanism (Springer & Schmitz

2017; Zhang et al. 2018). In the centromeric region, and repetitive sequences of the Arabidopsis genome DNA methylation is heavily abundant (Zilberman et al. 2007), of which one-third of the methylated loci are highly associated with siRNAs which also points to the fact that siRNAs are involved in DNA methylation (Lister et al. 2008). siRNAs are commonly associated with transcriptional silencing and targets CG or CHH cites for methylation and with each cycle of DNA replication, siRNAs preserves the pattern of CHH methylation in the daughter cells (Zhang & Zhu 2011). RdDM pathway directs DRM2, DNA methyltransferase 1 (MET1) and chromomethylase 3 (CMT3) to their target sites, as CHH methylation cannot be maintained by methyltransferases (Zhang & Zhu 2011). siRNAs play a key role in fine tuning any undesirable methylation pattern via RdDM pathway (Springer & Schmitz 2017) and furthermore deep sequencing of siRNAs at various stages of plant development will reveal other key roles characterized by them. RdDM is involved in various biological processes such as, paramutation, repression of transposons activity, biotic and abiotic stress responses, and creation of methylation patterns during reproduction (Zhang et al. 2018). This pathway was first discovered in transgenic tobacco plants infected with viroid, non-protein-coding RNA molecules of few base pairs long which produced recombinant viroid sequences in the plant genome after replication mechanism (Wassenegger et al. 1994). Although there are some pathways involved in silencing untargeted sequences, DNA methylation maintenance pathways alter the action of epigenome (Springer & Schmitz 2017). To sustain the silenced state of transposons and to conserve the cell identity, stability of established global DNA methylation pattern is crucial (Law & Jacobsen 2010). Maintenance of DNA methylation in plants depends on the susceptibility of the

methyltransferases towards different context of cytosine sequences such as CG, CHG and CHH (Zhang et al. 2018). CG methylation is maintained by DNA methyltransferase 1 (MET1 or DMT1), which is plant homologue of DNA methyltransferase 1 (DNMT1) (Law & Jacobsen 2010). MET1 identifies the hemi-methylated CG context after DNA replication and converts the unmethylated cytosine to 5-mC in the daughter strand (He et al. 2011). CHG methylation is maintained by CMT3 in a greater extent than by chromomethylase 2 (CMT2) (Cokus et al. 2008; Law & Jacobsen 2010). Methylated CHH context is maintained by DRM2 or CMT2 based on genomic region (Zhang et al. 2018; Gallego-Bartolomé 2020), for instance DRM2 preserves the RdDM target sites while CMT2 catalyses heterochromatin containing histone H1 which is prohibited from RdDM (Zhang et al. 2018). It was also found that in case of DDM1 (decrease in DNA methylation 1) mediated RdDM pathways CMT2 catalyze CHH methylation (Zhang et al. 2018). Although the maintenance of methylation status by these pathways is well studied, the removal of DNA methylation by the same is not outlined accurately (Law & Jacobsen 2010). DNA methylation in angiosperms is well maintained by the plant genome itself, new information on establishment and maintenance of this has extended our knowledge on regulation of DNA methylation (Springer & Schmitz 2017). It is still very important to have a clear understanding of these molecular mechanisms to use them for improvising the epigenome for crop improvement programs.

2.5. DNA methylation and gene expression

The molecular mechanism behind transcriptional control during plant development is not yet completely understood. Studies have shown that in plants, DNA methylation suppresses DNA-transcription factor associations, which in turn regulates gene expression and various cellular processes (Law & Jacobsen 2010). Recent studies on whole genome methylation regarding gene expression have emphasised the effect of DNA methylation on gene regulation. Methylation commits to the inactivation of transposable elements or foreign DNA, thus maintains the stability of whole genome over non-homologous recombination and controls gene transcriptions (Lukens & Zhan 2007). DNA methylation also affects the developmental characters in plants. For example, in linaria demonstrated variation in floral symmetry due to cytosine methylation (Cubas et al. 1999). The changes in floral symmetry resulted from epimutation and no changes were detected in the DNA sequence. Epimutations arise due to transcriptional repression and/or reactivation of a suppressed gene by DNA methylation. However, these epigenetic changes were unstable and reverted to the original form after a certain number of generations. Linaria epimutants produce radially symmetrical flowers instead of producing bilaterally symmetrical flowers. Due to the loss of function of linaria cycloidea-like gene (*Lcyc*), mutant flowers were produced in the same plant. It was found that in the mutant plants *Lcyc* gene persists in a highly methylated state, which causes the suppression of *Lcyc* gene function (Cubas et al. 1999). Although epigenetic variations are mostly non-heritable (Jain 2001; Miguel & Marum 2011), genes which are silenced by DNA methylation can be relocated to the alleles on sister chromatid or homologous genes

on the other chromosomes. The process by which gene gets suppressed by DNA methylation and transfer of that to the sister chromatid is referred as paramutation (Chandler 2010). Paramutation is unpredictable in nature and may lead to the production of variant phenotypes. In maize, paramutation has been well described in four genes, such as *b1*, *r1*, *p11*, and *p1* which are responsible for encoding the transcription factors and biosynthesizing flavonoid pigments (Sidorenko & Peterson 2001). Among these four genes paramutation at *b1* is extremely stable of all systems, because of which it is widely used as a model to study paramutation (Stam et al. 2002). Paramutants are generated due to methylated *B1* locus transferred to a non-methylated allele on the complementary gene, resulting in repression of gene expression (Soppe et al. 2000). DNA methylation can generate novel epiallelic state after transcription process which can provide a new avenue to give rise to phenotypic variation when it is lacking genetic mutation.

2.6. Transgenerational epigenetic memory and DNA methylation patterns

An organism's epigenetic information may affect their phenotype, which can be feasibly stored and inherited following segregation as cytosine methylation (Springer 2013). DNA methylation may activate a gene which has been silenced by other mechanisms during embryo development. On the contrary, embryonic transcription may result in complete exclusion of DNA methylation machinery (Bird 2002). Heritability of methylation state is a spontaneous phenomena which supports the concept that DNA methylation could be included or excluded for specific cellular memory during

development (Bird 2002). However, a growing number of evidences are available on heritable natural difference in the DNA methylation patterns of two individuals of the same species and it has a comprehensive capacity to confer to quantitative trait variation and crop improvement (Springer 2013). Evidences of epigenetic variations having an influence on natural variations can be seen in few classic examples, such as *peloric* mutant of linaria (Cubas et al. 1999), a *colorless non-ripening* variant of tomato (Manning et al. 2006), and sex determination of melon (Martin et al. 2009). These are the few cases where meiotically heritable epialleles give rise to morphological variations. Epigenetic alleles or epialleles are referred to the methylation level of certain region of genome which varies between individuals (Zhang et al. 2018). Within genetically diversified Arabidopsis population epiallelic variation has been discovered, however, it is still not clear if epialleles were emerged due to genetic variations (Vaughn et al. 2007). It has been also found that RdDM pathways can affect protein-coding genes by silencing their activity and giving rise to epialleles which could be heritable by mitosis/meiosis (Springer & Schmitz 2017). Epialleles can possibly be generated during DNA methylation maintenance or mutagenesis, which in turn creates an avenue for crop improvement by using artificially generated epialleles.

2.7. DNA methylation as molecular basis of tissue culture-induced variations (TCIV)

Recent researches have been focused on detecting the frequency of epigenetic variations in the tissue culture system to assess the stability of clonally propagated plants

(Miguel & Marum 2011). Epigenetic variations were reported to be stable for more than a hundred years but occasionally they revert back to the original form (Cubas et al. 1999). During callus formation, cultures are successfully being established as experimental model systems (Berdasco et al. 2008) to unveil the dynamics of epigenetic changes during cell dedifferentiation (Miguel & Marum 2011), and eventually the regulation of developmental reprogramming (Berdasco et al. 2008). Few well recognized examples of heritable and stable epigenetic modifications were already discussed, such as first natural plant variant linaria (Cubas et al. 1999) and melon (Martin et al. 2009). It has been speculated that the environment has an intense role in epigenetic variations (Us-Camas et al. 2014). For example, in Arabidopsis heat tolerance might get affected by CMT2-dependent CHH methylation, where it has been also showed that an allele at CMT2 locus exhibits a modified whole-genome CHH methylation pattern associated with temperature resilience (Shen et al. 2014). It has been found that the *Cmt2* mutants displayed higher level of heat tolerance, which in turn suggests genetic regulation of epigenetic mechanisms leading to natural adaptation to various levels of heat-stress (Shen et al. 2014). According to the epigenetic theories, the interaction between an organism's genes and environment affects an individual's developmental process and eventually it's heredity to the next generation (Springer 2013). In contrast to genetic alterations, epigenetic modifications may be heritable and can be influenced by the environment (Springer 2013). In the tissue culture system, the occurrence of epigenetic variations has been reported at several stages (Kaeppler & Phillips 1993; Miguel & Marum 2011; De-la-Pena et al. 2012). It was hypothesized by Phillips et al. (1990) that DNA methylation could be the prime factor in TCIV. Several studies have reported TCIV in plants due to

DNA methylation. For example, higher level of DNA methylation was noticed within maize callus and in vitro regenerated plants (Kaeppler & Phillips 1993; Stelpflug et al. 2014). Similarly, Brown et al. (1990), Müller et al. (1990), and Stroud et al. (2013) found that high frequency DNA methylation and sequence variation were present in the progeny of the tissue culture-derived rice plants. In lowbush and hybrid blueberries, higher level of methylated CCGG sites were found in callus (215 – 258), while in the leaves methylated tetranucleotides sites were present in comparatively low number (75 – 100) (Ghosh et al. 2017). However, analysis with methylation sensitive/insensitive restriction enzymes proved that DNA methylation does not play as the main factor behind all these changes.

It was reported that DNA methylation may affect gene expression by altering the chromatin structure via creating variation in methylation at specific sites which could result in alteration of gene expression sites in a positive or negative way (Jain et al., 2013). Higher expression of *OsSPL14* (*Squamosa promoter binding protein like – 14*) promoter during the reproductive stage in rice due to hypermethylation increase panicle branching leading to higher grain yield (Miura et al. 2010). In this study the authors have shown incorporation of the *OsSPL14* - *WFP* (*Wealthy farmer's panicle*) allele in the commonly used rice variety 'Nipponbare' improved rice grain yield. This is an example of positive gene expression due to DNA methylation, on the other hand classic example of gene expression in a negative fashion due to DNA methylation is mantled in vitro-derived variants of oil palm (Ong-Abdullah et al. 2015). Tissue culture-induced abnormality due to hypomethylation in oil palm fruit severely reduced the oil production

and mainly affected the production of elite hybrids for oil production via micropropagation. However, there are various examples available on attaining superior agronomic quality due to DNA methylation, many other studies also suggested that crop species which avoid DNA methylation might be agronomically superior to the ones which are vulnerable to DNA methylation (Lukens & Zhan 2007).

2.8. Stress induced in tissue culture system and DNA methylation

Even in the absence of an inducing stimulus, epigenetic mechanisms create an epigenetic memory during cell division to store the changes occurred in the surroundings (Springer 2013). It is well known that plant cells maintain their developmental plasticity during differentiation (Bednarek & Orłowska 2020) and need to go through reprogramming to switch from differentiation to toti-/pluripotency (Neelakandan & Wang 2012). Under tissue culture system, explants undergo either direct/indirect organogenesis or somatic embryogenesis (Neelakandan & Wang 2012). In case of indirect organogenesis process, the cells go through dedifferentiation, which incorporate chromatin level reprogramming to induce callus formation (Neelakandan & Wang 2012; Stelpflug et al. 2014). Eventually the proliferating cells in the callus go through redifferentiation with the application of plant growth regulators (PGRs) in the culture medium leading to organogenesis or plantlet regeneration (Miguel & Marum 2011). The dedifferentiation and redifferentiation process under an artificial condition during in vitro culture, imposes traumatic stress on the plant cells, which initiate mitotically and

meiotically heritable genetic and epigenetic variations (Kaeppler et al. 2000). Stresses which arise during plantlet regeneration via tissue culture process affect the normal functioning of cell organelles, but plasma membrane and cell wall sense the stress first and produce reactive oxygen species (ROS) (Das & Roychoudhury 2014). Overproduction of ROS by lipid autolytic peroxidation or antioxidative defences creates oxidative damages to the cells (Cassells & Curry 2001). Cellular homeostasis is not conserved in tissue culture system under stress like cold and heavy metal and excess production of ROS may influence the decadence of DNA, proteins, lipids and pigments affecting cellular functioning (Das & Roychoudhury 2014) leading to TCIV (Bednarek & Orłowska 2020). Cassells and Curry (2001) speculated that in plant tissue culture system various factors such as recalcitrance, hyperhydricity, poor physiological condition of explants are also involved behind inducing genetic and epigenetic variation, where oxidative-stress damage plays a major role (Cassells & Curry 2001). Not only that but also TCIV can occur due to epigenetic regulation as it may results in permanent alteration of DNA methylation level (Bednarek & Orłowska 2020). Although there are many reports available on this area, transgenerational responses leading to TCIV due to environmental stress in maize is one of the classic instances (Kaeppler et al. 2000). The sources of variations can be categorized according to the time of occurrence in the explants, such as pre-existing or variation induced during in vitro process (Bairu et al. 2010). Although pre-existing variations such as chimeras in the explants could eventually induce stress differently and leads to variation (Bairu et al. 2010), clear understanding which including methylation pattern is very important to comprehend the changes arise during tissue culture process.

Permanent variations in the regenerants are caused by the presence of pre-existing variations in the source plants or may be due to the expression of novel variations as an effect of an unknown mechanism(s) in the genome (Larkin & Scowcroft 1981). The genotype, explant type, propagation method, use of PGRs, ploidy level of explant tissue, and the age of culture are main determining factors of precedent variations in tissue culture system (Jain 1998). TCIV have been studied extensively to date, yet this phenomenon is far from complete understanding. Pre-existing variations arise separately from the effect of mutations, epigenetic changes such as alteration in DNA methylation pattern or by the combined effect of genetic and epigenetic factors (Kaeppler et al. 2000). However, qualitatively and quantitatively inherited mutations, use of chimeric tissues as explants, sequence variations, chromosomal aberrations, regulation of cell cycle, and transposable element activation in explants are also reasons behind the presence of pre-existing variations in the explant tissues (Bairu et al. 2010).

The frequency of appearance of somaclones and the nature of variation differ with the different explant sources used for clonal propagation (Sahijram et al. 2003). It has been noticed that in profoundly differentiating tissues such as roots, leaves, and stems normally produce an increasing number of variations if used as explants rather than tissues with pre-existing meristem, for example shoot tips and axillary buds (Duncan 1996). Regeneration from older and/or highly specialized structures generally recover higher amounts of variation in the regenerants (Krishna et al. 2016). Adventitious shoot regeneration directly from leaves, petioles, shoot internodes, segments of root, anthers, hypocotyls, cotyledons or indirectly through formation of callus from the same type of

explants shows higher amounts of variations in the regenerants (Pijut et al. 2012). The presence of chimeric tissue in the explants (Karp 1994), and the unstable behaviour of the explants in the tissue culture system due to in vitro-derived stresses are also involved in the induction of somaclonal variation (Bairu et al. 2010). Reportedly, type of explants as well as the age of the explants used in the tissue culture system are the major determinant factors of the plantlet regeneration rate (George et al. 2007). In general lower rate of DNA methylation was observed in the cultures, when the explants were obtained from young plants of *Pinus radiata* D. Don (Fraga et al. 2002). In contradiction, higher proportion of methylated cytosines (22.4%) was noticed in the young microshoots tissues of *Acacia mangium* in comparison to the mature microshoots with lower methylation rate (20.7%) (Baurens et al. 2004).

TCIV may also arise due to the presence of callus in the in vitro culture. Variants originating from the callus are sometimes denoted as “calliclones” and commercial laboratories try to avoid callus formation during micropropagation due to the occurrence of variation in in vitro-induced callus (Skirvin et al. 1994). Initiation of culture and subsequent subculture cycles make explants vulnerable to oxidative stress which might induce mutations (Cassells & Curry 2001). In addition to that, it is evident that unorganized growth in different levels of tissue culture process from highly organized meristem tip culture to callus formation in the ‘extreme’ process like protoplast culture also impose stress and causes somaclonal variation (Karp 1994). Generally, the extent of this stress depends on the technique of tissue culture (Krishna et al. 2016). Consequently, it was also found in eggplant that indirect regeneration via callus phase shows a higher

rate of mutation than plantlet production through axillary branching (Zayova et al. 2010). The extent of DNA methylation is also reported to be dependent on the level of differentiation. It was observed that, DNA methylation level changes radically as calluses go through the dedifferentiation and redifferentiation process (Huang et al. 2012).

In various plant species, callus formation starts with the application of exogenous auxin and cytokinin (Peschke & Philips 1992). Ideal concentration, and the ratio of auxin and cytokinin are most important for the efficient shoot, and root regeneration. Exogenously applied PGRs control primary events, which induce cell cycle disturbance leading to morphogenesis and the introduction of variations among the regenerants (Peschke & Philips 1992). In genetically abnormal cells, PGRs increase the rate of cell division (Bayliss 1980) and also the presence of PGRs in the media might induce somaclonal variation via cell-cycle disturbance (Peschke & Philips 1992). Morao et al. (2016) studied the comparison between DNA methylomes among various cell types where they found that mitotically active columella cells from root tips exhibits highest methylation level especially at CHH sites over transposons sequences implying strong RdDM activity. Comparative level of auxin and cytokinin influence the genetic composition of the cell population (d'Amato 1975). It has been observed that the application of 6-Benzylaminopurine in high concentration (15 mg/l), increases the chromosome number in the banana somaclone CIEN BTA-03 derived from a cultivar 'Williams' (Giménez et al. 2001). Similarly, a higher concentration of artificial auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) also triggers somaclonal variations in soybean (Gesteira et al. 2002) and cotton (Jin et al. 2008). This is possibly due to the frequent use

of 2,4-D in callus and cell cultures inducing genetic anomalies such as polyploidy and endo-reduplication of DNA (Bouman & De Klerk 2001). The effect of 2,4-D was also found in genome-wide cytosine methylation in carrots. The application of 2,4-D in the embryogenic culture of carrot promotes total cytosine methylation, therefore 2,4-D alters the pattern of methylation in the genome (LoSchiavo et al. 1989).

Induction of somaclonal variations during the in vitro condition also reportedly influenced by the number and length of the subculture cycles. With the increased number of subculture cycles, higher proliferation rate can be obtained in a relatively short period of time in tissue culture (Rodrigues et al. 1997; Bairu et al. 2006). In cell suspension and callus culture, the chances of somaclonal variation increases with a higher number of the subculture and duration in in vitro culture (Bairu et al. 2006). In micropropagated banana, somaclonal variation started to appear at 1.3% after the fifth subculture, and after 11^{th} subculture increased to 3.8% (Rodrigues et al. 1997). In addition to subculture cycle, the length of the culture period also affects the rate of somaclonal variation occurrence (Bairu et al. 2006). Four months of in vitro storage was suggested as a solution to the unstable ploidy level observed in long-standing coffee cell culture (Clarindo et al. 2012). The same phenomenon was observed in olive, where difference in the morphological characters was confirmed by randomly amplified length polymorphism (RAPD) analysis in long term cultures (Farahani et al. 2011).

2.9. DNA methylation and organogenesis

During organogenesis, regulation of specific genes is very important and DNA methylation is described as one of the regulatory mechanism during this process (Us-Camas et al. 2014). Several reports are available on effects of DNA methylation in direct organogenesis. In bush lily for example, the rate of DNA methylation was higher in shoot tip regenerated plants than the plants from young leaves or petals (Wang et al. 2012). However, along with the explants in vitro and ex vitro environmental factors also influence DNA methylation during organogenesis processes (De-la-Pena et al. 2012). In apple shoot tips organogenesis, it was observed that DNA methylation was influenced by the prolonged exposure of the tissue to low (4°C) temperature, though the DNA sequence or ploidy level did not change (Hao & Deng 2003). Effects of DNA methylation were also studied in indirect organogenesis along with direct organogenesis. In tissue culture-derived Arabidopsis callus, few chromatin modifying enzymes like *MET1*, *KRYPTONITE* (*KYP*), histone demethylase gene *JUMONJI 14* (*JMJ14*), and histone acetyltransferase *WUSCHEL* (*WUS*) were analyzed to identify their influences on degree of shoot regeneration (Li et al. 2011). Bisulfite sequencing (BS-seq) and ChIP results demonstrated that the methylation and histone modification at the promoter region of the gene resulted in altered expression of these genes and rate of shoot regeneration from the callus (Li et al. 2011).

2.10. DNA methylation and somatic embryogenesis

SE is one of the best studied plant tissue culture process (Karim et al., 2016) and it is affected by many factors. DNA methylation is regarded as a very crucial factor to control the induction and developmental process somatic embryos similarly to the zygotic embryos (Nic-Can et al. 2013). It is reported that DNA hyper and hypomethylation impact the SE process (Chakrabarty et al. 2003; Nic-Can et al. 2013; Kwiatkowska et al. 2014). For example, using HPLC/MS/MS method association of DNA hypermethylation was detected with somatic embryo development in *Acca sellowiana* (Fraga et al. 2012). Likewise, in *Coffea canephora* increase in DNA methylation level found to be positively correlated with rate of SE (Nic-Can et al. 2013). Furthermore, DNA hypomethylation has been found to be related to somatic embryo development from asynchronous T87 cell culture of Arabidopsis (Kwiatkowska et al. 2014). Ji et al. (2019) found that the level of genome-wide DNA methylation, most apparently for CHH context increases during globular-stage of somatic embryos collected from 6 weeks to 13 years of soybean continuous culture. It was also clearly shown that DNA hypomethylation was most abundant in the already silenced regions and it was coupled with the gene upregulation responsible for reinforcing RdDM pathway (Ji et al. 2019). On the contradiction, in Siberian ginseng lower level of DNA methylation was detected using MSAP technique in embryogenic callus in comparison to the non-embryogenic callus (Chakrabarty et al. 2003). The changes in DNA methylation pattern of plant cells and tissues arise during in vitro culture due to the exposure to different factors such as basal medium, PGR used, stages of culture, biotic and abiotic stresses due to wounding of the explant, nutrients used

in the growth medium, physical factors, photoperiod etc. (Karim et al. 2016; Neelakandan & Wang 2012). DNA methylation pattern found under these conditions regulate gene expression related to SE and plant regeneration processes (Karim et al. 2016). For instance, in carrot cell suspension cultures, 16% methylated cytosine with stable DNA methylation pattern was seen during SE upon applying exogenous auxin. However, the application of hypomethylating drugs such as 5–azacytidine (5-azaC) and ethionine, blocked SE (LoSchiavo et al. 1989). A similar trend was observed in alfalfa, application of 5-azaC in the embryogenic line decreased the formation of somatic embryos extensively (Santos & Fevereiro 2002). Fraga et al (2012) reported lower level of methylation in *A. sellowiana* cell culture when 5-azaC was added to the medium, but higher level of embryo induction was noticed when 5-azaC and 2,4-D were added in the culture in combination. It is also reported that at the preliminary stages of somatic embryo development, embryos contain comparatively lower levels of DNA methylation in comparison to the older stages (Sahijram et al. 2003). Occasionally, the SE pathway is selected as a desirable pathway for regeneration (Krishna et al. 2016) as direct embryogenesis provides genetically similar plants than shoot tip regeneration because at preliminary stage of SE, DNA contains comparatively lower level of methylation (Sahijram et al. 2003) .

2.11 Methods of detecting in vitro-induced DNA methylation

In large-scale commercial micropropagation detection of variants is a matter of challenge due to the presence of a huge number of clones in a large area. Therefore, detection and exclusion of negative agronomic traits at early stages is very important to reduce economic loss to the growers (Bairu et al. 2010). At later stages of any crop improvement program, it is essential to evaluate the variants at different environmental conditions for the successful establishment of desirable traits over generations (Duncan 1996). Although various methods are available to detect somaclonal variation, this review discusses the methods used for detection of DNA methylations in vitro. Modified AFLP techniques are mostly used to detect tissue culture-induced DNA methylation (Miguel & Marum 2011). The principal of MSAP technique is constructed on the susceptibility of the pair of isoschizomers *MspI* and *HpaII* instead of *MseI*, as 'frequent cutter' enzymes, and *EcoRI* as a 'rare cutter' enzyme same as that used in the original AFLP protocol (Vos et al., 1995; Reyna-Lopez et al. 1997; Fulneček & Kovařík 2014). *MspI* and *HpaII* restrict the 5-CCGG-3 recognition site based on methylation of external or internal cytosine. *MspI* cleaves methylated internal cytosine residues (C^mCGG) but not the external (^mCCGG) whereas *HpaII* cleaves the hemimethylated external cytosine but remains inactive for fully methylated sequences (Peraza-Echeverria et al. 2001). Although this is an economically feasible, fast, and easy practicable process for non-model organisms, the selection of these restriction enzymes may results in inconsistent data interpretation, which may not agree with the previously acquired data (Fulneček & Kovařík 2014). This technique was first developed in dimorphic fungi (Reyna-Lopez et al. 1997) and later

used to detect methylation in rice (Wang et al. 2013), banana (Peraza-Echeverria et al. 2001), apple (Li et al. 2002), Siberian ginseng (Chakrabarty et al. 2003), pepper (Portis et al. 2004), hop (Peredo et al. 2006), *Doritaneopsis* orchid (Park et al. 2009), freesia (Gao et al. 2010), and blueberry (Ghosh et al. 2017; Goyali et al. 2018). Quantification of global DNA methylation can be done by high-performance separation techniques such as HPCE, HPLC (Berdasco et al. 2009). These techniques involve genomic DNA digestion to nucleotide, nucleoside or nitrogenous bases through enzymatic hydrolysis, to isolate, and analyze 5-mC (Berdasco et al. 2009; Miguel & Marum 2011). Although both of this capillary hydrolysis based approaches are quite time consuming, they are highly specific and sensitive, which makes them useful for rapid quantification of global DNA methylation even from poorly isolated or low quality samples (Berdasco et al. 2009). In some ornamental crops, such as Cedrus (*Cedrus atlantica*, and *C. libani*) HPLC has been used to detect DNA methylation in axillary bud culture (Renau-Morata et al. 2005), whereas HPCE was used in in vitro shoot culture of pea to detect the global methylation pattern (Smýkal et al. 2007). Unlike in other eukaryotic organisms, in case of plants, genome-wide application using ChIP methodology is usually scarce (Kaufmann et al. 2010). ChIP techniques such as, (ChIP)-chip and ChIP-seq detect DNA methylation by mapping the point of interaction between DNA and the protein of interest. The ChIP-chip technique identifies the sites of DNA-protein interaction in DNA while ChIP-seq detects cytosine methylation by combining immunoprecipitation with shotgun sequencing technique (Kaufmann et al. 2010). However, the huge amount of data generated through the high-throughput sequence creates a great difficulty to identify the protein binding sites in case of ChIP-based techniques (Wang et al. 2010). ChIP-seq was used in Arabidopsis

to detect DNA-transcription factor binding site during DNA methylation process (Wang et al. 2010). Bisulfite modification is another efficient mechanism to identify methylated cytosine. During the process, genomic DNA treated with sodium bisulfite converts cytosine to uracil, while methylated cytosine does not change (Cokus et al. 2008). WGBS is believe to be the best procedure to detect DNA methylation in plant samples as this technique allows to detect single nucleotide resolution of 5-mC on a genome while other techniques fails to provide that (Cokus et al. 2008; Lin et al. 2019). Currently this is the most updated and direct approach which allows to identify and detect the pattern of methylated cytosine within the whole genome (Chwialkowska et al. 2017). However, this technique is still very costly for the plants with comparatively larger genome size than Arabidopsis or rice (Chwialkowska et al. 2017). So far WGBS has been used to detect level of DNA cytosine methylation on various plant genomes, which further proved the fact that DNA methylation varies across plant species. This technique was used in the detection of DNA methylation in the whole genome of Arabidopsis. An unknown side of the Arabidopsis methylome was revealed in WGBS procedure following next generation sequencing (Cokus et al. 2008). Recently, this technique has been used to detect altered methylation level on long-term in vitro shoot culture and regenerants of apple (Gulyás et al. 2019) and plants regenerated from pineapple callus culture (Lin et al. 2019). Methods detecting DNA methylation in some crops and plant species are listed in Table 2.1.

Table 2.1: Techniques used to detect tissue culture-induced variation due to DNA methylation in various plant species

Plant species	Major findings	Detection technique(s)	References
Alfalfa (*Medicago truncatula* Gaertn.) – R108, 'Jemalong'	*Medicago RetroElement 1-1* was detected in *Medicago* genome, which was found to be active only during culture and supplements favourably in genes in both accessions	BS-seq	(Rakocevic et al. 2009)
Apple (*Malus × domestica Borkh.)* – M. 26, MM – 106 rootstocks	Somaclones were detected with lower susceptibility to diseases	Isozymic analysis	(Rosati et al. 1990)
Apple – 'Gala'	Differential methylation pattern observed between in vitro and field grown plants	MSAP	(Li et al. 2002)
Apple – 'Gala'	AFLP detected no variation in DNA sequence, MSAP analysis confirmed insignificant difference in methylation level	AFLP/MSAP	(Hao & Deng 2003)
Apple – 'McIntosh', 'Húsvéti rozmaring'	Methylation change modifies gene expression in the in vitro shoot culture	WGBS	(Gulyás et al. 2019)
Arabidopsis (*A. thaliana*)	DNA methylation and histone modifications regulate i*n vitro* shoot regeneration by	BS-seq, ChIP	(Li et al. 2011)

	manipulating *WUS* expression and auxin signaling		
Bamboo (*Bambusa balcooa* Roxb.)	MSAP assays assured no changes in the methylation level in the somatic embryogenesis regenerants	MSAP	(Gillis et al. 2007)
Banana (*Musa* spp.) – 'CIEN BTA-03'	Somaclones were found to be Yellow Sigatoka disease resistant	RAPD	(Giménez et al. 2001)
Banana – 'Grand Naine'	DNA methylation polymorphism was reported only in the in vitro regenerated plants	MSAP	(Peraza-Echeverria et al. 2001)
Barley (*Hordeum vulgare* L.)	Developed met-AFLP based protocol to detect TCIV	MSAP	(Bednarek et al. 2007)
Blueberry (*Vaccinium angustifolium* Ait.) – Clones 'CL1', 'CL2', 'CL3', (*V. corymbosum* L. × *V. angustifolium*) – hybrid 'H1' ('Chippewa' × 'Patriot')	Higher level of DNA methylation was found in callus tissues	MSAP	(Ghosh et al. 2017)
Blueberry (*V. angustifolium*) – 'Fundy', 'QB9C'	Differential methylation pattern and methylation polymorphism was noticed in higher frequency in in vitro regenerated plants	MSAP	(Goyali et al. 2018)

Bush Lily (*Clivia miniata* Lindl.)	Altered DNA methylation profiles were reported from the somaclones and extent of DNA methylation depends on explant type, regeneration pathways and number of subculturing	MSAP	(Wang et al. 2012)
Cedrus (*Cedrus atlantica* Endl. and *C. libani* A.Rich.)	No significant differences were found in global DNA methylation level of in vitro shoot cultures	Reversed-phase HPLC, RAPD	(Renau-Morata et al. 2005)
Codonopsis (*Codonopsis lanceolate* Benth.)	Variant methylation pattern was found among the in vitro regenerants using MSAP assay profiles	MSAP	(Guo et al. 2007)
Doritaneopsis (*Doritis* × *Phalaenopsis*) orchid – 'Labios'	Somaclones derived from root tip cultures showed phenotypic variation through flower characteristics	MSAP	(Park et al. 2009)
Freesia (*Freesia hybrida* Eckl.)	SE regenerated plantlets from *Freesia* flowers exhibited higher level of cytosine methylation	MSAP	(Gao et al. 2010)
Grape (*Vitis vinifera* L.) – 'Chardonnay' clone 96, 'Syrah' clone 174	MSAP profile showed altered DNA methylation pattern in the regenerants	SSR, MSAP	(Schellenbaum et al. 2008)

Grape – ‘Müller Thurgao’, ‘Riseling’	Stress developed via nodal segments and in vitro thermotherapy during tissue culture process induce DNA methylation in plantlets	MSAP	(Baránek et al. 2010)
Hop (*Humulus lupulus* L.)	MSAP analysis confirm presence of DNA demethylation in organogenic in vitro culture	AFLP, MSAP	(Peredo et al. 2006)
Maize (*Zea mays* L.) – A188	High frequency DNA demethylation was observed during embryo culture of maize	MS-RFLP	(Kaeppler & Phillips 1993)
Maize – A188	A stable epigenetic footprint was detected in tissue culture regenerated	Methylated DNA immunoprecipitation (MeDIP)-ChIP, methylation, methylation sensitive qPCR, RNA sequencing	(Stelpflug et al. 2014)
Oil palm (*Elaeis guineensis* Jacq.)	Tissue culture-induced abnormality in oil palm fruit due to DNA hypomethylation severely reduces the oil production	BS-seq	(Ong-Abdullah et al. 2015)
Pea (*Pisum sativum* L.) – ‘Bohatýr’	MSAP detected differential DNA methylation pattern and polymorphism among in vitro shoot cultures	MSAP, HPCE	(Smýkal et al. 2007)

Pineapple (*Ananas comosus* L.)	WGBS results suggested that DNA methylation during tissue culture process induced variation	WGBS	(Lin et al. 2019)
Potato (*Solanum tuberosum* L.)	AFLP markers showed low level of methylation polymorphism in regenerants from SE and microtuberisation	AFLP	(Sharma et al. 2007)
Potato	*Stowaway* Miniature inverted-repeat transposable elements (MITEs) can be activated under in vitro culture process resulting changed tuber skin colour	Real-time PCR/southern blot analysis	(Momose et al. 2010)
Potato – 'Russet Burbank'	HPLC confirms escalated level of phytonutrients in more than 800 somaclones tested	HPLC	(Nassar et al. 2014)
Rice (*Oryza sativa* L.) – 'IR 40'	RFLP assays found higher level of genetic instability in the plants regenerated from callus culture which were related to DNA methylation	RFLP	(Müller et al. 1990)
Rice –'Norin N 10'	Methylation-sensitive and non-sensitive assays shown that DNA methylation was not the principle factor behind the TCIV in the protoplast culture	MS-RFLP	(Brown et al. 1990)
Rice – 'Nipponbare'	BS-seq tests in regenerated plants reported significant losses of methylation which was mostly stable across generations	BS-seq	(Stroud et al. 2013)

Rice (*O. sativa* spp. *japonica*) – 'Nipponbare', (*O. sativa* ssp. *indica*) – 93-11	Somaclonal variation was detected in rice pure-lines, F1-hybrids and polyploids via MSAP assays	MSAP	(Wang et al. 2013)
Siberian ginseng (*Eleuterococcus senticosus* Maxim.)	Lower level of DNA methylation was detected in embryogenic callus via MSAP technique in comparison to non-embryogenic callus	MSAP	(Chakrabarty et al. 2003)
Strawberry (*Fragaria* × *ana nassa* Duch.)	Selected somaclones were found to be distinct from each other in terms of fruit yield and other horticultural characters	RAPD	(Biswas et al. 2009)

2.12. Tissue culture-induced DNA methylation and it's implication on crop improvement

In any crop improvement program genetic variations are crucial factors. Conventional plant breeders develop new cultivars by combining genes of interest from well-established varieties or linked species by the process of sexual hybridization, thus developed new cultivars with better agronomic traits (Jain 2001). With an ever-increasing human population, the demand for sustainable food production came up as a challenge for conventional plant breeders. The aim of a crop improvement program is to select the improved varieties with heterosis and transgressive variations, including both genetic and epigenetic modifications (Springer 2013). Mutations caused by heritable epigenetic traits are known as epimutations and these epimutations are very difficult to detect without the

intervention of whole genome structure analysis (Law & Jacobsen 2010). Examples of epimutations found in various plants are listed in Table 2. Several recent studies on Arabidopsis have shown that in a population of epigenetic recombinant inbred lines (epiRILs) individual plants differ from each other on the basis of epigenetic information (Springer 2013). One of this studies demonstrated that the epiRILs were generated by exposing the genome to a mutation responsible for removal of DNA methylation and segregating away the mutation thus creating an altered methylation pattern in the segregated genomic segments (Bender & Fink 1995). Although there are only a few examples are available on using a genetic approach identical to epiRILs, it can be used as a technique to introduce variations in crop plants. Predominantly genetic mutations result in loss-of-function allele while epimutations often lead to gain-of-function alleles by the loss of epigenetic silencing (Springer 2013). Epialleles that originates from various genetic mutations such as transposon insertions are quite stable because of the continuous presence of reprogramming machinery of chromatin modification. On the other hand, naturally occurring epimutations are less stable due to the lack of assurance of reinstating epigenetic information (Springer & Schmitz 2017). Instability of the epimutants in comparison to the genetic mutants can be a potential drawback of using epimutation in a crop improvement program. During a breeding program for crop improvement, if the epigenetic state only displays partial heritability, it can be difficult to stabilize the epigenetic state within the population and generate epimutants (Springer 2013). However, epimutations in clonally propagated plant species may allow scrutinizing epiallelic alterations in favour of novel allelic variations without depending upon DNA recombination (Springer 2013; Telias et al. 2011). This would be specifically important in

case of clonally propagated species (Telias et al. 2011), with either germplasm bottlenecks or limited recombination in some genomic region (Springer 2013). Another drawback of using epimutations for crop improvement program would be activation of transposable elements (Mirouze et al. 2009), which can lead to generation of production of deleterious alleles and higher rate of mutation resulting into diminished utility of the epimutants (Springer 2013).

While some horticultural crop species are clonally propagated to maintain their trueness-to-type, in conventional crop improvement programs heritable genetic variations are important components, which often create stable inbred and hybrid varieties for further agricultural use (Springer 2013). Predominantly, plant breeders tend to select phenotypic variations over a specific molecular change, which occurs within a specific generation, e.g. non-heritable epigenetic variations (Jain 2001; Springer 2013). Stable genetic variations can arise due to the genetic changes, for example, gene duplication, and insertions of transposons in the genome (Jain 1998). However, as somaclones have widened the variability in crops, they can be used to improve many plant characteristics such as grain yield and quality, plant height, flowering, resistance to biotic and abiotic stresses such as insects-pests, diseases, cold, drought, salinity, and soil pH (Jain, 1998). Several reports are available demonstrating desirable variations in somaclones that are already used in plant breeding programs regularly. For instance, Indian mustard which has high yielding capacity and shattering resistance (Katiyar & Chopra 1995), mint with increased oil and herb yield (Kukreja et al. 1991), neurotoxin devoid Lathyrus (Yadav & McHta 1995), early blight disease resistant potato (Rodriguez et al. 2007), salinity and

drought tolerant sugarcane (Yadav et al. 2006), red rot disease resistant sugarcane (Singh et al. 2008), and aluminium toxicity tolerant rice (Roy & Mandal 2005).

DNA methylation is the only epigenetic factor for which conservation and stable inheritance pattern in the consecutive generations are well understood (Law & Jacobsen 2010), e.g. that heritable TCIV in rice regenerants for subsequent generations were often due to DNA hypomethylation which also sometime effect the expression of nearby genes (Stroud et al. 2013). In another study on maize, it was found that differentially methylated regions were developed due to tissue culture process and among these regions hypomethylation was prevalent in comparison to hypomethylation (Stelpflug et al. 2014). TCIV due to DNA methylation was detected in Arabidopsis lineages, where variations were successfully inherited and segregated within R1 progenies, which provided the opportunity to study variations induced during tissue culture system in plants (Jiang et al. 2011). DNA methylation also plays an important role managing the developmental events and response features in the in vitro system for example, in Arabidopsis callus and suspension cultures regulation of undifferentiated state due to gene repression by DNA methylation in the promoter region of a particular single copy gene has been reported earlier (Berdasco et al. 2008). In general, a typical crop improvement program takes 10 - 15 years to be fully completed and undergoes many stages from germplasm collection to crop production (Krishna et al. 2016). DNA methylation can be used in crop improvement programs in a controlled way to generate better agronomic traits without incorporating any foreign genes (Us-Camas et al. 2014) (Fig. 2.2). Although in few cases,

this method may not provide a extremely stable variant which will pass the long term conventional breeding procedure (Springer 2013).

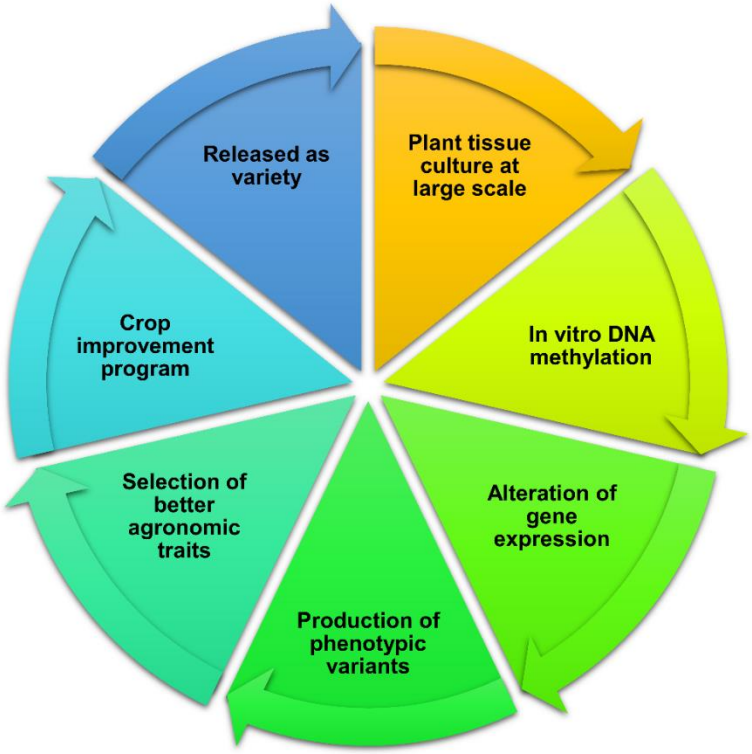

Figure 2.2: Potential implications of DNA methylation in the cycle of crop improvement via plant tissue culture

In Arabidopsis, diminished DNA methylation gives rise to plentiful morphological and phenotypic irregularities which includes reduced apical dominance, decreased plant size, leaf size and modification, lowered fertility, and irregular flowering time (Finnegan

et al. 1996). This may be due to the plants after going through the meiotic events, reportedly having decreased ability to reinstate the previous DNA methylation pattern (Finnegan et al. 1996). Some clonally propagated strawberry cultivars were observed with hyper flowering and abnormal fruit setting. Hyper flowering was reported from 'stipular buds' at a particular position of leaf petiole. These buds showed high multiplication rate in vitro and an increasing number of flower production per inflorescence in ex vitro condition. This habit was speculated to be due to the DNA methylation and not the influence of any true mutation (Boxus et al. 2000). In lowbush blueberry, it has been reported that micropropagated plants are higher in polyphenols and flavonoids than the softwood cutting plants (Goyali et al. 2015). However, number of flower clusters, berries, fruit weight per plants diameter and height of individual fruits were significantly reduced in clonally propagated plants (Goyali et al. 2015). Although molecular assay of the tissue culture regenerants with simple sequence repeat (SSR) markers established their genetic fidelity with the softwood cutting counterparts, it was hypothesized that variations originating in the blueberry clones probably due to epigenetic variations induced during the in vitro culture process (Goyali et al. 2015). Later methylation analysis in micropropagated lowbush blueberry callus and leaves collected from the greenhouse grown plants confirmed that, in vitro derived calluses contained higher amounts (14 - 30%) of methylated cytosine in the genome, while the percentage of DNA methylation in leaves is comparatively lower (13-18%) (Ghosh et al. 2017). Complete understanding of the molecular nature of TCIV is very important to exploit it further in crop breeding programs.

Table 2.2: Epimutations due to DNA methylation in various plant species

Plant Species	Epimutations	Induced during tissue culture	Phenotypic variant	References
African violet (*Saintpaulia* spp. H.Wendl.)	Retrotransposon activation of *VGs1*	Yes	Flower colour change	(Sato et al. 2011)
Alfalfa (*Medicago trunculata* Gaertn.)	Activation of *Medicago retroelement 1-1* (*MERE1-1)*	Yes	Curled root hair formation	(Rakocevic et al. 2009)
Arabidopsis (*Arabidopsis thaliana* L.)	Silencing of *Apetala 1 (AP1)* gene	No	Heavily methylated *AP1* gene	(Bender & Fink 1995)
Arabidopsis	Silencing of *SUPERMAN (SUP)* gene	No	Defective floral organ	(Jacobsen & Meyerowitz 1997)
Arabidopsis	Silencing of *FWA* gene due to DNA methylation in *SINE*	No	Late flowering	(Soppe et al. 2000)
Arabidopsis	Spreading of DNA methylation in *BSN* gene	No	Stunted growth	(Saze & Kakutani 2007)
Arabidopsis	Loss of *MET1* function	Yes	Altered shoot regeneration	(Li et al. 2011)

Arabidopsis	Loss of DNA methylation at 5' UTR of At5g43500	No	Phenotypic variant	(Schmitz et al., 2011)
Arabidopsis	Silencing of *Folate transporter 1* (*FOLT1)* gene due to DNA methylation	No	Reduced fertility	(Durand et al. 2012)
Maize (*Zea mays* L.)	Activation of *Ac1*	Yes	Cytogenetic variability	(Peschke et al. 1987)
Maize	Activation of *spm*	Yes	Coloured spot in kernel	(Peschke & Phillips 1991)
Maize	Paramutation at maize *b1* locus	No	Anthocyanin pigmentation	(Stam et al. 2002)
Melon (*Cucumis melo* L.)	Spreading of DNA methylation in *CmWIPI*	No	Promoted female flowering	(Martin et al. 2009)
Oil palm (*Elaeis guineensis* Jacq.)	Hypomethylation of *LINE* retrotransposon	Yes	Mantled phenotype	(Ong-Abdullah et al. 2015)
Potato (*S. tuberosum* L.)	Activation of *Tto1*	No	Change in tuber skin colour	(Momose et al. 2010)
Rice (*Oryza sativa* L.)	Silencing of *D1* gene due to DNA methylation	No	Dwarf variety	(Miura et al. 2009)

Rice	Hypermethylation at *OsSPL14* promoter	No	Higher grain yield and panicle branching	(Miura et al. 2010)
Rice	Hypermethylation at *OsFIEI* promoter	No	Dwarf variety	(Zhang et al. 2012)
Toadflax (*Linaria vulgaris* L.)	Silencing of *Lcyc* gene due to DNA methylation	No	Changed floral symmetry	(Cubas et al. 1999)
Tomato (*Solanum lycopersicum* L.)	Silencing of *Colourless non-ripening* (*CNR)* gene due to DNA methylation	No	Defective fruit ripening	(Manning et al. 2006)

2.13. Future prospects

For many years, researchers have been discussing various possible ways to include epigenetic variations in crop breeding programs. Although there are not many reports available on using epimutations in crop improvements program, Thieme et al. (2017) discussed inhibition of RNA polymerase II to decrease DNA methylation at heat responsive *copia*-like retrotransposon *ONSEN* to introduce novel genetic variation. However, there are some well-known cases on undesirable epimutations such as *colourless non-ripening* variant of tomato (Manning et al. 2006), reduction in palm oil yields due to DNA hypomethylation in *Karma* retrotransposon (Ong-Abdullah et al.

2015). Adaptation of tissue culture-induced (somaclonal) variation in crop improvement program have their advantages, for example, selection of somaclones in vitro may reduce the selection time and may fight biotic and abiotic stresses. TCIV has been successfully employed in many crop species with narrow genetic base and systems with limited genetic variations such as apomicts and vegetative reproducers (Krishna et al. 2016). Additionally, in the case of direct SE without any intervention of callus stage, embryos are formed from the individual somatic cell. Thus the chance of cellular mosaic formation is lower and originated plantlets are genetically similar with little variation (Deverno 1995). For example, in clonally propagated 'Grand Naine' banana plants, SE has been used to reduce the chance of somaclonal variation (Shchukin et al. 1996). As somatic embryos arise from single somatic cells, the chances of induction variation decrease. Later it has been confirmed that in banana variety, production of variants was lower (1.6% - 7.9%) when plantlets were produced via SE than from shoot tip culture (2.3% - 10.4%) (Shchukin et al. 1998). In general, clones originating from axillary branching are reported to be true-to-type to the donor plants (Debnath, 2018). However, disadvantages of this process include chances of negative agronomic traits production, the occurrence of unpredictable changes, and the need for extensive field trials of the somaclones before the release as a variety (Jain 2001). Moreover, the desirability of the somaclones is not possible to predict and there is no way to tell that the character of interest will always be modified in an advantageous way (Karp 1994). Predominantly, somaclonal variations in the micropropagated plants are random and lack reproducibility. Therefore, the main concern about TCIV is to make it reproducible so that it can be used in regular crop breeding programs (Jain 2001; Bairu et al. 2010). It is nt very easy to control TCIV as

more than one factor are responsible for the induction of variations in the tissue culture regenerants (Jain 2001). These variations can be controlled by avoiding longer duration in in vitro condition and reducing the number of subculture cycles (Clarindo et al. 2012). In micropropagated banana, increased rate of variation was reported with the increasing number of sub-culturing (Bairu et al. 2010). Not only the culture duration and several subculture cycles influence TCIV, mode of in vitro propagation method also affects the induction of variation. Reportedly, rice clones regenerated from protoplast culture gives rise to plant variants, which are different from conventionally propagated plants in terms of leaf morphology, flower characteristics, spikelet, and panicles (Vos et al. 1995). Cellular organization of the explants is also a very important factor for somaclonal variation (Karp 1994), generally the more breakdown of organizational structure (callus formation), the higher chances of occurrence of variation (Bairu et al. 2010). In clonally propagated 'Honeycrisp' apple, propagants shows some unexpected green and red colour patterns, which is found to be due DNA methylation at the promoter region of the *MYB10* transcription factor involved in anthocyanin production (Telias et al., 2011). Comprehensive knowledge of epialleles and epigenetic traits can help to crate a path of using DNA methylation profiling to identify the 'off-types' produced in tissue culture system (Springer & Schmitz 2017). Not only exclusion of deleterious alleles but also understanding epigenetic aspects of tissue culture regenerants will aid creating beneficial epialleles via epigenetic engineering.

2.14. Conclusion

Commercial micropropagation is based on trueness-to-type of the micropropagated plants to the donor plants. Various molecular approaches have been used to confirm the genetic fidelity in tissue culture plants. Although production of somaclones was thought to be originated from genetic variations, epigenetic factors are also found to be associated with phenotypic diversity. It is essential to understand the combining effects of genetic mutations and epigenetic variations on somaclonal variation to exploit it more efficiently in crop improvement programs. Even though somaclonal variation generates major problem regarding clonal fidelity in tissue cultured plants, it still provides a source for crop improvement in the plants with narrow genetic bases. Selection of the plants with desirable agronomic traits relies on induced variation during the tissue culture process. Recently, DNA methylation has been recognized as a major regulatory epigenetic mechanism which is associated with various regulatory gene functions during the tissue culture process. Although many studies are being performed on TCIV (including DNA methylation), the process is still far from being completely understood. Therefore, fully developed understanding of these processes will help to identify the hypervariable regions in the plant genome during tissue culture process, which could lead to the efficient control of somaclonal variations and their use in crop improvement programs on a regular basis.

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Chapter 3 - Thidiazuron-induced somatic embryogenesis and changes of antioxidant properties in tissue cultures of half-high blueberry plants

Abstract

An efficient protocol of somatic embryogenesis (SE) has been developed for the first time in four half-high blueberry (*Vaccinium corymbosum* L. × *V. angustifolium* Ait.) cultivars. Thidiazuron (TDZ), a plant growth regulator with potential activities for shoot regeneration and shoot proliferation, was found most effective for somatic embryo formation when added to a nutrient medium at high concentration (9 µM). Although TDZ was also best for embryo germination at low concentration (2.3 µM), it was followed by zeatin at 4.6 µM for the same. Plantlets developed from SE were removed from the nutrient medium and transferred on a peat: perlite medium where 100% survival rate was acquired following the acclimatization process in a greenhouse. The concentrations of total phenolic and flavonoid contents were higher in greenhouse-grown conventionally cutting-propagated donor mother plants than those of respective SE plants for 'St. Cloud', 'Patriot' and 'Northblue' but not for 'Chippewa'. The effect of propagation method and/or the older age of donor mother plants were clearly visible; as the 15-year-old donor plants showed higher level of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity than the eight-weeks-old SE plants in all four cultivars.

3.1. Introduction

Blueberry is a health-promoting small fruit crop, belonging to the genus *Vaccinium* (family: *Ericaceae*) (Vander Kloet 1988). It has very high commercial value and is cultivated across the world although the majority of the production is in the United States and Canada (Debnath 2016). There are five different types of blueberries: northern highbush (*Vaccinium corymbosum* L.), southern highbush (*V. darrowii*), low-bush (*V. angustifolium* Ait.), half-high (*V. corymbosum* x *V. angustifolium*) and rabbiteye blueberries (*V. ashei*) (Vander Kloet 1988). Half-high blueberries are hybrids between highbush (*V. corymbosum* L.) and lowbush blueberries *(V. angustifolium* Ait.) (Debnath 2016). These berries are greatly valued due to their superior quality and have gained immense popularity among the consumers. Among the 400 – 500 species in the genus *Vaccinium* (Vander Kloet 1988), blueberries contain higher levels of phenolic compounds, which are high in antioxidant activities (Prior et al. 1998; Howell et al. 2001). Presence of phenolic components in berry crops and their activities have been studied thoroughly due to the apparent relationship between the phytochemicals present in the plant product, and their association with the prevention of chronic diseases such as cancer, heart, vascular and neurodegenerative diseases (Ames et al. 1993; Shaughnessy et al. 2009; Adams et al., 2010). Antioxidants act to prevent these diseases by scavenging free radicals (Wang et al. 1996; Ehlenfeldt & Prior 2001).

Although blueberry plants can be propagated conventionally by stem cutting, it is a labour-intensive and time consuming process. Micropropagation is a rapid and efficient method for mass propagation of blueberries which can be done all the year round. This

can be obtained either by axillary bud proliferation and differentiation to mature plants or re-differentiation of newly formed meristematic tissues to fully grown plants (Debnath 2018). There are two morphogenic pathways in plant regeneration: (1) organogenesis – the formation of root or shoot (2) somatic embryogenesis (SE) – formation of root and shoot meristem simultaneously (Steward et al. 1970). In SE, somatic cells undergo a number of morphological and biochemical changes to form somatic embryos (Zimmerman 1993). These include the formation of globular, oblong, heart, enlarge, torpedo, and cotyledonal-shaped structures (Zimmerman 1993). The steps are: initiation, proliferation, maturation and plantlets formation (von Arnold et al. 2002). SE is a well-recognized powerful tool for clonal propagation and has been explored in several agronomically and horticulturally important crop species (Zimmerman 1993). The process can also be used for genetic transformation and artificial seed production (von Arnold et al. 2002). Typically, the success of SE depends on explant types and the culture media containing an optimum plant growth regulator (PGR) regime. Based on the mode of occurrence, SE can be direct, without the intervention of callus stage or indirect SE, in which they are developed via callus phase (von Arnold et al. 2002).

During past decades, thidiazuron (N-phenyl-N′-1,2,3-thiadiazol-5-ylurea) (TDZ) gained a lot of attention due to its prominent role on in vitro culture, with both auxin and cytokinin like effects, in different plant species (Murthy et al. 1995; Debnath 2018). Although limited reports on SE are available in few fruit crops such as grape (Vidal et al. 2009; Dhekney et al. 2016;) and strawberry (Biswas et al. 2009), none in detail with *Vaccinium* species. The present study aimed at whether somatic embryos can be

regenerated in vitro, and the plantlet formation is possible from four half-high blueberry cultivars, in a TDZ containing medium. TDZ was also found effective to induce SE in grapes (Dhekney et al. 2016) and for shoot proliferation and/or adventitious shoot regeneration of highbush (Debnath 2017), lowbush (Debnath 2009), and in half-high blueberries (Debnath 2017).

In vitro-derived plants frequently show a phenotypic variation known as somaclonal variation (Larkin & Scowcroft 1981). This variation in tissue culture plants from their respective donor plants during the micropropagation is of significant important for commercial propagation and germplasm conservation (Debnath et al. 2012). The study also verified if there is any difference for the antioxidant properties between the SE plants and their corresponding donor mother plants. Investigation of the effects of embryogenesis on antioxidant properties in tissue culture plants compared to their respective donor plants has not been reported before in small fruit crops. However, this is of significant importance to establish this regeneration pathway as a reliable option of commercial blueberry production.

3.2. Materials and Methods

3.2.1. Plant material

The four half-high blueberry cultivars ‘St. Cloud’, ‘Patriot’, ‘Northblue’, and ‘Chippewa’, used for this study, were grown and maintained in a greenhouse at the St.

John's Research and Development Centre, Agriculture and Agri-Food Canada (AAFC), St. John's, Newfoundland and Labrador, Canada in plastic pots (10.5 × 10.5 × 12.5 – cm^3) containing 3 : 1 (peat : perlite, v/v) under the natural light source of photosynthetic photon flux density (PPFD) at a maximum light intensity of 90 µmol m^{-2} s^{-1}, 20 ± 2 °C at 85 % of relative humidity with an automated sprinkler system for more than 15 years (Debnath 2017).

3.2.2. Effect of TDZ concentration on somatic embryo induction

Young and actively growing leaves of 2 - 3 weeks age were collected from the greenhouse-grown plants, surface sterilized (Debnath 2009) and cultured on a blueberry basal medium (BM) containing three-quarter micro salts and macro salts of Debnath and McRae (Debnath & McRae 2001) supplemented with (per litre) 3.5 g Sigma (Catalogue No. A 1296) agar, 25 g sucrose, and 1.25 g Gelrite™ (Sigma Chemical Co., St. Louis, MO, USA) for embryo induction. Leaves were placed, abaxial surface touching the medium, on 100 × 25 -mm Fisherbrand™ Petri dishes with clear lids (Fisher Scientific, Fair Lawn, NJ, USA) containing 25 ml of BM with 0 (control), 2.3, 4.5, 6.9, or 9 µM TDZ. The Petri dishes were then sealed along the rims with two layers of Paraflim™. The pH of the medium was adjusted to 5.0 prior to the autoclaving at 121 °C for 20 min. TDZ was added in the medium before it was autoclaved. Each Petri dish contained five explants and three Petri plates were used per treatment. The cultures were placed in dark for 2 weeks at 20 ± 2 °C and then transferred to the diffused light (PPFD at 30 µmol m^{-2} s^{-}

^{1}, 16 h photoperiod) provided by cool-white fluorescent lamps under same culture conditions. Data for somatic embryo formation were taken after 10 weeks of culture. The experiment was conducted three times within two-week-intervals. A 4×5 completely randomized factorial experiment was conducted to compare all treatment combinations of four half-high blueberry cultivars and 5 TDZ concentrations.

3.2.3. Effect of type and concentrations of plant growth regulators (PGRs) on the maturation of somatic embryos

For embryo maturation and elongation of plantlets, 10-week-old somatic embryos developed on perti plates with 9 µM TDZ were transferred to 175-mL Sigma glass baby-food jars (Sigma Chemical Co., St. Louis, MO, USA) containing 35 mL BM supplemented with gibberellic acid (GA_3) (0,1.4, 2.9, 4.2, or 5.8 µM), indole3-butyric acid (IBA) (0, 2.5, 4.9, 7.5, or 9.8 µM), zeatin (ZEA) (0, 2.3, 4.6, 6.9, or 9 µM), or TDZ (0, 2.3, 4.5, 6.9, or 9 µM). Filter sterilized GA_3 and ZEA were added to the cooled medium after autoclaving, while IBA and TDZ were added to the medium before autoclaving. Cultures were maintained under 16-h photoperiod at 30 µmol m^{-2} s^{-1} for shoot and root elongation. There were three jars per treatment and each jar contained five explants. The experiment was repeated three times. Data on embryo maturation and plantlet elongation was recorded after 10 weeks of transfer in glass jars. A completely randomized factorial experiment was laid out to compare all treatment combinations of four blueberry cultivars and 16 PGR concentrations with controls (void of PGR).

3.2.4. Acclimatization

After 8 weeks of culture, five plantlets (4 – 5 cm long) with 8 – 10 leaves developed on all concentrations of TDZ and ZEA were removed from the glass jars, rinsed off in sterilized water, and planted in plastic pots (25 × 18 × 6*;* East-Chem Inc. Mount Pearl, NL, Canada) containing peat: perlite (3: 1, v/v). Plantlets developed on IBA or GA_3 were not selected for acclimatization due to their poor vigour. The pots were maintained in a humidity chamber with a vaporizer at 20 ± 2 ºC, 16-h photoperiod at 55 µmol/m^2/s, humidity 95%. Acclimatization was performed by lowering down the humidity over 2 – 3 weeks to 85% which was found suitable for tissue culture-derived plantlet survival in a growth chamber (Debnath 2017). Data on the survival rate of the plantlets were recorded after the hardening off process was complete (6 weeks). Hardened off plants were transferred in 6 cm^3 plastic pots containing the same peat-perlite medium and grown in a greenhouse under natural light condition (temperature approx. 20 ± 2 ºC, 16-h photoperiod, maximum PPFD = 90 µmol m^{-2} s^{-1}, humidity approx. 85%) (Debnath 2017).

3.2.5. Extraction of polyphenolics from leaves of tissue culture and donor plants

The purpose of the biochemical assays was to compare the total phenolics, flavonoids, and antioxidant capacity in the leaves of SE plants with those of donor plants to investigate the effect of SE and/or physiological age of plants on biochemical compounds, as well as to explore the potential of SE plants to synthesize phenolic and

flavonoid compounds and evaluate the changes in antioxidant activity due to the use of TDZ in the growth medium during this process. Leaves collected from eight-weeks-old greenhouse-grown SE plants and four more than 15-year-old donor cultivars (Debnath, 2014) were shock-frozen, immediately flash-frozen in liquid nitrogen and stored at –80 ºC for chemical analyses. Antioxidant capacities in blueberries were found to be much higher in leaves than fruits (Vyas et al. 2013). At least three plants were selected randomly for each cultivar, and the leaves from each cultivar were collected in a replication of three from each plant. Five hundred milligrams of pre-frozen leaves from each plant were homogenized in a FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Irvine, CA, USA) in 80% aqueous acetone solution with 0.2% formic acid (1 : 4 g/mL) (Goyali et al. 2015). The homogenate was kept for slow agitation at 4 ºC for 30 min and then centrifuged at 13,000 rpm for 15 min at 4 ºC using Allegra 64R (Beckman Coulter Inc., Palo Alto, CA, USA) before collecting the supernatant. Extraction was done two more times with the pellets and the supernatant was combined with the initial crude extract. The final volume of the crude extraction solution for polyphenolics was 6 mL. The extracts were preserved at ultralow freezer (Thermo Scientific, Burlington, ON, Canada) for further use to determine the contents of total phenolics, and flavonoids and the antioxidant capacity. All chemical analyses were carried out thrice with each sample and mean values were used for analysis.

3.2.6. Determination of the total phenolic content (TPC)

Total phenolic content was determined following photometric technique using the Folin-Ciocalteu method (Singleton & Rossi 1965), following Goyali et al. (2015). To obtain the final concentration of 200 µg/mL, the crude extracts of polyphenolics were dissolved in methanol. Folin-Ciocalteu reagent (100 µL) was mixed to the diluted leaf extract (100 µL) and 20% saturated (w/v) sodium carbonate (200 µL) was added to it after 5 min. The mixture was added to 1.5 mL distilled water, kept in dark for 35 min at room temperature, and subjected to centrifugation at 4000 × g for 10 min in Allegra 64R. To identify the best wavelength, calibrated standards were processed according to the Folin-Ciocalteu index at 4 different wavelengths: 725 nm, 750 nm, 760 nm, and 765 nm using an Ultrospec 4300 pro UV/Visible Spectrophotometer (Amersham Biosciences Corp. San Francisco, CA, USA). The absorbances of the test samples were measured with UV/Visible Spectrophotometer at the wavelength of 725 nm, as according to mathematical calculations (Bancuta et al. 2016), wavelength of 725 nm was found to be the best to determine Folin-Ciocalteu index (Goyali et al. 2013; Abeywickrama et al. 2016). Gallic acid solution with a concentration of 5 mg/mL with ≥ 98% purity was used as calibration solution. The linearity of the standard calibration curve for gallic acid was obtained in the range of 2.5 – 10 µg/mL and results were expressed as milligrams gallic acid equivalents (GAE) per gram of leaf weight (mg GAE/ g lw).

3.2.7. Determination of the total flavonoid content (TFC)

Total flavonoid content of blueberry samples was determined according to colorimetric method (Zhishen et al. 1999) following Goyali et al. (2015). 500 µL sample extract was added to the distilled water (2 mL) and 5% (w/v) sodium nitrate (150 µL). After 5 min, 150 µL of 10% (w/v) aluminium chloride was added to the mixture, incubated for 6 min at room temperature and 1 mL of 1 M sodium hydroxide solution was added to the mixture. To dilute the mixture, 1.2 mL distilled water was added to it and absorbance was measured at 510 nm using Ultrospec 4300 pro. Catechin solution (1 mg/mL) with ≥ 98% purity was used for standard curve calibration. As the flavonoid compound is present at a very high concentration and, anthocyanidins and flavonols are the most abundant flavonoids in blueberries (Rodriguez-Mateos et al. 2012), and as reports are available that the main flavanol in blueberries is catechin (Harnly et al. 2006), it has been used as an ideal standard for this assay. Catechin has also been used by other researchers for the estimation of total flavonoid content in blueberries (de Souza et al. 2014; Wang et al. 2017; Sharif et al. 2018). Linearity of the catechin standard calibration curve was obtained in the range of 20 – 200 µg/mL and results were expressed as milligrams catechin equivalents (CE) per gram leaf weight (mg CE/ g lw).

3.2.8. Determination of antioxidant activity (AA)

2,2-diphenyl-1-picrylhydrazyl (DPPH), an artificial stabilized free radical, was used to determine the AA (Goyali et al. 2015). The free radical scavenging activity was measured as percentage inhibition of DPPH radicals. 100 mL diluted extract was thoroughly mixed with 0.06 mM DPPH methanolic solution (1.7 mL) and incubated in dark for 60 min at room temperature and the absorbance of the mixture was monitored at 517 nm at 5 min interval to perform a saturation curve. Steady state of the DPPH reaction was observed at 45 min which was used to continue the experiment. Aqueous acetone (80%) mixed with DPPH solution was the blank. To calibrate the standard curve, gallic acid at 5 mg/mL (≥ 98% purity) was used. The linearity of the gallic acid standard curve was obtained in the range of 20 – 80 µg/mL and the results were expressed as mg GAE/ g lw. Percentage inhibition was calculated using the following formula (Mishra et al. 2012):

$$\% \text{ Radical scavenging activity} = [(\text{Absorbance}_{(\text{Blank})} - \text{Absorbance}_{(\text{Extract})}) / \text{Absorbance}_{(\text{Blank})}] \times 100$$

3.2.9. Statistical data analysis

All experiments were conducted following completely randomized design with three replications and the data were subjected to a two-way analysis of variance (ANOVA), employing general linear model for main effect ANOVA using STATISTICA version 10, data analysis software (Statsoft Wipro, East Brunswick, NJ, USA). Medium

with no PGR did not show any response in terms of somatic embryo formation and were excluded from the analysis. Similarly, IBA at 2.5 µM was found ineffective for the maturation of somatic embryos in all four cultivars and the relevant data were also excluded from analysis. Tukey's Test was used to compare treatment means at a critical difference (P) of ≤ 0.05. Results are presented as mean ± standard error. Correlation coefficient (r), coefficient of determination (r^2) and linear regression between TPC and TFC, TPC and DPPH, TFC and DPPH were analysed using STATISTICA version 10 and the relationships were significant at a confidence interval of 95% ($\alpha = 0.05$).

3.3 Results

3.3.1. Effects of TDZ concentration on somatic embryo induction

At all TDZ concentrations, within 2 weeks of incubation in the dark, clear oblong-shaped protuberances started emerging at the edge of the leaves (Fig. 3.1a). Medium without TDZ did not show any response for embryo induction. Protuberances turned into globular shaped embryos within another 3 – 4 weeks of culture (Fig. 3.1b) under the diffused light. An additional week was needed for the formation of heart (Fig. 3.1c) or torpedo shaped embryos (Fig. 3.1d). Embryo formation was more at the edges of the leaf, than the middle part (Fig. 3.1b). Epicotyl development was observed within 7 – 8 weeks of culture initiation (Fig. 3.1e). Shoot and roots were formed from the meristem after another 2 – 3 weeks of culture (Fig. 3.1f).

An interaction ($P \leq 0.05$) between cultivar and TDZ concentrations was observed for the percentage SE formation. The cultivars differed significantly ($P \leq 0.05$) for this trait. Across TDZ concentrations, 'St. Cloud' (69%) was the best followed by 'Patriot' (64 %), 'Northblue' (62%), and 'Chippewa' (58%). Across cultivars, highest percentage of embryo formation was observed at 9 µM (99%) and it was followed by 6.9 µM (76 %), 4.5 µM (56%), and 2.3 µM (22%); and they were significantly different at $P \leq 0.05$ from each other. At 2.3 µM of TDZ, the percent embryo formation values were the lowest in all four cultivars ranging from 17% to 27% for ''Patriot' and 'Northblue', respectively. The percentage of SE formation increased with the increasing concentrations of TDZ and at 9 µM TDZ these values ranged from 98% to 100% in all four cultivars (Fig. 3.2).

ANOVA showed that only TDZ concentrations affected the number of somatic embryo formation ($P \leq 0.05$). As was for the percentage of embryo formation, the number of embryos per explant also increased with the increase of TDZ concentration in all four cultivars (Fig. 3.2). While across cultivars, the frequency of embryo formation varied from 1.4 at 2.3 µM TDZ to 44 at 9 µM TDZ, the cultivars had 22 – 26 embryos per explant across all TDZ concentrations. At 9 µM TDZ, the number of embryos per explant varied from 42 – 45 in four cultivars.

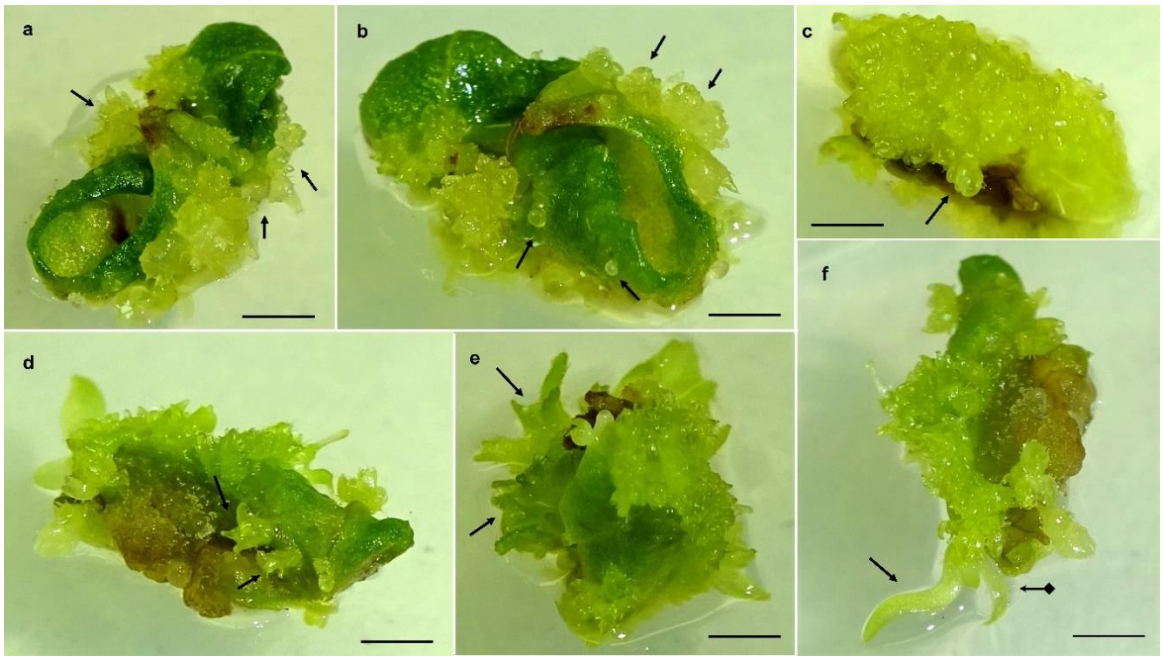

Figure 3.1: Somatic embryogenesis from leaf explants of 'St. Cloud' on a basal medium supplemented with 9.0 µM TDZ. (a) Formation of protuberances (arrows) after 2 weeks of incubation, (b) globular embryo development (arrows) from the protuberances after 4 weeks of culture, (c) heart-shaped embryo induction (arrow) after 6 weeks of culture, (d) torpedo-shaped somatic embryos (arrows) at 6 weeks in culture, (e) epicotyl development from embryos (arrows) after 8 weeks and (f) germination of somatic embryos and shoot (arrow with square end) and root apex development (arrow) after 10 weeks of culture. (Bars = 0.5 cm).

3.3.2. Effects of type and concentration of plant growth regulators (PGRs) on the maturation of somatic embryos

Somatic embryos with elongated shoots and apical root meristem developed on a BM with 9 µM TDZ were transferred on BM supplemented with 5 different concentrations of GA_3, IBA, ZEA, or TDZ to promote maturation. The medium without PGR and at 2.5 µM IBA was found ineffective for the maturation of somatic embryos in all four cultivars. The relevant results for rest of the treatment combinations are presented in Table 3.1. Analysis of variance revealed that the PGR type and concentration combinations affected significantly ($P \leq 0.05$) the maturation of somatic embryos. GA_3 at 1.4 µM promoted maturation of embryos only in 'Chippewa'. All other PGR combinations enhanced embryo maturation in all four cultivars with the highest percentage being at 2.3 µM TDZ (27 – 31%) followed by 4.6 µM ZEA (20 – 23%). Maturation percentage of the embryos was very poor in BM with GA_3 and IBA, it varied from 0 – 7% for GA_3 and from 0 – 13% for IBA. Plantlets formed after maturation in BM containing GA_3 and IBA grew very poorly and eventually did not survive after more than 8 weeks of culture. Elongation of roots started after 4 – 5 weeks of culture (Fig. 3.3a) at 2.3 µM TDZ and 4.6 µM ZEA, and a profuse rooting system was developed after 6 – 7 weeks of culture (Fig. 3.3b). After 8 – 9 weeks of culture in maturation medium, plantlets of 4 – 5 cm height were produced that were ready for transfer onto peat-perlite medium for acclimatization.

3.3.3. Acclimatization

The plantlets when transferred to plastic pots containing a peat: perlite medium (3:1, v/v) and grown in a greenhouse where they acclimatized readily and all plants survived. The hardened-off plantlets have normal growth without any morphological variation (Fig. 3.3c).

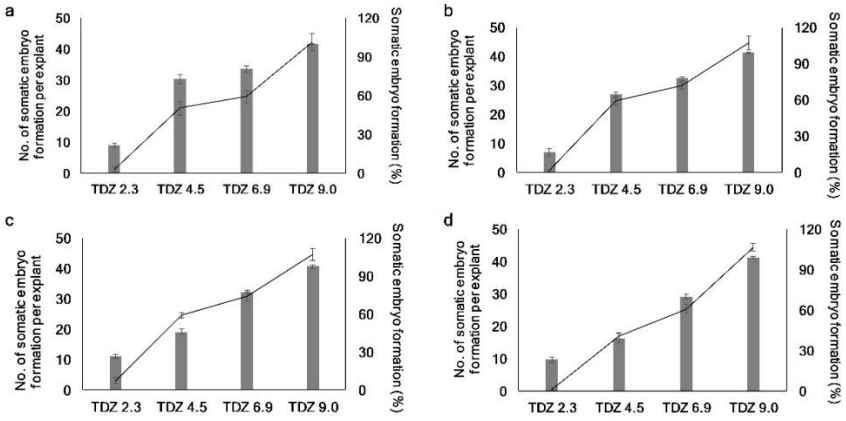

Figure 3.2: Effects of TDZ concentrations (µM) on the number of somatic embryos per explant (primary axis, line graph) and the percentage of embryo formation (secondary axis, column graph) in half-high blueberry cultivars after 10 weeks of culture. (a) 'St. Cloud', (b) 'Patriot', (c) 'Northblue', and (d) 'Chippewa'. Each experiment was repeated three times. Tukey's test was used to detect significant difference at $P \leq 0.05$.

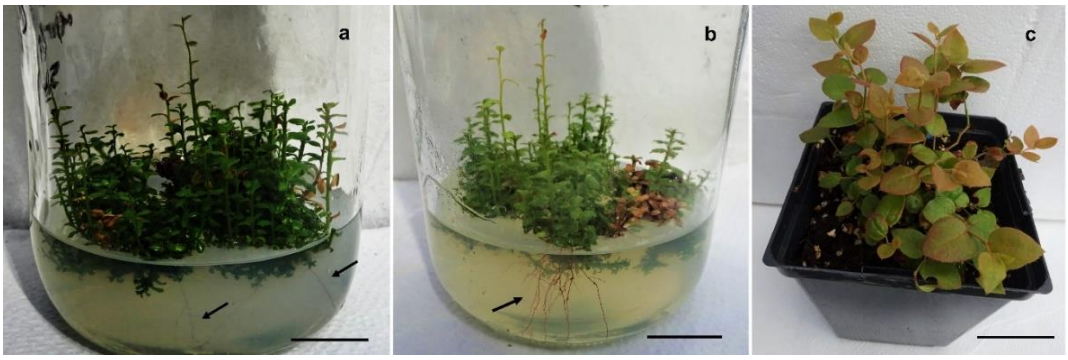

Figure 3.3: Shoot and root elongation and plantlet acclimatization of 'St. Cloud'. (a) Root elongation (arrows) in a nutrient medium containing 2.3 µM of TDZ, after 4 weeks of culture in a glass jar. Bar = (2 cm). (b) Development of rooting system (arrows) in a nutrient medium with 2.3 µM of TDZ after 6 weeks of culture in a glass jar. (Bar = 2 cm). (c) Two-month-old greenhouse-grown plant in a 6-cm^3 plastic pot containing peat-perlite medium. (Bar = 3.5 cm).

3.3.4: Total phenolic content (TPC)

The genotypes, propagation methods and their interactions affected significantly ($P \leq 0.05$) the phenolic contents in cutting-propagated donor and tissue culture plants (Table 3.2). Phenolic contents in blueberry plants followed the decreasing order of donor 'Northblue' (0.45 ± 0.00 mg GAE/ g lw) > SE 'Chippewa' (0.43 ± 0.01 mg GAE/ g lw) > donor 'Chippewa' (0.37 ± 0.01 mg GAE/ g lw) and donor 'Patriot' (0.37 ± 0.02 mg GAE/ g lw) > donor 'St. Cloud' and SE 'Patriot' (0.35 ± 0.01 mg GAE/ g lw) > SE 'St. Cloud' (0.31 ± 0.00 mg GAE/ g lw) > SE 'Northblue' (0.26 ± 0.00 mg GAE/ g lw). 'Chippewa' tissue culture plants showed higher content of phenolics than those of the donor plants although in 'St. Cloud' and 'Northblue', donor plants had more phenolic contents. In 'Patriot', the greenhouse-grown donor plants and the SE plants had similar amount of total phenolics (Table 2).

Table 3.1: Effect of different concentrations of PGRs on somatic embryo maturation and root elongation of four half-high blueberry cultivars. Each experiment was repeated three times. Maturation data was collected after 18 weeks of culture. Standard error associated with different letters indicates significant differences according to Tukey's test at $P \leq 0.05$.

PGR and concentration (µM)	Percent of somatic embryo maturation			
	'St. Cloud'	**'Patriot'**	**'Northblue'**	**'Chippewa'**
GA_3				
1.4	0.00 ± 0.00^{i}	0.00 ± 0.00^{h}	0.00 ± 0.00^{h}	1.50 ± 0.29^{g}
2.9	1.11 ± 0.11^{hi}	1.78 ± 0.78^{gh}	1.83 ± 0.44^{h}	1.67 ± 0.67^{g}
4.2	4.33 ± 0.67^{efghi}	5.33 ± 1.45^{fgh}	3.44 ± 0.30^{fgh}	5.67 ± 1.20^{fg}
5.8	5.11 ± 1.06^{efghi}	4.17 ± 0.83^{fgh}	7.00 ± 1.15^{defgh}	7.00 ± 0.58^{efg}
IBA				
4.9	2.17 ± 0.60^{ghi}	3.00 ± 1.15^{gh}	2.44 ± 0.73^{gh}	1.83 ± 0.83^{g}
7.5	7.67 ± 1.20^{defgh}	10.00 ± 1.73^{def}	9.67 ± 1.45^{cdefg}	8.00 ± 1.15^{defg}
9.8	11.00 ± 1.53^{cde}	13.33 ± 0.88^{cd}	13.00 ± 1.00^{cde}	11.67 ± 0.88^{cdef}
ZEA				
2.3	15.00 ± 1.73^{bc}	16.89 ± 0.95^{bc}	10.56 ± 2.25^{cdef}	16.00 ± 1.00^{bc}
4.6	19.67 ± 1.45^{b}	22.67 ± 0.88^{ab}	20.67 ± 1.20^{b}	21.33 ± 0.67^{b}
6.9	8.67 ± 0.88^{cdefg}	7.00 ± 1.15^{efg}	9.33 ± 2.40^{cdefg}	7.33 ± 1.45^{efg}
9.2	4.00 ± 1.15^{fghi}	6.00 ± 0.58^{fg}	5.67 ± 0.88^{efgh}	3.00 ± 0.58^{g}

TDZ				
2.3	27.33 ± 1.20^{a}	27.67 ± 1.86^{a}	31.11 ± 1.25^{a}	29.33 ± 0.88^{a}
4.5	15.3 ± 2.03^{bc}	14.33 ± 0.67^{cd}	12.67 ± 2.40^{cde}	14.67 ± 2.60^{bcd}
6.9	13.00 ± 2.31^{bcd}	12.33 ± 1.45^{cde}	16.33 ± 1.76^{bc}	13.00 ± 2.31^{cde}
9.0	10.33 ± 0.88^{cdef}	9.67 ± 1.20^{def}	13.67 ± 1.45^{bcd}	12.00 ± 1.73^{cdef}

3.3.5. Total flavonoid content (TFC)

From the results, it is evident that, not only the propagation method and genotypes, but also their interactions played a major role on the varying content of flavonoids present in the leaf extracts of the four half-high blueberry cultivars (Table 3.2). SE derived ‘Chippewa’ plants exhibited the highest TFC (11.65 ± 0.1 mg CE/ g lw) followed by SE ‘St. Cloud’, donor ‘Northblue’, donor ‘St. Cloud’, SE ‘Patriot’, and donor ‘Chippewa’ while ‘Northblue’ tissue culture plants had the lowest TFC (4.88 ± 0.18 mg CE/ g lw). Tissue culture plants had lower TFC than the donor plants in all cultivars except ‘St. Cloud’ where tissue culture plants had higher TFC than the donor plants (Table 3.2).

3.3.6. Total antioxidant activity (AA)

Similarly, as TPC and TFC results, the genotype, propagation methods and their two-way interaction effects were significant ($P \leq 0.05$) for DPPH (Table 2). In case of radical scavenging activity, greenhouse grown donor plants in all cultivars exhibited higher AA than those of their respective SE counterparts. In this study, donor plants showed much high level of AA ranging from 15.05 ± 0.11 (‘Northblue’) to 14.46 ± 0.32 (‘Patriot’) mg GAE/ g lw, while leaves from SE regenerated plants displayed comparatively lower level of activity that varied from 6.48 ± 0.18 (‘Chippewa’) to 0.08 ± 0.00 (‘Northblue’) mg GAE/ g lw (Table 3.2).

Table 3.2: Effects of cultivar, and propagation method on total phenolic (TPC), flavonoid (TFC), and antioxidant activity (DPPH scavenging activity) of four half-high blueberry cultivars. GAE = gallic acid equivalent, lw = leaf weight, CE = catechin equivalent, DPPH = 2,2-diphenyl-1-picrylhydrazyl radical, D = donor, SE = somatic embryogenesis. Data is expressed as means ± SE. Values with the different letters in the same column are significantly different at $P \leq 0.05$ by Tukey's test.

		Parameters		
Cultivars (C)	**Propagation method (P)**	**TPC (GAE/ g lw)**	**TFC (CE/ g lw)**	**DPPH scavenging activity (GAE/ g lw)**
'St. Cloud'	D	0.35 ± 0.01^{bc}	9.97 ± 0.42^{b}	14.85 ± 0.70^{a}
	SE	0.31 ± 0.00^{c}	10.90 ± 0.10^{ab}	1.96 ± 0.23^{c}
'Patriot'	D	0.37 ± 0.01^{b}	10.89 ± 0.06^{ab}	14.46 ± 0.32^{a}
	SE	0.35 ± 0.01^{bc}	8.03 ± 0.34^{c}	1.34 ± 0.10^{cd}
'Northblue'	D	0.45 ± 0.00^{a}	10.89 ± 0.06^{ab}	15.05 ± 0.11^{a}
	SE	0.26 ± 0.00^{d}	4.88 ± 0.18^{d}	0.08 ± 0.00^{d}
'Chippewa'	D	0.37 ± 0.02^{b}	7.93 ± 0.02^{c}	14.85 ± 0.18^{a}

	SE	0.43 ± 0.01[a]	11.65 ± 0.70[a]	6.48 ± 0.18[b]
Significant effects		C, P, C × P	C, P, C × P	C, P, C × P

3.3.7. Relationship among antioxidant properties

The antioxidant activity determined by DPPH assay, had a significant linear correlation ($r^2 = 0.345$) with TPC of the leaf extracts of four SE and donor plants. This shows that phenolic compounds of this extracts provide considerable amount of antioxidant activity. To evaluate the relationship between DPPH assay and TFC, linear regression was performed, which suggests a significant relationship between these two components ($r^2 = 0.175$). The correlation coefficient was higher between DPPH assay and TPC (r) = 0.587, compared to antioxidant capacity and TFC (r = 0.418). TPC showed positive correlation with TFC ($r^2 = 0.453$, r = 0.673) (Fig. 3.4).

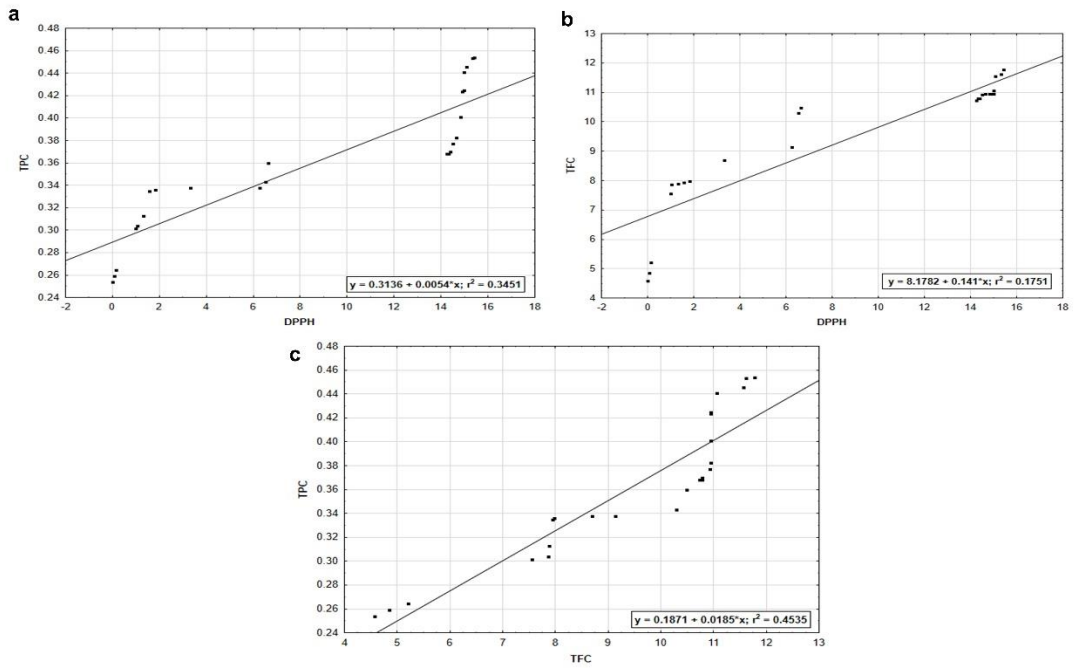

Figure 3.4: Linear regression between antioxidant properties in tissue culture and cutting propagated donor plants of four half-high blueberry cultivars. (a) Total antioxidant activity by DPPH (mg gallic acid equivalent (GAE)/ g leaf weight (lw) and total phenolic content (mg GAE/ g lw). (b) Antioxidant activity and total flavonoid content (mg catechin equivalent (CE)/ g lw). (c) Total flavonoid (mg CE/ g lw) and total phenolic contents (mg GAE/ g lw).

3.4. Discussion

This is the first report of SE in half-high blueberries. Some preliminary work on blueberry SE without any detail have been reported by Cui et al. (2008). In this study, TDZ was found effective for SE from leaf explants of half-high blueberries on a semi-solid medium. TDZ, although classified under the group of cytokinins due to its natural cytokinin-like response (Murthy et al. 1998), in in vitro culture, it exhibits both auxin and cytokinin-like activities in various plant species (Mok & Mok 1985; Murthy et al. 1995). There are some hypotheses available on the mode of action of TDZ, but it is still unclear (Radhakrishnan et al. 2009). A possible explanation to the action mechanism of TDZ is that it may help in the accumulation and/or synthesis of endogenous plant growth hormones (Mok & Mok 1985). The promotion of growth such as callus formation, shoot regeneration, and somatic embryo formation take place when applied at higher concentration, and axillary proliferation at comparatively lower concentrations which might be due to its similar biological activity like other N6-substituted cytokinins such as N-N-diphenylurea and N-(2-chloro-4-pyridyl)-N′-phenylurea (Mok & Mok 1985; Huetteman & Preece 1993).

Among different TDZ concentrations, the highest number and percentage of somatic embryo formation was recorded in 9 µM TDZ after 10 weeks of culture. The same phenomenon was also observed when TDZ was applied in higher concentrations (10 – 20 µM), giving rise to direct somatic embryos from the intact seedlings of pigeonpea instead of adventitious shoot regeneration (Singh et al. 2003). Similarly, the use of TDZ in a range of 0.5 – 10 µM has been reported to induce SE in grape (Dhekney et al. 2016)

and in *Rubus* (Fiola et al. 1990). Somatic embryo development within 2 weeks of culture from the hypocotyl explants of geranium (*Pelargonium* × *hortorum*) was observed in culture medium supplemented with 0.2 – 1 µM TDZ (Visser et al. 1992). In *Saintpaulia ionantha* (H. Wendl.), TDZ in higher concentrations (5 – 10 µM) proved to be more effective for somatic embryo formation than the cytokinins benzyladenine (BA) and N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU) used in that study (Mithila et al. 2003). The percentage of embryo formation per explant was genotype dependent but in all four cultivars, increased SE was noticed with increasing the TDZ concentration. Similar results were observed during SE of eight pigeonpea cultivars, where percent and average number of somatic embryo formation varied with genotypes, and embryo induction started at the higher concentration of TDZ (10 – 20 µM) (Singh et al. 2003).

In the present study, maturation of somatic embryos was recorded on BM containing GA_3 and IBA, but the plantlets were very poor in vigour and did not survive. Similar results were observed in geranium where exogenous application of GA_3 on MS medium inhibited induction and expression of different SE stages and was found to be detrimental on TDZ-derived SE formation (Hutchinson et al. 1997). IBA is a preferably used PGR for adventitious root induction from in vitro grown or cutting plants in woody plant species (James 1983). IBA was added in the medium prior to autoclaving as it is co-autoclavable with other media components, however it may lose some activity due to autoclaving (Nissen & Sutter 1990). IBA when added to the liquid medium before autoclaving approximately 20% loss of activity of IBA was observed in comparison to filter sterilization (Nissen & Sutter 1990). Additionally, IBA is sensitive to

photooxidation and degrades in the tissue culture media during light incubation (Nissen & Sutter 1990; Drew et al. 1991). However, during incubation in dark for 28 – 30 days, IBA concentration decreases in agar solidified medium from 10% – 38% respectively (Nissen & Sutter 1990; Drew et al. 1991). Instability of IBA in light has several other effects on tissue culture system, which may be crucial during designing a plant tissue culture experiment (Drew et al. 1991). It is also reported that direct SE from leaf explants of *Oncidium* 'Gower Ramsey' was completely inhibited when cultured on the medium supplemented with IBA (Chen & Chang 2001).

My results demonstrated the effects of TDZ and ZEA on in vitro maturation of somatic embryo maturation in half-high blueberries. It has been reported that lower concentrations of cytokinin may initiate root formation while higher concentrations inhibit the rooting process and accumulate for shoot proliferation (Gaspar & Coumans 1987). Although there is no report available on involvement of TDZ and ZEA on maturation of somatic embryos during multi-step embryogenesis, effect of these two PGRs are available on in vitro or ex vitro rooting of various plant species. For example, in vitro rooting with ZEA was recorded in 'Bounty' strawberry (*Fragaria ananassa* Duch.), where sepal-derived adventitious shoots when cultured in the medium supplemented with 1 – 2 µM of ZEA, provide maximum rooting (Debnath 2006). It was also reported that in vitro and ex vitro rooting can be obtained from the pre-treatment of microshoots of cranberry (Qu et al. 2000), strawberry (Debnath 2005) and blueberry (Debnath 2009) in a TDZ containing medium. In soybean, the maximum number of root formation was reported from cotyledonary nodal explants in B5 medium supplemented

with 3.5 – 4.6 µM of TDZ (Radhakrishnan et al. 2009). Plantlets regenerated via SE were transferred to the greenhouse where complete survival was obtained after acclimatization. Due to the improper development of cuticle, epicuticle and cuticular waxes and retarded functioning of stomatal apparatus during tissue culture process, in vitro grown plantlets at the time of acclimatization transpire in a high rate through the stomata and cuticles present on the leaves (Chandra et al., 2010). To avoid this excess water loss via the cuticle and stomata, micropropagated plantlets should be transferred slowly from high humid to low humid environment (Chandra et al., 2010). Similar results were also reported by Debnath (2017) where 80 – 90 % survival rate was observed in in vitro-derived half-high blueberry plantlets when transferred to the greenhouse. In this study, the plantlets developed via SE without any intervention of callus phase, reducing the probability of occurrence of somaclonal variance to the minimum (Singh et al. 2003).

Antioxidant properties of blueberries are well recognized due its medicinal importance to inhibit the detrimental effects on human health caused by free radicals. Plants can produce and accumulate health-promoting secondary metabolites in in vitro culture (Matkowski 2008). In this study, we have compared the changes of antioxidant properties among the cutting-propagated donor plants and SE regenerated plants. From the results of the biochemical assays, it was evident that propagation method and genotype have an impact on half-high blueberries under certain condition to synthesize phenols and flavonoids in the leaves. Leaf extracts from donor plants of ‘St. Cloud’ and ‘Northblue’ contains higher levels of TPC than SE plants, while donor plants of ‘Patriot’

did not show any significant difference than the SE counterparts. On the other hand, 'Chippewa' donor plants contain lower concentrations of TPC than the SE regenerated plants. The effects of the propagation methods on genotypes were also observed in the TFC profile, where SE regenerated 'St. Cloud' and 'Chippewa' plants showed higher level of TFC than greenhouse grown donor plants, and in 'Patriot' and 'Northblue' SE plants contains more TFC than mother plants. Plant tissue culture system has an important role on phenolic content biosynthesis, as the growth hormones used in the system may up or down-regulate the genes involved in the biosynthesis pathway (Sakakibara et al. 2006). Cytokinins found to be positively correlated with TPC, TFC, and condensed tannin concentration when applied individually or combined with auxin during micropropagation of aloe species (Amoo et al. 2012). In lowbush blueberries, higher levels of polyphenols were reported in the leaves of conventionally grown plants than tissue culture (TC) plants, which could be due to the nutritional level difference affected by the propagation methods (Goyali et al. 2013). In that same study, it was also observed that the phytochemical profile of a wild lowbush clone was affected more by the propagation method than the cultivar 'Fundy' (Goyali et al. 2013), which further supports the fact that propagation method and genotype have cumulative effects on the phytochemical components of the plant species. Phenolic compounds are the most abundant secondary plant metabolite derived from phenylalanine. Different environmental factors such as low light conditions and lower concentrations of nutrient increase the activity of phenylalanine ammonia lyase, which is a crucial regulatory factor of phenol metabolic pathway (Taiz & Zeiger 2006). In the greenhouse, prolonged culture

of plants could initiate low nutrient stress in the donor plant system which might act as an enhancer of TPC (Goyali et al. 2013).

I observed the changes of antioxidant properties in the TDZ-induced SE plants. Although there is no report on comparative study of antioxidant properties between SE and donor plants, drop in TPC level was noticed during SE in cacao (Ndoumou et al. 1997). In another study, no direct relationships between presence and absence of polyphenolics with embryogenic or non-embryogenic condition of cacao callus were detected but presence of TPC and tannins were observed during somatic embryogenic response (Alemanno et al. 2003). In *Artemisia absinthium* callus cultures, production of phenolic contents was positively related with the presence of TDZ and cultures with lower concentration (0.5 – 3.0 mg/L) displayed maximum TPC and antioxidant potential (Ali & Abbasi 2014).

The determination of antioxidant activity is a complex procedure as it is a combination of synergistic and antagonistic effects of various environmental factors (Hassimotto et al. 2005). To measure the AA, DPPH radical scavenging method was used, which is a widely used, rapid, and easy method to detect antioxidants (Mensor et al. 2001). DPPH is a stable free radicle, when dissolved in ethanol it produces a violet solution and in the presence of antioxidant molecules it is reduced to a uncoloured solution (Mensor et al. 2001). The propagation method as well as the physiological age had a clear effect on the antioxidant activity of the four cultivars, as all donor plants showed significantly higher levels of activity than SE plants. These results align with the TPC profile of ‘St. Cloud’ and ‘Northblue’, and TFC profile of ‘Patriot’ and ‘Northblue’

where the leaves of SE plants from the same cultivar contained more polyphenols and flavonoids. This indicates that TPC and TFC may not adequately explain the antioxidant activity of the leaf extracts, which are admixture of different components with diverse activities. Additionally, total AA represented by the DPPH value is the sum of various antioxidant compounds, which also relies on the chemicals used during the extraction process from the leaves (Abeywickrama et al. 2016). Similarly, in lingonberry (*V. vitis-idaea* L. ssp. *vitis-idaea* Britton) higher AA in the leaves of greenhouse-grown cutting propagated plants in comparison to the TC plants were reported earlier, although in fruits of the same species, contained higher phenolic and anthocyanin components than the cutting plants (Foley & Debnath 2007). The reasons behind the presence of higher content of antioxidant in the conventionally grown plants are not very clear, but the possible reason could be the age of the plants as well as the culture condition. In the present study, the donor plants were more than 15-years-old, while the SE plants were eight-week-old. Although reports on the effects of the age of tissue culture and donor plants on antioxidant properties are lacking, young leaves of various varieties of blackberries, raspberries and strawberries were found to contain higher TPC and AA than the older leaves (Wang & Lin 2000). Similar results were observed in tea leaves, where comparatively younger leaves showed higher ORAC values and contained more polyphenols, predominantly (–) -epigallocatechin 3-galate, than the older leaves (Lin et al. 1996).

I have observed a positive correlation between AA with TPC and TFC of the SE and donor plants in four cultivars. Various studies on blueberries showed a significant

positive correlation between TPC profile and AA (Goyali et al. 2013; Goyali et al. 2015; Prior et al. 1998) and of TFC with AA, evaluated by DPPH radical scavenging activity (Goyali et al. 2013; Goyali et al. 2015). The results showed that AA measured by DPPH assay were slightly better correlated to total phenolics than to total flavonoids components. These results are in agreement with the previous findings on blueberries (Prior et al. 1998; Goyali et al. 2013).

3.5. Conclusion

In conclusion, this study, for the first-time reported SE in half-high blueberries and the changes in antioxidant properties due to this process in the tissue culture regenerated plants. Results of this study indicate that these findings could lead to tremendous labour and time savings and SE can be used as a useful means of commercial blueberry propagation.

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Chapter 4 - Detection of DNA methylation pattern in thidiazuron induced blueberry callus using methylation-sensitive amplification polymorphism

Abstract

During the normal developmental process, programmed gene expression is an essential phenomenon in all organisms. In eukaryotes, DNA methylation plays an important role in the regulation of gene expression. The extent of cytosine methylation polymorphism was evaluated in leaf tissues collected from the greenhouse grown plants and in in vitro-derived callus of three lowbush and one hybrid blueberry genotypes, using methylation-sensitive amplification polymorphism (MSAP) technique. Callus formation started from the leaf segments after 4 weeks of culture on a thidiazuron (TDZ) containing medium. Maximum callus formation (98%) was observed in the hybrid blueberry at 4.5 µM of TDZ. Although noticeable changes in cytosine methylation pattern were detected within the MSAP profiles of both leaf and callus tissues, methylation events were more polymorphic in calluses than in leaf tissues. The number of methylated CCGG sites varied significantly within the genotypes ranging from 75 - 100 in leaf tissues and 215 - 258 in callus tissues. Differences in the methylation pattern were observed not only in a tissue-specific manner but also within the genotype in a treatment specific manner. These results demonstrated the unique effect of TDZ and the tissue culture process on DNA methylation during callus development.

4.1. Introduction

Successful establishment of in vitro propagation systems enables rapid production of genetically similar plants. Although tissue culture propagated plants are intended to be identical, this is not always the case. Somaclonal variation (Larkin & Scowcroft 1981) occurs invariably during cell and tissue culture processes regardless to the regenerating system (Duncan 1997). Due to the occurrence of somaclonal variation in the micropropagation system, clones are not true-to-type to the donor plants. Continuous subculturing and changes in the tissue culture microenvironment contribute to the additional stress to regenerating plant cells and induce a variety of genetic and epigenetic instabilities in the genome of the regenerants leading to the production of plant variants (Peredo et al. 2006; Smýkal et al. 2007). In plants, epigenetic modification such as DNA methylation is associated with gene regulation, chromatin inactivation, genomic imprinting and cell differentiation (Park et al. 2009). DNA methylation induced changes have been hypothesized as the fundamental mechanism of tissue culture-induced mutations which involves activation of transposable elements, chromosome breakage, and/or DNA sequence changes, and finally high frequency phenotypic variation (Kaeppler et al. 2000; Park et al. 2009). Across different taxa, methylation of the nucleotides is the most commonly found covalent modification of DNA (Hernando-Herraez et al. 2015). During post replicative DNA modification, specific DNA methylases generate several methylated bases (Wion and Casadeús 2006). Among these, 5-methylcytosine (5-mC) is predominant in higher plants and eukaryotes (Finnegan et al. 1998; Zemach et al. 2010).

Methylation-sensitive amplification polymorphism (MSAP), a modification of the amplified fragment length polymorphism (AFLP) (Vos et al. 1995), is a cost effective, rapid and easy to carry out process for non-model organisms and does not require information of the sequenced genome (Umer & Herceg 2013; Fulneček & Kovařík 2014). The technique was used to determine DNA methylation events in dimorphic fungi (Reyna-Lopez et al. 1997) and some plant species including rice (Xiong et al. 1999), banana (Peraza-Echeverria et al. 2001; Baurens, Causse, & Legavre 2008), Siberian ginseng (Chakrabarty et al. 2003) and in orchid *Doritaneopsis* (Park et al. 2009). MSAP technique is based on the sensitivity of the pair of isoschizomers *MspI* and *HpaII* instead of *MseI*, which was used in the original AFLP protocol, to detect the methylation at the tetranucleotide recognition site CCGG (Peraza-Echeverria et al. 2001; Portis et al. 2004; Fulneček & Kovařík 2014). *MspI* and *HpaII* restrict differently the same recognition site depending on the methylation state of the external and internal cytosine residues. *MspI* cleaves methylated internal cytosine residues (C^mCGG) but not the external (^mCCGG), whereas the cleaving activity of *HpaII* is still under controversy. *HpaII* is most probably inactive for any methylation of CCGG site including hemimethylated external cytosine (^mCCGG, (Peraza-Echeverria et al. 2001) and specific *HpaII* bands represent more likely fragments with internal C^mCGG site(s) which can easily verified by combined digestion with both *HpaII* and *MspI* enzymes as suggested by Fulneček and Kovařík (2014).

Blueberry (*Vaccinium* spp. L., family: *Ericaceae*) is a widely accepted economically important health-promoting small fruit crop. In Newfoundland and Labrador, Canada, naturally growing wild lowbush blueberries are managed and

harvested commercially to meet the increasing demand of high quality blueberries. Lowbush blueberry (*Vaccinium angustifolium* Ait.; 2n=4x=48) plants are hermaphroditic and predominantly cross-pollinated in nature (Vander Kloet 1978) and reproduces sexually by bee-pollination or asexually by underground rhizome system (Bell et al. 2009). Half-high blueberries are hybrids between highbush (*V. corymbosum*) and lowbush blueberries and are outcrossing in nature (Harrison et al. 1993). Being genetically heterozygous in nature, blueberry plants grown from the seeds are not true-to-type to the donor plants. Although the species is successfully propagated by conventional methods, it is labour-intensive and time consuming. With the advent of plant tissue culture techniques, they have been extensively employed to multiply plants rapidly which provides year around production. However, occurrence of somaclonal variation in micropropagated plants is a matter of concern as it affects clonal fidelity. Goyali et al. (2015) found that micropropagated lowbush blueberry contain a higher amount of polyphenols and flavonoids in fruits than softwood cutting counterparts, although number of flower clusters, berries, fruit mass per plants as well as diameter and mass of individual fruits were significantly lower in clonal propagated plants. Variation originated in the lowbush blueberry clones might be due to the epigenetic changes occurred during tissue culture process, as molecular analysis of the micropropagated plants with simple sequence repeat (SSR) markers confirmed their genetic uniformity with the softwood cutting plants. The objective of this study was to estimate the altered methylation pattern at the tetranucleotide CCGG sites induced exclusively due to the tissue culture process at the stage of callus development with the application of TDZ, having both the effects of auxin and cytokinin, using MSAP technique. To the extent of my knowledge, this is the

first report on tissue culture induced DNA methylation in the genus *Vaccinium*.

4.2. Materials and methods

4.2.1. In vitro culture

Actively growing young leaves were collected from wild lowbush blueberry (*Vaccinium angustifolium* Ait.) clones designated as 'CL1', 'CL2', 'CL3' and a hybrid ('H1') between highbush and lowbush blueberry (*V. corymbosum* L. × *V. angustifolium* Ait.) cvs. 'Chippewa' and 'Patriot'. The plants were maintained in plastic pots containing peat: perlite (2:1, v/v) medium under the natural irradiance (photosynthetic photon flux density, PPFD of 90 µmol m^{-2} s^{-1}), at 20 ± 2 °C, and 85 % relative humidity in a greenhouse of St. John's Research and Development Centre, Agriculture and Agri-Food Canada, St. John's, Canada (Debnath 2009). Surface sterilized leaves (Debnath 2009) of each genotype were segmented into basal, middle and upper portion and inoculated abaxial surface touching the media in Fisherbrand™ Petri dishes with clear lids (Fisher Scientific, Fair Lawn, NJ, USA) containing 25 g of basal medium [BM, three-quarter micro salts and macro salts of Debnath and McRae (2001) supplemented with 25 g sucrose, 1.25 g Gelrite, and 3.5 g Sigma agar (Catalogue number A 1296, Sigma Chemical Co., St. Louis, USA)]. The pH of the medium was adjusted to 5.0 before autoclaving at 121 °C for 20 min. BM was supplemented with four concentrations of

TDZ, i.e., 0 (control), 0.45, 2.3, and 4.5 µM. The inoculated cultures were kept under dark for 2 weeks at 20 ± 2 °C, 60 - 70 % of relative humidity and then transferred to continuous PPFD of 30 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps under same culture conditions. Calli were subcultured in the same media after every 4 weeks. Three explants per plate and three plates per treatment were used. The experiment was repeated three times.

4.2.2. DNA extraction

Genomic DNA was extracted from the leaf samples of greenhouse grown plants and from 12-week-old pale green clump of calli of all four genotypes using DNeasy plant mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instruction. The purity of the DNA was inferred from the absorbance ratio A_{260}/A_{280} and concentration from A_{260}. For MSAP analysis, the dilution of DNA used was 80 ng μL^{-1} with A_{260}/A_{280} of 1.8 - 2.1 (Debnath 2014).

4.2.3. Methylation sensitive amplification polymorphism (MSAP) assay

The MSAP assay was adopted from the AFLP protocol established by Vos et al. (1995) and modified by Reyna-Lopez et al. (1997). *EcoRI* (Thermo Scientific) and the methylation-sensitive restriction enzymes (isoschizomers), *MspI* and *HpaII* (Thermo Scientific) were used. To detect MSAP, digestion reactions were set up in two steps. In

the first step, 400 ng of the genomic DNA was digested with 2 U of *EcoRI* in a final volume of 50 µL containing 10× FastDigest buffer (Thermo Scientific) for 1.5 h at 37 °C and the reaction was stopped by incubating at 65 °C for 15 min. In the second step, two digestion reactions were carried out simultaneously. In the first reaction, 25 µL *EcoRI* digested DNA was restricted with 2 U of *MspI* enzyme in a final volume of 50 µL containing 10× FastDigest buffer. The second reaction was carried out in the same way except *HpaII* enzyme instead of *MspI* enzyme. Both reaction mixtures were incubated at 37 °C for 3 h followed by stopping the reaction at 65 °C for 15 min. In another reaction, 400 ng of the genomic DNA was digested with 2 U of each of *EcoRI*, *MspI*, and *HpaII* enzymes in a final volume of 50 µL containing 10× FastDigest buffer for 3 h at 37 °C. The reaction was stopped by incubating the mixture at 65 °C for 15 min.

All digested fragments were ligated to the adapters by adding 50 µL of ligation mixture containing 1× T4 DNA ligase buffer (Thermo Scientific), 10 µM *EcoRI* adapter, 100 µM *MspI-HpaII* adapter (Thermo Scientific), 50% (m/v) polyethylene glycol solution and 5 U T4 DNA ligase (Thermo Scientific) and incubated at 23 °C for 5 h. The reactions were stopped at 65 °C for 10 min.

Pre-selective amplification was conducted by using 4 µL of the ligated product with *EcoRI* and *MspI-HpaII* primers in a volume of 50 µL containing 1× Taq buffer (Qiagen), 10 mM dNTPs (Amresco LLC, Solon, OH, USA), 10 µM of each primer and 5 U of Taq polymerase (Qiagen). PCR reactions were performed with the following cycle: *65* °C for 2 min, 94 °C for 5 min, 25 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 2

min with a final extension at 72 °C for 10 min. After checking for the smear of fragments (100 - 1000 bp in lengths) in 1.8% agarose gel electrophoresis, the amplification product was diluted to 15 times in 1× Tris-borate-EDTA (TBE) buffer and stored at -20 °C until used for selective amplification.

Selective amplification reactions were conducted in volumes of 25 µL containing 2 µL of the preamplified DNA, 10 µM of *EcoRI* primer and 10 µM of *MspI-HpaII* primer along with the same other components as used in pre-selective amplification step. Selective amplification involved 94 °C for 5 min, 13 cycles of 94 °C for 30 s, 65 °C for 1 min, 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 at 72 °C for 10 min.

4.2.4. Methylation sensitive amplification polymorphism (MSAP) electrophoresis and autoradiography

The selective PCR products were mixed with equal amount of formamide dye [98% (v/v) formamide, 10 mM EDTA, 0.01% (m/v) bromophenol blue and 0.01% (m/v) xylene cyanol)], denatured at 95 °C for 4 min and immediately cooled on ice. Aliquots (6 µL) of each reaction were separated by electrophoresis on 5 % denaturing polyacrylamide sequencing gels (5% acrylamide 19:1, 7 M urea) in 1× TBE buffer. The gels were pre run at 100 V for 30 min. 6 µL of each of the PCR product and formamide dye mix was loaded and the gels were run at 115 V for 5 h in Owl gel separation system (Thermo Scientific). Silver staining was done following Bassam et al. (1991). The gel was fixed in the fixer

solution (7.5% acetic acid) for 30 min, washed twice with deionized double distilled water and incubated in the silver solution (0.1% $AgNO_3$) for 30 min. The silver impregnated gel was washed twice with ultrapure water collected from Barnstead Mega-Pure D2 (Thermo Scientific). Image development was carried out in the developer solution (30 g/L sodium carbonate, 0.056 % formaldehyde, 40 g L^{-1} sodium thiosulfate) for 1 - 2 min. To stop the image development, developer stop solution (7.5% acetic acid) was added immediately and incubated for 3 min. All steps were performed with slow agitation on Rocker 25 shaker (Labnet International, Edison, NJ, USA). The gel was then rinsed with deionized double distilled water and dried at room temperature. Band separation pattern was visualized using the InGenius 3 gel documentation system (Syngene, Frederick, MD, USA).

4.2.5. Data analysis

Callus formation experiments were conducted following completely randomized design (CRD) and the data on callus formation were subjected to analysis of variance (ANOVA) employing the general linear model. The significance of the TDZ treatments was assessed by Duncan Multiple Range Test (DMRT) (Duncan, 1955) using STATISTICA data analysis software version 7 (Statsoft Wipro, East Brunswick, NJ, USA) at a critical difference of $P \leq 0.05$.

In the MSAP analysis, DNA methylation events were detected on the basis of the presence or absence of the bands in the autoradiograph. Methylated tetranucleotide sites (CCGG) were detected when *MspI* cleaved only internal methylated cytosine (C^mCGG) residues. Any extra pure *HpaII* bands were not detected for the digestion of hemimethylated external cytosine (^mCCGG) and the fact was supported by the third lane of the samples digested with *MspI* + *HpaII* enzyme combinations, which showed almost same profile as *MspI* (Fulneček and Kovařík 2014). Polymorphic bands for DNA methylation events were recorded in all reactions with *EcoRI* + *MspI/HpaII/MspI+HpaII* following Chakrabarty et al. (2003).

4.3 Results and discussion

Leaf explants of all genotypes, when cultured in vitro on medium with TDZ, formed callus (Fig. 4.1). Pale green callus started emerging at the leaf margin after 4 weeks of culture on BM containing 0.45, 2.3, and 4.5 µM TDZ. Cultures on BM without TDZ did not form any callus and were excluded from data analysis. After 12 weeks of culture, highest percentage of callus formation was observed in 'H1' (98%) at 4.5 µM TDZ. The callus formation increased with the increase of TDZ concentration in the culture medium for 'H1' but not for the clones where highest percentage of callus formation was observed at 2.3 µM TDZ (Fig. 4.2). Although in all four genotypes maximum number of methylated CCGG sites were recorded in the callus with 4.5 µM TDZ, the number of methylated sites detected varied among the genotypes. For example,

in hybrid ‘H1’, the total number of methylated sites were 239 (Table 4.1), among which highest number of CCGG methylation detected were 93 in the callus formed on BM with 4.5 µM TDZ, followed by 81 at 0.45 µM TDZ and 65 at 2.3 µM TDZ (Table 4.2). In ‘CL1’, ‘CL2’, and ‘CL3’, total number of methylated tetranucleotide sites were recorded 215, 237, and 258 respectively (Table 4.1). In the callus tissues of these three clones, the maximum methylated sites were 81, 80, and 94, respectively, at 4.5 µM TDZ followed by 77, 79, and 86, respectively, at 0.45 µM TDZ. At 2.3 µM TDZ, these values were 57, 78, and 78, respectively (Table 4.2). From these observations it is clear that all genotypes followed the same trend in relation to DNA methylation at different TDZ concentrations (Table 4.2). It is reported that in dedifferentiation stage (callus formation), explants change dynamically and cytokinins are apparently effective for genome modification during that stage (Huang et al. 2012). In support of that fact, LoSchiavo et al. (1989) observed that upon applying exogenous cytokinin, a stable methylation pattern with 16% methylated cytosine was expressed in cell suspension culture of carrot. The same trend was reported by Arnholdt-Schmitt et al. (1995) where freshly inoculated tissues of carrot showed a higher rate of methylation than established cell culture with the application of kinetin. In our study, the reason behind the hypermethylation status observed in the in vitro formed callus might be TDZ present in the media.

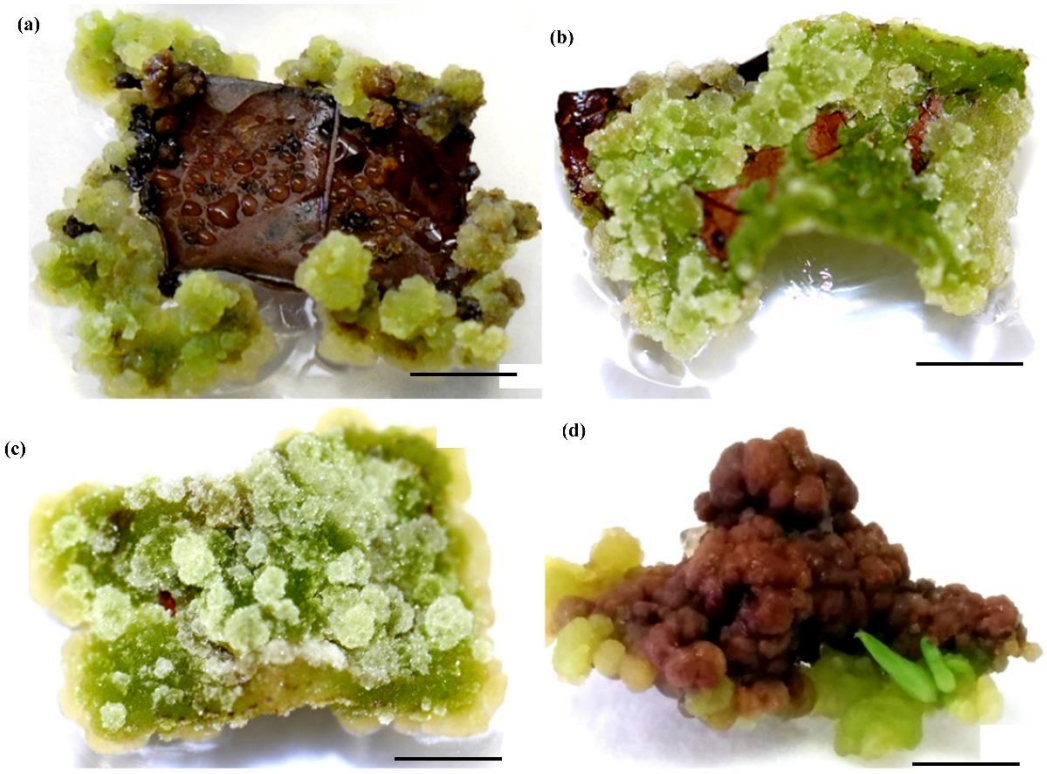

Figure 4.1: Callus and shoot formation on hybrid blueberry 'H1' ('Chippewa' × 'Partriot') leaf explants. Callus forming after 4 (a), 8 (b), and 12 weeks of culture (c). Formation of shoots from the callus clump after 16 weeks of culture (d). Bar = 2 cm.

Although all living cells of the plants are theoretically totipotent in nature, to acquire dedifferentiation, redifferentiation, and competency, application of plant growth regulators is essential (Murthy et al. 1998). During explant culture in vitro, TDZ mimics the effects of auxin and cytokinin and helps in the differentiation process alone or in combination with other phytohormones (Murthy et al. 1998, Ghosh et al. 2014). With the application of different concentrations of TDZ in the medium, callus formation from the leaf segments up to 78% was reported in lowbush blueberry by Debnath (2005). Callus formation due to the application of TDZ was observed earlier in woody species, such as grape. In Kyoho grapes (*Vitis vinifera* × *Vitis labruscana* cv. Kyoho), with linear increment of TDZ concentration, maximum callus growth was obtained after 12 d of culture in medium supplemented with 100 µM TDZ (Lin et al. 1989).

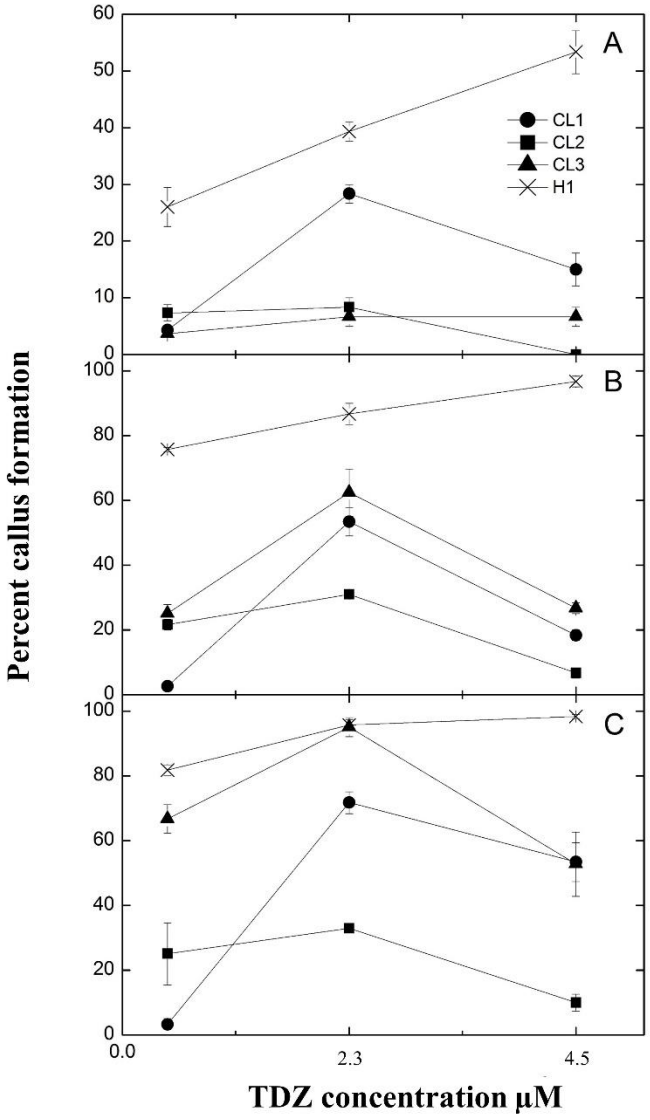

Figure 4.2: Percentage of callus formation of lowbush blueberry clones 'CL1', 'CL2', 'CL3' and hybrid 'H1' after 4 (A), 8 (B), and 12 weeks of culture (C) on BM supplemented with 0.45, 2.3, and 4.5 µM TDZ (Means ± SEs, *n* = 3).

Leaves from greenhouse grown plants and *in vitro*-derived callus of four blueberry genotypes were used for MSAP analysis. We used eight primer combinations (Suppl. Table S1) to detect the cytosine methylation at the tetranucleotide (CCGG) recognition sites. The adapter and primers were designed following Xiong et al. (1999). Leaf and callus DNA digested with *MspI* and *HpaII* enzymes, can produce three fragment classes: 1) cleaving non-methylated CCGG tetranucleotide sites, both *MspI* and *HpaII* produce identical fragments, 2) *MspI* specific fragments result from digestion of fully or hemimethylated internal cytosine ($C^{m}CGG$) in the recognition site; and 3) *HpaII* specific fragments assumed to represent hemimethylated external cytosines ($^{m}CCGG$) (Portis et al., 2004) are more likely fragments with CCGG site at the end and one or more internal $C^{m}CGG$ sites which can be verified by adding one or more lane after simultaneous *MspI* + *HpaII* digestions as recommended by Fulneček and Kovařík (2014). The reproducibility of the methylation patters was confirmed by repeating the experiments and all the bands were repeatedly detected in each case (Fig. 4.3).

Table 4.1: Total number of methylated CCGG sites and the number of methylation polymorphism for each primer detected by methylation-sensitive amplification polymorphism in tissue culture derived calli of four blueberry genotypes. Each experiment was repeated twice with three independently induced calluses for each treatment and same bands were detected in each case.

Oligonucleotide combination	Total number of methylated CCGG sites				Methylation polymorphism			
	'CL1'	'CL2'	'CL3'	'H1'	'CL1'	'CL2'	'CL3'	'H1'
E-TT/MH-AAC	9	16	20	17	5	2	2	6
E-TT/MH-ATG	8	9	10	8	2	2	2	2
E-TT/MH-AAG	6	3	14	8	3	0	4	4
E-TT/MH-ACA	46	35	24	26	8	3	3	4
E-TG/MH-AAC	65	61	67	64	7	4	6	8
E-TG/MH-ATG	16	24	36	31	3	4	6	7

E-TG/MH-AAG	38	46	50	44	5	7	7	8
E-TG/MH-ACA	27	43	37	41	7	4	2	7
Total	215	237	258	239	40	26	32	46

The primer combinations produced a total of 75 fragments in 'H1' leaves which were methylated at CCGG sites, out of which 10 sites were polymorphic. In 'CL1', 'CL2', and 'CL3', total number of polymorphic sites were 10, 9, and 8, respectively (Table 3). Calli from all genotypes and concentrations of TDZ were also analyzed for altered methylation pattern where highest methylation was recorded in 'CL3' (258 fragments) and the lowest in 'CL1' (215 fragments produced) for the eight specific primer combinations. 'CL2' and 'H1' were differentially amplified with 237 and 239 bands, respectively (Table 4.1). The number of polymorphic events increased significantly in in vitro grown callus compared to leaves. These values were 40, 26, 32, and 46, respectively for 'CL1', 'CL2', 'CL3', and 'H1' calli (Table 4.1). The increased polymorphism of callus tissues compared to those in leaves might be due to the stress induced by tissue culture process and the influence of TDZ on methylation. Banding pattern detected in leaf tissues and in callus cultures were genotype dependent (Table 4.2). Our results have demonstrated the effect of TDZ concentrations on number of methylation events in each genotype (Table 4.2). All genotypes responded in the same

manner, in terms of total number of methylated tetranucleotide sites in the callus, treated with 0.45, 2.3, and 4.5 µM TDZ. The number of methylated CCGG sites varied from 80 ('CL2') to 94 ('CL3') in callus cultures at 4.5 µM TDZ. However, the presence of polymorphic sites among the methylated CCGG sites was highest (16.7 to 27.7%) at 2.3 µM TDZ for all four genotypes (Table 4.2).

Table 4.2: Effect of thidiazuron (TDZ) concentrations on the total number of methylated CCGG sites, number of methylation polymorphism, and percentage of methylation polymorphism for CCGG sites detected within four blueberry genotypes. Each experiment was repeated twice with three independently induced calluses for each treatment and same bands were detected in each case.

Genotype	TDZ [µM]	Total number of methylated CCGG sites	Number of methylation polymorphism	Percentage of methylation polymorphism
'CL1'	0.45	77	9	11.7
	2.3	57	13	22.8

	4.5	81	18	22.2
'CL2'	0.45	79	7	8.9
	2.3	78	15	19.2
	4.5	80	4	5.0
'CL3'	0.45	86	8	9.3
	2.3	78	13	16.7
	4.5	94	11	11.7
'H1'	0.45	81	14	17.3
	2.3	65	18	27.7
	4.5	93	14	15.1

The present results are in agreement with those found in micropropagated banana (Peraza-Echeverria et al. 2001) and in seedlings and flag leaves of rice (Xiong et al.

1999), where the methylation pattern was found to be tissue specific. This is most likely due to the reason that DNA methylation is a tissue specific developmentally regulated phenomenon (Chakrabarty et al. 2003). Evidences are available on spontaneous DNA methylation variants that arise in tissue culture system and do not follow Mendelian inheritance (Niederhuth and Schmitz 2014). As callus go through the dedifferentiation and redifferentiation process during regeneration, the level of methylation changes drastically (Huang et al. 2012). The reason behind changed methylation pattern during the developmental process is for the failure of maintaining the methylation status during DNA replication due to *de novo* methylation and passive demethylation (Matsuo et al. 1998; Hsieh, 1999). This might have caused higher frequency of methylation polymorphism in in vitro grown callus compared to greenhouse-grown leaves. Plants predominantly do not undergo the cycle of remethylation following demethylation in the tissue culture system and thus produce variants. For example, in *Arabidopsis thaliana*, decreased methylation gave rise to numerous morphological and phenotypic abnormalities including decreased apical dominance, reduced plant size, modified leaf size and shape, diminished fertility, and altered flowering time as the plants were unable to restore the previous methylation pattern after the passage through meiotic event (Finnegan et al. 1996).

Table 4.3: Total number of methylated CCGG sites and the number of methylation polymorphism for each primer detected by methylation-sensitive amplification polymorphism in the greenhouse-grown leaves of four blueberry genotypes. Each experiment was repeated twice, and same bands were detected in each case.

Oligonucleotide combination	Total number of methylated CCGG sites				Methylation polymorphism			
	'CL1'	'CL2'	'CL3'	'H1'	'CL1'	'CL2'	'CL3'	'H1'
E-TT/MH-AAC	14	10	10	12	0	0	1	1
E-TT/MH-ATG	15	14	11	17	1	1	2	1
E-TT/MH-AAG	12	2	10	6	3	1	1	1
E-TT/MH-ACA	9	12	12	2	1	0	1	0
E-TG/MH-AAC	15	13	14	10	0	2	1	2
E-TG/MH-ATG	6	7	10	7	2	1	0	2

E-TG/MH-AAG	15	15	14	9	1	1	2	2
E-TG/MH-ACA	14	15	16	12	2	3	0	1
Total	100	88	97	75	10	9	8	10

In the current study, calli for all genotypes were maintained under same in vitro condition except different TDZ treatments. Altered methylation pattern in the calli might be due to the effect of TDZ concentration in the culture. Phytohormones have been proposed to play a central role to control the signal transduction cascade leading to the reprogramming of the gene expression (von Arnold et al. 2002). With the three different concentrations of TDZ, the variable pattern of cytosine methylation originated within the same genotype, has similarities with the cases of tissue cultured carrot plants, where different treatments of phytohormones altered methylation pattern (LoSchiavo et al. 1989; Arnholdt-Schmitt 1993; Arnholdt-Schmitt et al. 1995).

DNA methylation is an epigenetic phenomenon associated with somaclonal variation, which has potential use in the plant breeding program (Karp 1995). However, it has negative implications for clonal propagation and affects the production of true-to-type plants to the donor plants. So, the maintenance of clonal fidelity is of prime interest in tissue culture system (Karp 1995). For example, production of ‘an array of agronomically inferior genotypes’ of spring wheat (*Triticum aestivum* cv. ‘HY320’)

through tissue culture has been reported by Qureshi et al. (1992). Till date, the molecular basis of somaclonal variation is not completely understood. The most accepted hypothesis is that during in vitro culture, breakdown of normal cellular process results in genetic and epigenetic instabilities and altered gene expression that produces plant variants (Kaeppler et al. 2000). This may be due to the exposure to the stressful conditions such as wounding, pathogen attack, or the application of growth regulators in tissue culture media. Callus formation is a huge commitment for a fully-grown plant system as during this phase, plants give up their established developmental program and switch to a new one (Ikeuchi et al. 2013). In tissue culture system, plant cells undergo dedifferentiation and redifferentiation stimulated with the introduction of a plant growth regulator in the culture medium. During this phase, formation of callus is induced by the termination of current gene expression program and is turned on the callogenic gene expression process. As a result, DNA methylation is induced in the callus formation and differentiation stage (Huang et al. 2012). Several authors demonstrated the changes of the methylation pattern in tissue culture plants (Gao et al. 2010).

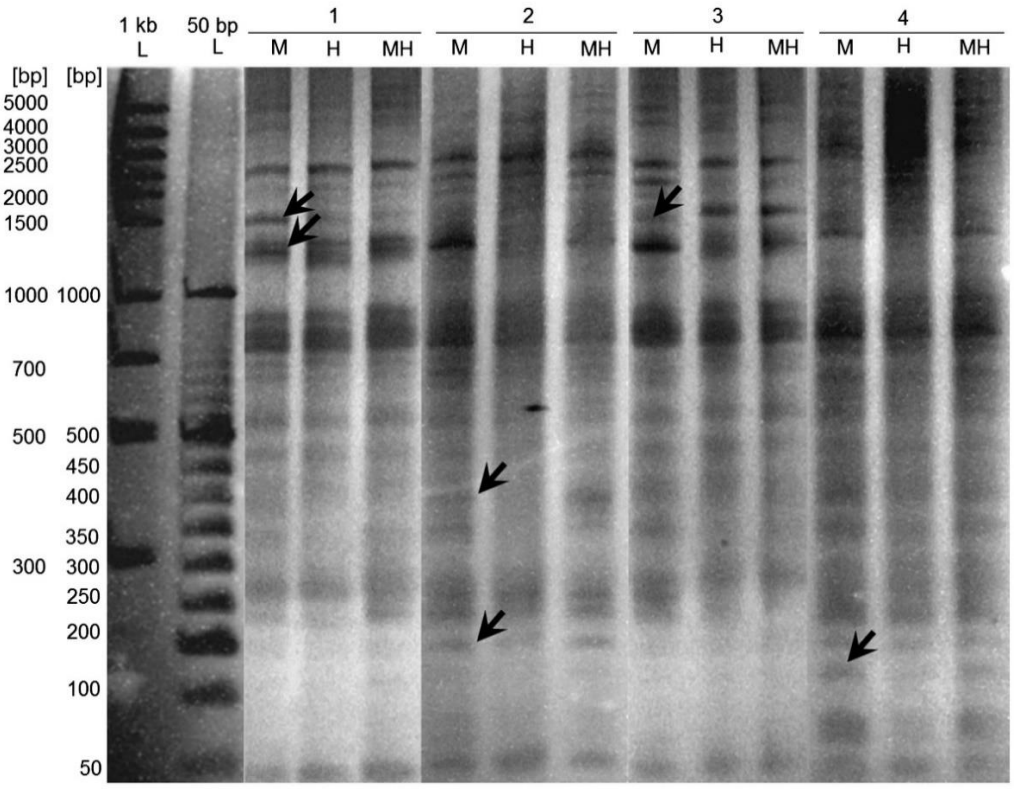

Figure 4.3: Methylation pattern detected in lowbush blueberry clones 'CL1' and 'CL2'. Pattern detected in in vitro grown callus of 'CL1' (1), 'CL2' (3) and greenhouse-grown leaves of 'CL1' (2) and 'CL2' (4) using the primer combination E-TG/MH-ACA. M, H and MH refers to the digestion with *EcoRI* + *MspI*, *EcoRI* + *HpaII* and *EcoRI* + *MspI* + *HpaII* enzymes, respectively. 1 kb L = MidRanger 1 kb DNA ladder (Norgen, Biotek Corp., Thorold, ON, Canada), 50 bp L = O'RangeRuler 50 bp DNA ladder (Thermo Scientific, Mississauga, ON, Canada). Arrows show differentialy methylated CCGG sites occurred in the enzyme digested DNA sequences of 'CL1' callus treated with 2.3 µM TDZ (1), 'CL1' leaves (2), 'CL2' callus treated with 2.3 µM TDZ (3) and 'CL2' leaves (4).

However, manipulation of the tissue culture conditions during in vitro culture induces altered methylation status. LoSchiavo et al. (1989) showed in carrot cultures that global methylation patterns vary with the changed hormone concentration in the media. In this study, it was noticed that the level of methylation was developmentally regulated and changed with the induction of somatic embryogenesis. Arnholdt-Schmitt et al. (1995) observed that the DNA methylation varied in different developmental stages of tissue culture regenerated carrot plants.

4.4. Conclusion

This study provides evidence that tissue culture process during callus formation is solely responsible for the induction of the hypermethylation pattern as the leaves from conventionally propagated plants showed lower DNA methylation compared to the in vitro-derived callus. To the best of my knowledge, this is the first report of DNA methylation at callus stage in blueberry species cultured in vitro. Further studies are required at the plantlet stage to identify whether DNA methylation is changing from callus to the plantlet formation. Moreover, the characterization of the gene expression during callus formation and maturation, and during different stages of plantlet formation will lead to the identification of several developmental regulatory genes that will further help in better understanding the methylation induction process in a plant tissue culture system.

4.5. References

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Chapter 5 - Half-high blueberry plants from bioreactor culture display elevated levels of DNA methylation polymorphism

Abstract

Blueberry (*Vaccinium* spp. L.) plants exhibit high potential for regeneration via adventitious shoot formation on a semi-solid medium followed by shoot elongation on a liquid medium in a bioreactor system. To find out whether DNA methylation plays a role during shoot elongation, we compared DNA methylation levels in the regenerants of two in vitro-grown half-high blueberry (*Vaccinium corymbosum* L.× *V. angustifolium* Ait.) cultivars, 'Patriot' and 'Chippewa', on a semi-solid medium (SSM) in glass bottles and in a liquid medium in temporary immersion bioreactor (TIB) system, via the methylation-sensitive amplification polymorphism (MSAP) technique. The SSM was separately fortified with different combinations of two plant growth regulators (PGRs), zeatin (ZEA) and thidiazuron, for shoot regeneration but elongation was carried out using the same medium under both SSM and TIB systems with only ZEA. In both in vitro culture systems, the number of shoots per explant was higher in SSM in both cultivars, but shoots proliferated in TIB were more vigorous. Noticeable changes in the methylation profiles were detected using MSAP in the regenerants grown in each type of culture system. The TIB system exhibited a significant increase in total methylation percent and methylation polymorphism in comparison to SSM. Additionally, differential methylation patterns were observed within the same genotype depending on the culture system. Here I have

described the effects of culture in vitro on DNA methylation that induced during the process of shoot elongation in two half-high blueberry cultivars.

5.1. Introduction

Plant tissue culture technique depends on the ability of a cell to differentiate, proliferate, and eventually regenerate into a fully grown plantlet under optimum culture conditions (Skoog & Miller 1957). Although it is a challenging process, as a result of the hormonal stimulation, plant cells initiate regeneration and elongation from a diverse array of tissues (Neelakandan & Wang 2012). In the presence of ideal culture conditions, explants develop into an identical copy of the donor plant, which is referred to as a clone. However, the adaptability of the explants cultured in vitro relies on reprogramming of their genetic and epigenetic makeup in tissue culture microenvironment (Neelakandan & Wang 2012). Due to continuous subculturing and artificial hormonal environment, explant tissues reset their genetic and epigenetic makeup for better adaptability to the surrounding environments, which ultimately leads to the production of variant phenotypes (Smýkal et al. 2007; Neelakandan & Wang 2012). Variants with stable genetic and epigenetic alterations produced in a clonally propagated plant population are referred to as somaclonal variants (Larkin & Scowcroft 1981). Somaclonal variation occurs in a tissue culture system imposes a major problem where the main goal is to achieve the production of identical progenies (Jain 2001).

Somaclonal variation can arise in the tissue culture system due to various reasons among which DNA methylation is hypothesized as one of the fundamental mechanism (Kaeppler & Phillips 1993). It is associated with transposable elements activation, chromosome breakage, DNA sequence changes, and ultimately resulting in phenotypic variation (Park et al. 2009; Kaeppler & Phillips 1993). DNA methylation is an epigenetic phenomenon that occurs due to the conversion of cytosine to methylcytosine, by adding a methyl group on the fifth position of the cytosine base on an aromatic ring (Chwialkowska et al. 2016). Among all the heritable epigenetic modifications, DNA methylation is the best-studied process (Karim et al. 2016). It is extensively presumed that cytosine methylation works mainly as a negative gene expression regulator and stops the movement of repetitive elements, thus maintaining genome integrity (Chwialkowska et al. 2016). DNA methylation arises at a higher rate in plants and varies over 30% among various genera (Cokus et al. 2008; Law & Jacobsen 2010). DNA cytosine methylation typically appears in CpG islands in CG or CHG (where H = A, T or G), and CHH contexts in plants, among which CG methylation occurs most frequently superseded by CHG and CHH sequences (Karim et al. 2016). This was supported by a study of genome-wide cytosine methylation in *Arabidopsis thaliana* where 24% of cytosine methylation was observed from CG context, while 6.7 and 1.7% were from CHG and CHH contexts (Cokus et al. 2008).

Blueberry (*Vaccinium* spp., family: Ericaceae) is a small fruit crop with enormous economic importance and grows on acidic soil which in other respects does not have agronomic value (Vander Kloet 1988; Rowland et al. 2012). In recent years, there has

been an increased market demand on consumption of blueberries due to their many recognized health-promoting properties (Rowland et al. 2012). Although blueberry has been cultivated worldwide, the majority of the production has been done in North America (Rowland et al. 2012; Debnath 2016). Half-high plants which are either hybrids or backcross derivatives of high and lowbush blueberry, have small-scale commercial plantings in the United States and Canada (Debnath 2017). Typically, blueberry plants are propagated asexually by stem cutting, but it requires time and skilled labour. Mass propagation of blueberries via micropropagation is much rapid and can be done year-round (Debnath 2007). There have been some reports of in vitro propagation of half-high blueberry plants (Debnath 2009; Debnath 2017; Ghosh et al. 2017; Ghosh et al. 2018). Although in vitro propagation was reported mostly using a semi-solid medium, it was problematic to automate and the production cost higher which makes it less suitable for large-scale commercial propagation (Debnath 2009). On the contrary, automated temporary immersion bioreactors are unparallel for large-scale industrial micropropagation (Debnath 2009). According to Paek et al. (2005) bioreactors are defined as independent, free of contamination, based on liquid nutrient medium or inflow and outflow systems of liquid and air, designed for exhaustive culture in controlled microenvironment such as temperature, agitation, aeration, dissolved oxygen and pH of the nutrient medium. Use of bioreactors in micropropagation of horticultural plants was introduced by Levin and Vasil (1989) and was used in various plants including blueberries (Debnath 2009; Debnath 2017).

Methylation Sensitive Amplification Polymorphism (MSAP) is the universally used technique among the various methods available to detect tissue culture-induced genome-wide methylation changes (Ghosh et al. 2017). I have used MSAP to detect genome wide DNA methylation level of the regenerants cultured on semi-solid and liquid media in a bioreactor system. In plant epigenomics, several detection methods are being used. Among them whole genome bisulfite sequencing is the most comprehensive approach of detection as it provides single base pair resolution of methylated cytosine (Cokus et al. 2008). However, this technique is costly up till now, especially for plants with larger genome size and this technique also requires knowledge of sequenced genome which makes it ideal for detecting cytosine methylation in model organisms (Chwialkowska et al. 2016). On the other hand, MSAP technique gives an overall idea about the methylation status of the genome and is a fast and feasible process. MSAP technique does not require any information of the sequenced genome, and can be used to study methylation profiles of non-model organisms (Fulneček & Kovařík 2014). It is adopted from the amplified fragment length polymorphism technique (AFLP) (Vos et al. 1995). MSAP method was first developed in dimorphic fungi (Reyna-Lopez et al. 1997) and it is based on the sensitivity of two isoschizomers *MspI* and *HpaII* to detect the methylation at the tetranucleotide recognition site CCGG, unlike *MseI* used in the original AFLP protocol (Fulneček & Kovařík 2014). Among these two enzymes, *MspI* only restricts internal methylated cytosine ($C^{m}CGG$) but not the external methylated one ($^{m}CCGG$), while the restriction activity of *HpaII* is still not known clearly due to the continuing controversy that it is inactive for hemi-methylated external cytosine ($^{m}CCGG$) including any CCGG methylated site and its digestion ability of the hemi-methylated

tetranucleotide site depends on the incubation time of the enzyme and concentration (Peraza-Echeverria et al. 2001; Fulneček & Kovařík 2014). This technique has been used to detect altered DNA methylation pattern in several plants including rice (Xiong et al. 1999), banana (Peraza-Echeverria et al. 2001), Siberian ginseng (Chakrabarty et al. 2003), *Doritaneopsis* orchid (Park et al. 2009), tobacco (Fulneček & Kovařík 2014), blueberry (Ghosh et al. 2017; Goyali et al. 2018), and barley (Chwialkowska et al. 2016). The rationale of this study was to compare the altered DNA methylation pattern of the half-high blueberry regenerants derived from two different in vitro culture systems. Furthermore, the effect of two plant growth regulators (PGRs; zeatin, ZEA and thidiazuron, TDZ) on shoot regeneration on a SSM and of ZEA concentrations on the proliferation of adventitious shoots on a semi-solid and in a liquid medium were also investigated. Although there are only two reports available on tissue culture-induced DNA methylation in blueberries (Ghosh et al. 2017; Goyali et al. 2018), no reports are available so far on the impact of different types of in vitro culture systems on global DNA methylation.

5.2 Materials and methods

5.2.1 In vitro culture establishment, callus formation and shoot regeneration

Two half-high blueberry cultivars ‘Patriot’ and ‘Chippewa’ were grown and maintained in the greenhouse of St. John’s Research and Development Centre, Agriculture and Agri-food Canada, St. john’s, NL, Canada for more than 15 years in a plastic pots (10.5 × 10.5 × 12.5 cm^3) containing 3 : 1 (peat : perlite, v/v) at a maximum light intensity of 90 μmol m^{-2} s^{-1}, under the natural light source (photosynthetic photon flux density (PPFD) at 20 ± 2 °C) with a maximum relative humidity of 85%. Young leaf explants of these two cultivars were collected from the greenhouse for in vitro culture establishment and surface sterilization was done following Debnath (2009). Sterilized leaves were sectioned into three pieces (approx. 5 × 5 mm) and were inoculated abaxial side facing the media in petri dishes with clear lids (Fisherbrand™, Fisher Scientific, Fair Lawn, NJ, USA) with 25 g of a basal medium [BM; three-quarter micro salts and macro salts of Debnath and McRae (2001)]. BM was fortified with 25 g sucrose, 1.25 g Gelrite, and 3.5 g Sigma agar (Catalogue no. A 1296, Sigma Chemical Co., St. Louis, USA) and the pH was adjusted to 5.0 before autoclaving at 121 °C for 20 min.

Four concentrations of ZEA (2.3, 4.6, 6.9 or 9.2 μM) and TDZ (2.3, 4.5, 6.9 or 9.2 μM) were added to the medium for callus induction and shoot regeneration experiments. BM with no PGR was treated as control. TDZ was supplemented in the medium before autoclaving while filter sterilized ZEA was added in the medium after autoclaving

following Ghosh et al. (2018). After adding the PGRs, rims of petri dishes were sealed with a double layer of Paraflim™. Three Petri plates were used per treatment and each Petri dish contained nine (Fig. 5.1a) leaf explants. The cultures were placed in the dark for 2 weeks at 20 ± 2 °C and afterwards they were transferred under cool-white fluorescent lamps with a PPFD of 30 µmol m^{-2} s^{-1}, 16 h photoperiod (at 20 ± 2 °C). The experiments were repeated three times within two-week intervals. Two 2 × 9 completely randomized factorial experiments were employed to compare two half-high blueberry cultivars and five treatment combinations (including control) for ZEA or TDZ concentrations. The stock culture was maintained in 4.6 µM ZEA for micropropagation studies and axillary shoots from stock culture were inoculated in BM with no PGR to neutralize the effect of PGRs. Data on callus formation and shoot regeneration were taken after 4 weeks and 12 weeks of culture, respectively.

5.2.2 Shoot elongation on a semi-solid medium (SSM) and in a temporary immersion bioreactor (TIB) system containing a liquid medium

Regenerated shoots of ‘Patriot’ and ‘Chippewa’ developed on Petri plates without any PGRs were transferred to 175-mL baby-food jars (Sigma Chemical Co., St. Louis, MO, USA) after 8 weeks of culture initiation. Each jar contained 35 mL BM supplemented with ZEA (2.3, 4.6, 6.9, or 9.2 µM). BM without any ZEA was treated as control for this experiment. The explants were taken from the stock culture which was maintained in BM with no PGR. Each jar contained five explants and there were three

jars per treatment. Similarly, regenerated shoot maintained for 8 weeks in SSM without any PGR were transferred to temporary immersion RITA® bioreactor vessels (VITROPIC, Saint-Mathieu-de-Treíviers, France; (Teisson & Alvard 1995) with 200 mL liquid media Debnath (2009). There were eight explants per RITA vessel and three vessels per treatment. Shoot elongation experiments were repeated three times in both culture systems. Baby food jars and RITA vessels with cultures were maintained for shoot elongation under 16-h photoperiod at 30 µmol m^{-2} s^{-1}. Data on shoot elongation in both SSM and TIB systems were collected after 16 weeks of culture.

5.2.3 Rooting and acclimatization

Elongated shoots (4 – 5 cm long) regenerated from both SSM and TIB systems (at 9.2 µM ZEA) were excised right above the original explant, then coated with 39.4 mM indole-3-butyric acid (IBA) powder (Stim-Root #3, Plant Products Co. LTD., Brampton, ON, Canada), and planted in 45-cell plug trays (cell diameter = 5.9 cm, cell depth = 15.1 cm; Beaver Plastics, Edmonton, AB, Canada). Plug trays containing 2 peat:1 Perlite (v/v) were kept in humidity chamber at 22 ± 2 °C, 95 % of relative humidity, PPFD of 55 µmol m^{-2} s^{-1} and 16 h photoperiod for rooting.

Plantlets were transferred to 25 (L) × 18 (D) × 6 (W) cm^3 plastic pots *(*East-Chem Inc. Mount Pearl, NL, Canada) containing peat: perlite (3: 1, v/v). Acclimatization of the plantlets was done by reducing the humidity over 2 – 3 weeks to 85% (Debnath, 2017). The hardening off process was complete after 6 weeks and data on plantlets survival were

recorded. Later, hardened off plants were transferred in plastic pots (6 cm^3) containing the same medium and grown under the natural light condition at maximum PPFD 90 µmol m^{-2} s^{-1}, 20 ± 2 ºC, 16-h photoperiod and humidity approx. 85% in a greenhouse (Debnath 2017).

5.2.4 DNA extraction

Leaf samples were collected from three randomly selected hardened off plants of both the cultivars and genomic DNA was extracted. These plants were grown on the SSM in jars and in liquid medium (in RITA bioreactors) supplemented with 9.2 µM ZEA. DNA extraction was done using DNeasy plant mini kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. DNA purity was measured from the absorbance ratio A_{260}/A_{280}. The dilution of DNA used for the MSAP analysis was 80 ng μL^{-1} with A_{260}/A_{280} of 1.8 – 2.1.

5.2.5 Methylation sensitive amplification polymorphism (MSAP) assay

Vos et al. (1995) established the AFLP protocol MSAP assay were modified from the AFLP protocol and modified by Reyna-Lopez et al. (1997). Unlike *MseI*, in the modified protocol methylation-sensitive restriction enzymes *MspI* and *HpaII* (Thermo Scientific) two isoschizomers were used as "frequent cutter" and *EcoRI* (Thermo Scientific) was used as "rare/hexa cutter". To detect MSAP digestion reactions were set

up in two steps following (Ghosh et al. 2017). 400 ng of the genomic DNA was digested with 2 U of *EcoRI* in a final volume of 50 µL containing 10× FastDigest buffer (Thermo Scientific) for 1.5 h at 37 °C and the reaction was deactivated by incubating at 65 °C for 15 min. Three digestion reactions were carried out where DNA already digested with *EcoRI* was restricted with 2 U of *MspI/HpaII/MspI+ HpaII* enzymes in a final volume of 50 µL containing 10× FastDigest buffer. After that, the reaction was incubated for 3 h at 37 °C and then it was inactivated at 65 °C for 15 min. The digested DNA fragments were ligated to the *EcoRI* and *MspI-HpaII* adapters (Ghosh et al. 2017).

The ligated products (4 µL) of each reaction were pre amplified with two *EcoRI* and eight *MspI-HpaII* primers (Table S1) in a total volume of 50 µL comprise of 1× Taq buffer (Qiagen), 10 mM dNTPs (Amresco LLC, Solon, OH, USA), 10 µM of each primer and 5 U of Taq polymerase (Qiagen). PCR reactions were set up following Ghosh et al. (2017) in Eppendorf Mastercycler Gradient thermocycler (Eppendorf AG, Hamburg, Germany). The quality of the pre-amplified fragments was checked by electrophoresis in 1.8% agarose gel and a smear of fragments was visible between 100 – 1000 bp. After that, the amplification product was 15 times diluted to in 1× Tris-borate-EDTA (TBE) buffer and stored at -20 °C until further use.

Selective amplification reactions were done using 2 µL of the pre-amplified DNA, 10 µM of *EcoRI* primer and 10 µM of *MspI-HpaII* primer along with the same other components as used in pre-selective amplification step in a total volume of 25 µL. Selective amplification was achieved by involving a set of reactions in Eppendorf

Mastercycler Gradient thermocycler with the following profile: 94 °C for 5 min, 13 cycles of 94 °C for 30 s, 65 °C for 1 min, 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 at 72 °C for 10 min.

5.2.6 Methylation sensitive amplification polymorphism (MSAP) gel electrophoresis and silver staining

PCR products from the selective amplification stage were mixed with equal amount of formamide dye, prepared by mixing 98 % (v/v) formamide, 10 mM EDTA, 0.01% (m/v) bromophenol blue and 0.01% (m/v) xylene cyanol). After this step, the aliquot was denatured at 95 °C for 4 min, and instantly cooled down on ice. Each reaction aliquots were then separated by electrophoresis on 5% denaturing polyacrylamide sequencing gels following Ghosh et al. (2017) . After pre-running at 100 V for 30 min, the PCR product and formamide dye were mixed at an equal amount (6 µL) and loaded in the wells. Then the gels were run at 115 V for 5 h in Owl gel separation system (Thermo Scientific). Silver staining was done on the DNA fragments in gel follow*ing* (Bassam, Caetano-Anollés, & Gresshoff, 1991). All steps were performed on Rocker 25 shaker (Labnet International, Edison, NJ, USA) with slow agitation. A 50 bp ladder (New England Biolabs LTD., Whitby, ON, Canada) was used as a molecular marker to identify the DNA bands in the gel. Band separation pattern in the polyacrylamide gel was pictured using the InGenius 3 gel documentation system (Syngene, Frederick, MD, USA).

Experiments were repeated twice to confirm then reproducibility of the methylation pattern.

5.2.7 Data analysis

Shoot regeneration and elongation experiments were done following completely randomized design (CRD) and the data was subjected to analysis of variance (ANOVA) employing the general linear model. For shoot regeneration, a 2 × 9 completely randomized factorial experiment was used to study all 9 treatment combinations in two half-high blueberry cultivars and significant effects were tested at a critical value (P) of 0.05. The significance of the ZEA and TDZ treatments was assessed by Tukey's test using STATISTICA software version 10 (Statsoft Wipro, East Brunswick, NJ, USA). Tukey's test was used to compare treatment means at a critical difference (P) of ≤ 0.05. Shoot vigour at elongation stage was measured on a scale of 1 to 8 where 1 meant completely vitrified, necrotic and/or malformed shoots; 2, comparatively less vitrified, necrotic and/or malformed shoots; 3, with no sign of very poor vigour; 4, poor shoot vigour but no vitrification; 5, better shoot vigour; 6, with good shoot vigour; 7, very good shoot vigour; and 8, with fully normal-healthy shoots with high vigour (Debnath 2017).

DNA methylation events were detected in the MSAP assays on the basis of the presence or absence of the bands in the autoradiograph. Four types of methylated sites were detected following (Chwialkowska et al. 2016), where methylated tetranucleotide sites (CCGG/GGCC) were detected when demethylated or hemi-methylated at internal

cytosine (CCGG/GGCC or C^{m}CGG/GGCC) restricted by *MspI* + *HpaII* enzyme (type I), when hemi-methylated tetranucleotide at external cytosine or one strand hemi-methylated (^{m}CCGG/GGCC or ^{m}C^{m}CGG/GGCC) were cleaved by *HpaII* only (type II), and *MspI* cleaved only internal methylated cytosine (C^{m}CGG/GG^{m}CC) residues (type III) and no bands were detected for both the enzymes (type IV). Polymorphic banding patterns were detected in all reactions with *EcoRI* + *MspI*/*HpaII*/*MspI*+*HpaII* following (Chakrabarty et al., 2003). The total number of methylated bands was obtained by adding up type II, III and IV.

5.3 Results

5.3.1 Effect of different concentrations of zeatin (ZEA) and thidiazuron (TDZ) on callus formation and shoot regeneration

Leaf explants of both the genotypes started initiating pale green calluses at the leaf margin on BM supplemented with various concentrations of ZEA and TDZ after 4 weeks of culture (Fig. 5.1a). The highest percentage of callus formation was observed at 2.3 µM TDZ (91 ± 0.6% to 93 ± 0.9%) irrespective of culture media type in both cultivars. 'Chippewa' showed the highest percent callus formation on 2.3 µM TDZ (93 ± 0.9%) and 6.9 µM ZEA (93 ± 1.5%; Table 5.1). In 'Patriot', the maximum percentage of callusing (91 ± 0.6%) was noticed at 2.3 µM TDZ following 87 ± 1.3% at 6.9 µM ZEA. Although a

higher percentage of callus formation was noticed at 2.3 µM TDZ, with the increasing concentration of TDZ, shoot formation decreased significantly (Table 5.1).

Shoot regeneration was started after 8 weeks of culture (Fig. 5.1b) and the highest number of regenerations was noticed at 9.2 µM ZEA in both 'Chippewa' and 'Patriot' in SSM system after 12 weeks of culture (Fig. 5.1c). The maximum numbers of shoots 'Chippewa' and 'Patriot' developed were 24 ± 1.2 and 19 ± 1.5, respectively. Data of percent shoot regeneration was recorded after 12 weeks of culture, where highest percentage in 'Chippewa' (95 ± 2.6) and 'Patriot' (87 ± 1.5) was reported on 9.2 µM ZEA (Table 5.1). The lowest shoot regeneration percentage was noticed in control, where media was not supplemented with any PGR (Table 5.1). There were no significant effects of cultivars found on mean callusing percentage, mean number of shoots per explants or percent shoot regeneration. However, there were prominent effects of different concentrations of PGR on callus percentage and percent shoot regeneration. Also, effects of PGR and interaction effects of cultivars with PGR concentrations was clearly visible on the mean number of shoot formation.

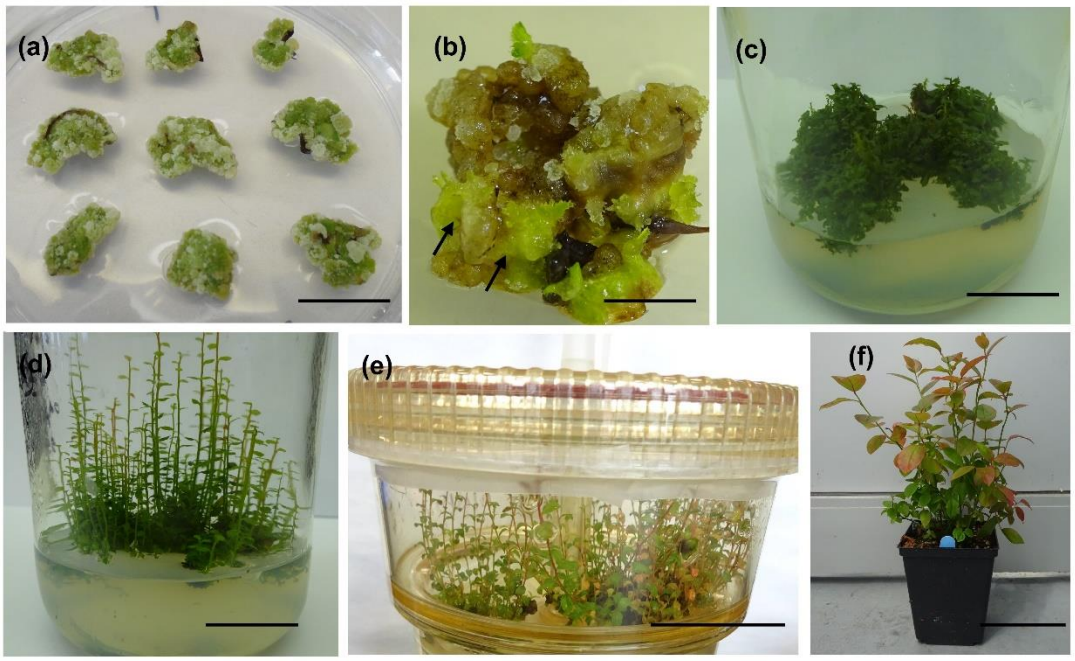

Figure 5.1: In vitro callus formation, shoot regeneration, shoot elongation and plantlet formation from 'Chippewa' leaf explants. (a) Callus formation after 4 weeks of culture on a semi-solid medium (SSM) with 2.3 µM zeatin (ZEA) (bar = 2 cm), (b) bud initiation from callus (arrows) after 8 weeks of culture on SSM with 9.2 µM ZEA (bar = 2 cm), (c) initiation of shoot regeneration after 12 weeks of culture on SSM with 9.2 µM ZEA (bar = 2 cm), (d) shoot elongation after 16 weeks of culture on SSM with 9.2 µM ZEA (bar = 2 cm), (e) shoot elongation after 16 weeks of culture in a temporary immersion bioreactor (TIB) containing liquid medium with 9.2 µM ZEA (bar = 3 cm), (f) one year old hardened-off plants in a greenhouse (bar = 8 cm).

Table 5.1: Effects of zeatin (ZEA) and thidiazuron (TDZ) on callus formation and shoot regeneration from leaf explants of half-high blueberry cultivars 'Patriot' and 'Chippewa' on a semi-solid medium. Each experiment was repeated three times. Standard errors associated with different letters indicate significant differences within each group according to by Tukey's test at $P \leq 0.05$. Significant effects (0.05): C = cultivars, P = PGR concentrations

ZEA/TDZ (µM)	Callus formation (%)		Shoots regenerated per explant (no.)		Shoot regeneration (%)	
	'Patriot'	'Chippewa'	'Patriot'	'Chippewa'	'Patriot'	'Chippewa'
Control	8.7 ± 0.6^{i}	13 ± 1.1^{i}	1.5 ± 0.3^{h}	1.6 ± 0.2^{gh}	1.0 ± 0.6^{f}	1.2 ± 0.7^{f}
ZEA 2.3	36 ± 1.2^{h}	33 ± 0.9^{h}	3.3 ± 0.9^{efgh}	2.0 ± 0.6^{fgh}	7.0 ± 1.2^{de}	6.3 ± 1.3^{d}
ZEA 4.6	75 ± 0.6^{bc}	73 ± 1.7^{cd}	12 ± 1.2^{c}	10 ± 1.2^{cd}	32 ± 1^{c}	36 ± 2.1^{c}

ZEA 6.9	87 ± 1.3^{a}	93 ± 1.5^{a}	18 ± 1.2^{b}	19 ± 2.1^{b}	72 ± 2.5^{b}	73 ± 3.4^{b}
ZEA 9.2	65 ± 1.8^{de}	63 ± 2.0^{g}	19 ± 1.5^{ab}	24 ± 1.2^{a}	87 ± 1.5^{a}	95 ± 2.6^{a}
TDZ 2.3	91 ± 0.6^{a}	93 ± 0.9^{a}	8.4 ± 0.8^{cde}	8.3 ± 0.7^{cde}	12 ± 0.3^{def}	13 ± 1.0^{def}
TDZ 4.5	79 ± 2.2^{b}	81 ± 3.8^{bc}	5.7 ± 0.7^{defgh}	6.7 ± 0.9^{defg}	5.7 ± 1.8^{f}	5.3 ± 0.3^{def}
TDZ 6.9	64 ± 1.0^{ef}	65 ± 0.8^{fg}	3.8 ± 0.6^{efgh}	7.0 ± 1.2^{cdef}	3.7 ± 0.7^{f}	4.0 ± 1.0^{ef}
TDZ 9.2	35 ± 0.3^{h}	36 ± 1.7^{h}	3.3 ± 0.9^{efgh}	4.0 ± 0.6^{efgh}	3 ± 1.2^{f}	2.3 ± 0.7^{f}
Significant effects	P	P	P, C × P	P, C × P	P	P

5.3.2 Effects of various concentrations of zeatin on shoot elongation on a semi-solid and in a liquid medium

The concentrations of ZEA were found to affect the shoot elongation in both SSM and TIB systems. With the increasing concentration of ZEA, number of shoots per explant increased significantly in both cultivars under SSM and TIB systems as some of the buds which were dormant during the shoot regeneration process started regeneration at shoot elongation phase. At 9.2 µM ZEA supplemented SSM, the greatest number of shoot formation was noticed in both 'Chippewa' (33 ± 0.9) and 'Patriot' (30 ± 0.6) (Fig. 5.2a, 5.3a). It was followed by 6.9 µM ZEA, where in 'Chippewa' 28 ± 0.9 shoots proliferated per explant and 27 ± 1.2 were formed in 'Patriot' (Fig. 5.2a, 5.3a). On average, 4.0 ± 0.6 to 5.3 ± 0.3 shoots formed per leaf explant in BM with no PGR in SSM (Fig. 5.2b), while 3.7 ± 0.3 to 5.0 ± 0.6 in TIB (Fig. 5.3b). The average height of the shoots did not follow the same trend. Shoots grown on/in BM with 9.2 µM ZEA were with lowest average height. In SSM, shoot height varied from 3.1 ± 0.5 ('Patriot') – 2.5 ± 0.3 ('Chippewa') (Fig. 5.2c), while in TIB it varied from 3.5 ± 0.1 ('Patriot') – 3.9 ± 0.2 ('Chippewa') (Fig. 5.3c). It was evident from the experiments that even though shoot number increased with ZEA concentration, longer shoots were obtained at 2.3 µM ZEA and decreased linearly with increasing ZEA concentrations in both the culture systems (Fig. 5.2c, 5.3c). Similarly, the highest number of leaves per shoot was noticed in 2.3 µM ZEA and lowest number of leaves was recorded in 9.2 µM ZEA. In both the cultivars higher number of leaves was observed in SSM system, which varied from 12.3 ± 1.5 ('Patriot') – 18.3 ± 1.2 ('Chippewa') (Fig. 5.2d), followed by 13 ± 1.5 ('Patriot') – 16 ±

1.2 (‘Chippewa’) in TIB (Fig. 5.3d). Additionally, shoot vigour was best (2.3 – 4.6 µM) of ZEA irrespective of culture type and cultivar. Average shoot vigour varied from 2.7 ± 0.9 – 6.7 ± 0.7 in ‘Patriot’ and 3.3 ± 0.9 – 7 ± 1.0 in ‘Chippewa’ in TIB system (Fig. 5.3d), while it was in the range of 3 ± 0.6 – 6.7 ± 0.9 in ‘Patriot’ and 2.7 ± 0.9 – 7 ± 0.6 in ‘Chippewa’ in SSM system (Fig. 5.2d).

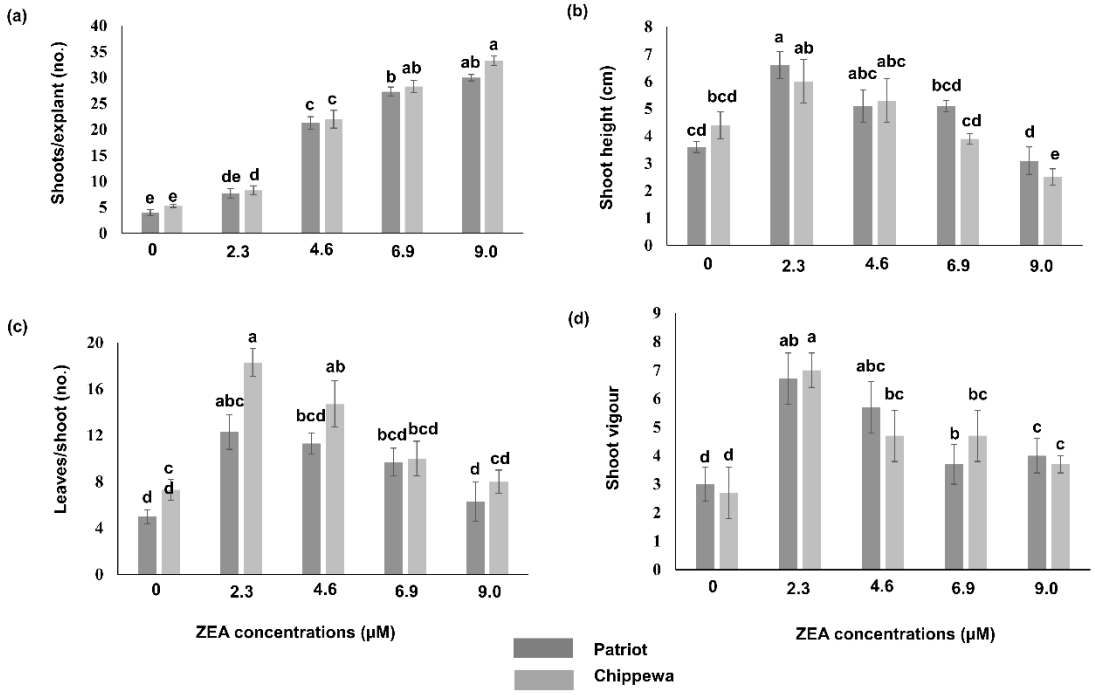

Figure 5.2: Effects of zeatin (ZEA) concentrations on in vitro shoot elongation of half-high blueberry cultivars 'Patriot' and 'Chippewa' on a semi-solid medium (SSM) for (a) number of shoots per explant, (b) shoot height (cm), (c) number of leaves per shoot and (d) shoot vigour supplemented with 0 (control), 2.3, 4.6, 6.9 and 9.2 µM zeatin (ZEA) after 16 weeks of culture. Each bar represents mean ± SE. The experiment was repeated three times. Significant difference at $P \leq 0.05$ was detected using Tukey's test.

5.3.3 Rooting and acclimatization

Microshoots derived from SSM and TIB systems rooted easily after 4 weeks of transfer to plastic pots containing peat: perlite medium (3: 1 v/v). Plantlets from the SSM system when transferred in the pots, 80 – 90% survived while 70 – 80% of the plantlets survived from the TIB system. Hardened-off plants grew well in a greenhouse with no apparent morphological abnormalities (Fig. 5.1e).

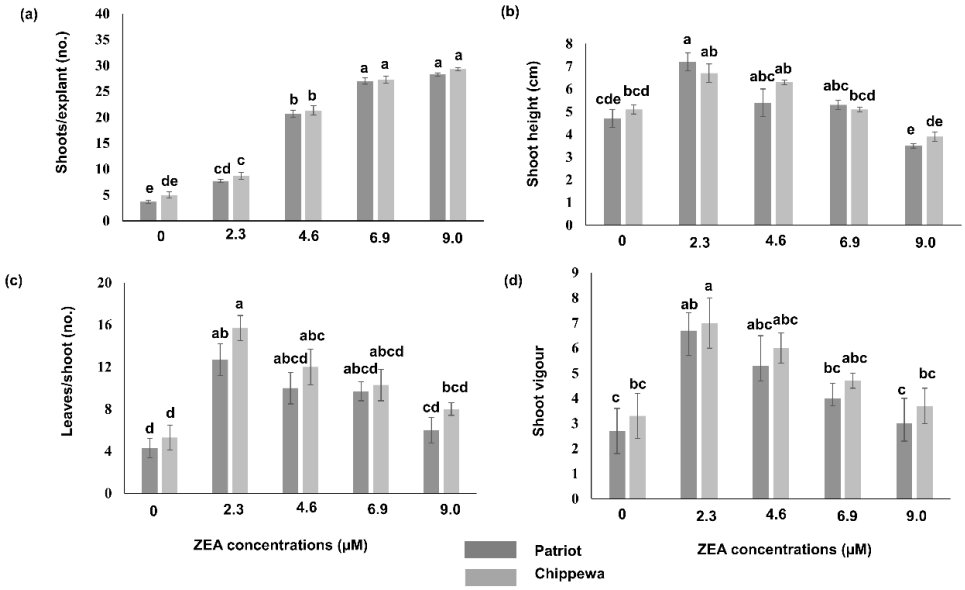

Figure 5.3: Effects of zeatin (ZEA) concentrations on in vitro shoot elongation of half-high blueberry cultivars 'Patriot' and 'Chippewa' in a temporary immersion bioreactor (TIB) containing a liquid medium for (a) number of shoots per explant, (b) shoot height (cm), (c) number of leaves per shoot, and (d) shoot vigour supplemented with zeatin (ZEA) 0 (control), 2.3, 4.6, 6.9 and 9.2 µM after 16 weeks of culture. Each bar represents mean ± SE. The experiment was repeated three times. Significant difference at $P \leq 0.05$ was detected using Tukey's test.

5.3.4 MSAP assay

In this present course of investigation, MSAP primers were used to detect changes in methylation status of DNA, type of methylated bands, and polymorphism in methylated DNA in micropropagated 'Patriot' and 'Chippewa' plants (Fig. 5.4) regenerated from SSM and TIB systems.

In SSM eight selective combinations of MSAP primers were used to produce a total of 741 bands in 'Patriot' among which 25% were methylated and 2.4% were polymorphic bands (Table 5.2). Similarly, in 'Chippewa' total numbers of generated DNA bands were 812, within which 32% were methylated and 2.5% were polymorphic in nature (Table 5.2). The E1MH2 primer produced the highest percentage of methylation (48%) and E1MH3 produced the highest percentage of methylated polymorphic bands in 'Patriot' (SSM) (25%) (Table 5.2). Among all the primers used to detect DNA methylation in 'Chippewa' (SSM), E1MH3 detected the highest percentage of methylated bands (59.4%) and E1MH4 produced the highest methylation polymorphism (3.3%) (Table 5.2). From the results, it was clearly visible that micropropagated plants originating from TIB were more methylated irrespective of cultivar. 'Patriot' plants regenerated from TIB produced 864 total bands among which 435 (50%) were methylated and 43 (5.0%) were polymorphic (Table 5.2). Likewise, 'Chippewa' grown from TIB system produced 864 total bands in which 407 (40%) were methylated and on average 47 (4.6%) were polymorphic (Table 5.2). E2MH2 produced 59% methylated bands which was highest among all the primers, E1M'H1' produced the highest level of methylation polymorphism (10%) in 'Patriot' (TIB) (Table 5.2). In 'Chippewa' (TIB) E1MH4

detected the highest percentage of methylated bands (66%) and E1M'H1' produced highest polymorphic bands (12%) (Table 5.2). Although effect of culture systems was clearly visible on the methylation events, effects of cultivars on methylation was not significant.

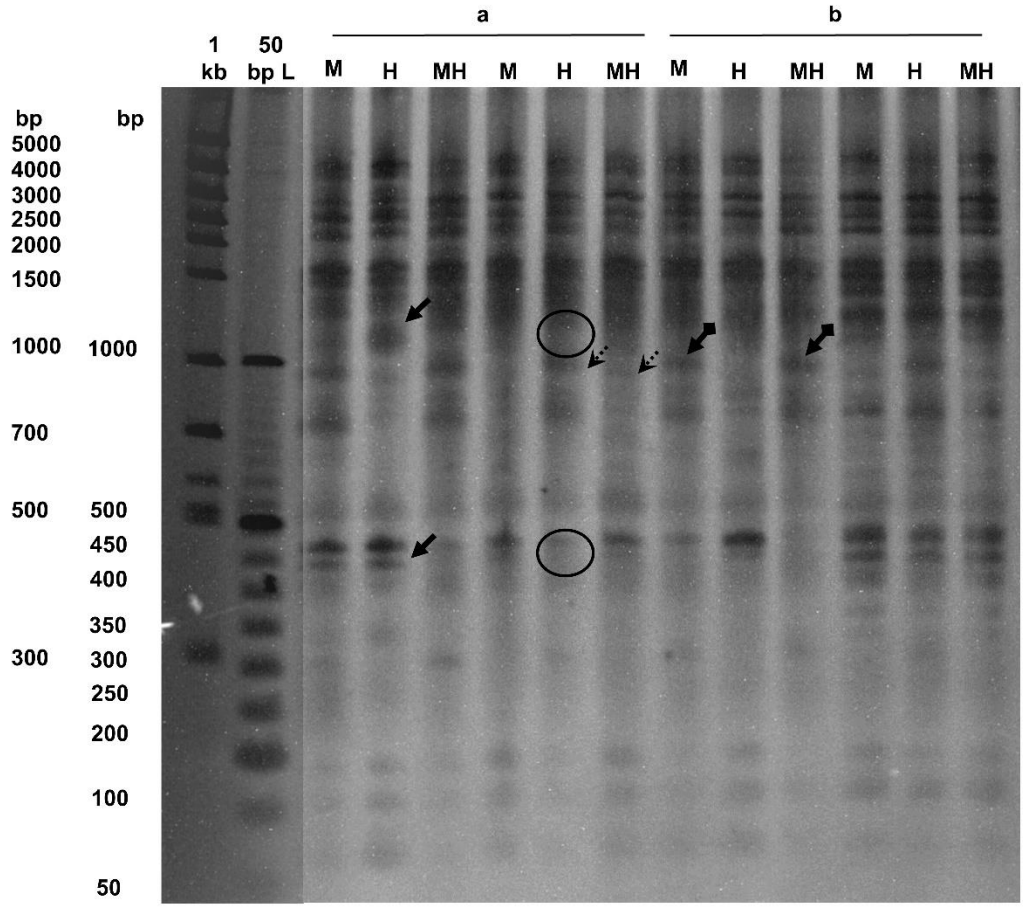

Figure 5.4: Methylation pattern detected in blueberry cultivar 'Chippewa'. Pattern detected in hardened off plants derived from (a) semi-solid medium and (b) temporary immersion bioreactor with 9.2 µM zeatin, using E-TT/MH-ATG (E1MH2) primer combination. M, H and MH refers to the restriction with *EcoRI* +

***MspI, EcoRI + HpaII* and *EcoRI + MspI + HpaII* enzymes, respectively. 1 kb L = MidRanger 1 kb DNA ladder (Norgen, Biotek Corp., Thorold, ON, Canada), 50 bp L = O'RangeRuler 50 bp DNA ladder (**Thermo Scientific, Missisauga, ON, Canada**).** DNA methylation polymorphisms in 'Chippewa' plants regenerated from SSM are represented with banding pattern (marked by arrows) in H lanes that are absent in one plant (circled). Bands (marked by broken arrows) that are present in H restriction lanes but not in M lanes indicate hemi-methylated external cytosine at CCGG sites in genomic DNA, while bands (marked by arrowheads) present in M restriction lanes but not in H lanes indicate methylated internal cytosine at the CCGG sites.

Table 5.2: Total number of methylated CCGG sites and the number of methylation polymorphism for each primer detected by methylation-sensitive amplification polymorphism in semi-solid medium (SSM) and temporary immersion bioreactor system (TIB) containing a liquid medium-derived plants of half-high blueberry cultivars 'Patriot' and 'Chippewa'. Each experiment was repeated twice for each treatment and the same bands were detected in each case.

Primer combinations	**Total no. of bands**		**No. of methylated bands**		**Percent methylation**		**No. of polymorphic methylated bands**		**Percent polymorphism methylation**	
	SSM	**TIB**	**SSM**	**TIB**	**SSM**	**TIB**	**SSM**	**TIB**	**SSM**	**TIB**
'Patriot'										
E1M'H1'	50	50	11	14	22	28	2	5	4	10

E1MH2	44	50	21	27	48	54	0	3	0	6
E1MH3	24	55	11	30	46	55	6	3	25	5.5
E1MH4	100	208	25	93	25	45	2	7	2	3.4
E2MH1	181	97	18	59	9.9	61	2	11	1.1	11
E2MH2	83	182	28	107	34	59	4	3	4.8	1.6
E2MH3	140	112	34	46	24	41	0	4	0	3.6
E2MH4	119	110	35	59	29	54	2	7	1.7	6.4
Total	741	864	183	435	25	50	18	43	2.4	5.0

‘Chippewa’										
E1M’H1’	38	49	10	16	26	33	0	6	0	12
E1MH2	37	46	14	15	38	33	1	3	2.7	6.5
E1MH3	32	38	19	11	59	29	2	3	6.3	7.9
E1MH4	91	92	41	61	45	66	3	5	3.3	5.4
E2MH1	192	177	74	99	39	56	4	4	2.1	2.3
E2MH2	115	151	39	75	34	50	5	9	4.3	6.0
E2MH3	174	174	33	79	19	45	2	5	1.1	2.9

E2MH4	133	137	26	51	20	37	3	5	2.3	3.6
Total	812	864	256	407	32	47	20	40	2.5	4.6

The DNA methylation fingerprints were grouped into four classes as bands were detected after the digestion of DNA with *EcoRI* in combination with *MspI* and/or *HpaII*. From the results it was evident that type I methylation events were most frequent in 'Patriot' plants (1605 bands) regenerated from both the culture systems, followed by type III (262 bands), II (244 bands) and IV (112 bands), in the same way in 'Chippewa' it was type I (1676 bands) > type III (272bands) > type II (235 bands) > type IV (94 bands) (Table 5.3). In these assays, type I was defined as the bands obtained from demethylation or hemi-methylation at the internal cytosine of the tetranucleotide site and restricted by both isoschizomers; type II restriction sites were hemi-methylated at the external cytosine and digested by only *HpaII*. Type III was methylated internal cytosine restricted by only *MspI* and type IV was the case where no bands were detected in either of the enzyme digestion. In 'Patriot' grown in SSM, 183 total methylated bands were amplified among which 80 (10.8%) were type III, following 67 (9.0%) type II, 36 (4.9%) type IV (Table 5.3). Similarly, in TIB system total methylated bands detected were 435, among which methylation varied from 21 (type III) – 8.8% (type IV) (Table 5.3). Among both the culture systems regenerated 'Chippewa' plants number of methylated bands varied from 192 (22%) type III – 58 (6.7%) type IV in TIB and 80 (10%) type III – 36 (4.4%) type IV in SSM system (Table 5.3).

Table 5.3: Analysis on the methylation level and type of bands obtained by eight primer combinations as revealed by methylation-sensitive amplification polymorphism assay of 'Patriot' (PAT) and 'Chippewa' (CHIP) plants derived from a semi-solid medium (SSM) and a temporary immersion bioreactor system (TIB) containing a liquid medium. Each experiment was repeated twice for each treatment and same bands were detected in each case

Primer combinations	**Total no. of bands**		**No. of methylated bands**		**Type I**		**Type II**		**Type III**		**Type IV**	
SSM	**PAT**	**CHIP**	**PAT**	**CHIP**	**PAT**	**CHIP**	**PAT**	**CHIP**	**PAT**	**CHIP**	**PAT**	**CHIP**
E1M'H1'	50	38	11	10	39	28	3	4	7	5	1	1
E1MH2	44	37	21	14	23	23	8	4	10	6	3	4
E1MH3	24	32	11	19	13	13	4	9	3	7	4	3

E1MH4	100	91	25	41	75	50	10	13	12	21	3	7
E2MH1	181	192	18	74	163	118	7	26	9	38	2	12
E2MH2	83	115	28	39	55	76	12	17	10	14	6	8
E2MH3	140	174	34	33	106	141	7	18	18	13	9	2
E2MH4	119	133	35	26	84	107	16	11	11	12	8	3
Total	741	812	183	256	558	556	67	192	80	116	36	40
TIB												
E1MH1	50	49	14	16	36	33	5	6	7	8	2	2

E1MH2	50	46	27	15	23	31	9	4	14	7	4	4
E1MH3	55	38	30	11	25	27	15	4	10	6	5	1
E1MH4	208	92	93	61	115	31	45	19	33	27	15	15
E2MH1	97	177	59	99	38	78	25	51	27	47	7	12
E2MH2	182	151	107	75	75	76	39	38	45	32	23	5
E2MH3	112	174	46	79	66	95	14	31	25	37	7	11
E2MH4	110	137	59	51	51	86	25	15	21	28	13	8
Total	864	864	435	407	429	457	177	168	182	192	76	58

All eight primer combinations in SSM produced a total of 1556 fragments, among which 455 (22%) were methylated at the CCGG tetranucleotide sites, out of which 38 (2%) were polymorphic sites in 'Patriot' and 'Chippewa' cumulatively (Fig. 5.5a). The number of methylation and polymorphic events increased significantly when plants were regenerated from TIB system, where out of a total of 1728 bands, 32% (842) were methylated and 3% (83) were found to be polymorphic (Fig. 5.5b).

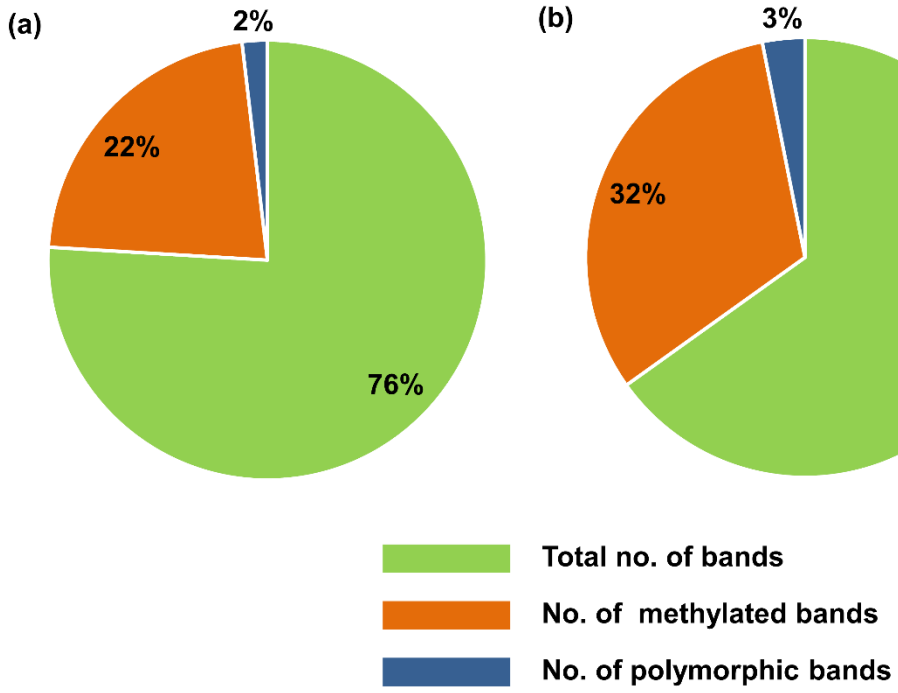

Figure 5.5: Pie charts representing the total number of bands, number of methylated bands, and number of polymorphic bands detected in plants derived from (a) semi-solid medium and (b) liquid medium in a temporary immersion bioreactor containing. Each experiment was repeated twice for each treatment and the same bands

were detected in each case.

5.4. Discussion

The present study compares the global DNA methylation level in two half-high blueberry cultivars ‘Patriot’ and ‘Chippewa’. These cultivars were grown in a greenhouse that were derived from adventitious shoots regenerated on a SSM and in a liquid medium contained in a TIB. Callus formation and shoot regeneration were obtained at the highest rate using 2.3 µM TDZ and 9.2 µM ZEA, respectively. Although highest percentage of callus formation was obtained on TDZ, the rate of shoot regeneration decreased with increasing concentration of this PGR. Theoretically, all plant cells are totipotent, and each cell can regenerate into a full-grown plantlet. However, to attain dedifferentiation, redifferentiation and competency, PGRs plays an important role (Murthy et al. 1998). TDZ imitates the effect of auxin and cytokinin both during the in vitro culture process and promotes the differentiation process individually or in association with other PGRs (Murthy et al. 1998; Ghosh et al. 2014). TDZ has been used previously in many woody species to promote callus formation such as grapes, blueberries, lingonberries. In four lowbush and hybrid blueberry genotypes, up to 98% callus formation was observed from young leaf explants after 12 weeks of culture in a blueberry culture media fortified with 2.3 – 4.6 µM TDZ (Ghosh et al. 2017). A similar phenomenon was observed in shoot-tip cultures of two lingonberry clones, with the increasing concentration of TDZ in the media (0.1 - 5.0 µM), callus size increased from 1.2 – 7.4 (vertically) and 1.6 – 7.7 (horizontally) when measured in a scale of 0 – 8 (Debnath 2005a). After 5 – 6 weeks of

culture of lingonberry cultivar Erntedank in TDZ fortified media, the highest level of callus formation (78%) was noticed in 5 µM TDZ (Debnath 2005b). In two lowbush blueberry clones, ‘QB1’ and ‘QB2’ up to 79% bud regeneration were observed from the base of the leaf explant in the medium supplemented with 1.2 – 2.3 µM TDZ (Debnath 2011). In Kyoho grapes maximum callus growth was observed in the medium with 100 µM TDZ after 12 days of culture (Lin et al. 1988).

Among all the different concentrations of ZEA and TDZ, at 9.2 µM ZEA highest shoot regeneration was noticed in both the cultivars. Two lowbush blueberry cultivars when cultured in vitro displayed 35 – 50% more shoot regeneration in media supplemented with 1.0 – 4.0 µM ZEA (Debnath 2009). Similarly, in two blueberry cultivars ‘Bluejay’ and ‘Pink lemonade’, the best shoot regeneration was obtained in 13.68 and 9.12 µM ZEA supplemented Anderson media respectively (Fan et al. 2017). Debnath (2017) found basal medium supplemented with 4.6 µM ZEA to be optimum for maintenance and elongation of shoot culture in highbush, half-high and hybrid blueberry in vitro cultures. However, our results show that with the linear increments of TDZ concentration, the BM in vitro cultures started showing less regeneration, unlike for the ZEA concentrations. Shoot regeneration increased up to 2.3 µM TDZ and then gradually decreased when TDZ concentration was increased to 9.2 µM for both the cultivars. A similar trend was observed in two lingonberry clones, where the number of shoots per explant decreased when TDZ concentration exceeded 1.0 µM, which was eventually overcome with the application of ZEA in the medium (Debnath 2005a). In another case of lingonberry in vitro shoot-tip culture, shoot regeneration was inhibited when TDZ

concentration increased from 0.1 – 10.0 µM (Debnath 2005b). Inhibition of shoot elongation was previously reported in several studies (Huetteman & Preece 1993) which was hypothesized to be due to high cytokinin activity of TDZ (Huetteman & Preece 1993).

From our results, it was clearly visible that shoot elongation was dependent on the culture media type. However, for most of the treatments, SSM produced a greater number of shoots per explant, shoot height, number of leaves and shoot vigour were similar in both the culture systems. Although there are previous reports available in highbush, half-high and hybrid blueberries where shoots elongated in TIB systems were detected with high vigour (Debnath 2017). Axillary shoots regenerated from both media types did not show any sign of hyperhydricity regardless of PGR concentrations with either of the cultivars. The effect of genotype on shoot elongation was not prominent from our results. Although plantlets regenerated from liquid media are more prone to hyperhydricity, TIB systems are found to be more able to prevent this problem from the regenerants (Preece 2010). It was observed that axillary shoot elongation started with the adding of ZEA in the media up to a certain concentration (Debnath 2017). This further supports the fact that cytokinin promotes apical dominance, thus accelerating the rate of shoot multiplication (Huetteman & Preece 1993). Our results agree with the previous studies that ZEA is the cytokinin most suited for blueberry shoot elongation (Debnath 2005a, 2005b; Debnath 2009; Debnath 2017; Fan et al. 2017). Debnath & McRae (2001) found ZEA extremely effective for shoot elongation in lingonberry. In the current study, micropropagation using TDZ was not successful in either SSM or TIB. Inhibition of shoot elongation has been

overcome after culturing the regenerated shoots in BM supplemented with ZEA for four weeks. Similarly, Debnath et al. (2005a) reported TDZ induced inhibition during shoot elongation of lingonberry was altered by transferring them in a medium containing 1.0 µM ZEA.

In eukaryotic plants, DNA methylation is associated with various important cellular processes such as genomic imprinting, gene regulation, chromatin activation, and cell differentiation (Park et al. 2009). Global analysis of two half-high blueberry methylome was carried out using the MSAP technique. This procedure allows for the identification of altered DNA methylation level only within the CCGG tetranucleotide sequences. However, CG, CHG and CHH context can only be determined within the CCGG sites not at the whole-genome level. During the developmental process, it was found that changes in methylation pattern. It is due to the failure of maintenance of methylation status during DNA replication as a result of to *de novo* methylation and passive demethylation (Hsieh 1999). Although there are previous reports on tissue culture-induced DNA methylation in blueberry (Ghosh et al. 2017; Goyali et al. 2018), effects of culture type on tissue culture-induced DNA methylation has not been previously reported in small fruit crop. Our results mainly address the relationship between DNA methylation and culture medium type. Among the two cultivars, the highest level of DNA methylation measured by methylated cytosine in the CCGG sites was found in ‘Patriot’ cultured in TIB compared to SSM. Plants cultured in TIB also showed a higher level of methylation polymorphism in case of both the cultivars. The total percentage of methylated bands for both the cultivars was much higher in TIB in comparison to the SSM system. During the

micropropagation of lowbush and hybrid blueberry leaves, methylated CCGG sites were present in callus (215 – 258) in a higher number in comparison to the leaves where methylated CCGG sites were found in relatively low numbers (75 – 100) as detected by MSAP technique (Ghosh et al. 2017). In the same study, in vitro grown callus was highly polymorphic: 19.24% bands were reported to be polymorphic, while in leaves 10% of the bands were polymorphic (Ghosh et al. 2017). Another study on lowbush blueberry clones 'QB9C' and 'Fundy' showed the presence of higher-level DNA methylation in micropropagated plants in contrast to plants grown by stem cutting in the greenhouse. Micropropagated 'QB9C' and 'Fundy' showed 29% and 20% methylated CCGG sites respectively while stem cutting plants showed 25 ('QB9C') – 19% ('Fundy') methylated tetranucleotide sites (Goyali et al. 2018). From the results it was clear that in the above-mentioned study in vitro regenerated plants showed higher level of methylation alteration. In *Doritaneopsis* orchid, plantlets regenerated from shoot tip culture showed up to 22.9% methylation alteration leading to phenotypic variants (Park et al. 2009). Peraza-Echeverria et al. (2001) observed in in vitro propagated banana cultivar 'Grand Naine' displayed 107 methylated bands (23%) among 465 total amplified bands; on the contrarily 84 bands were methylated of total 456 bands (18.4%) in conventionally grown plants. Although there are various reports available on tissue culture-induced DNA methylation, the effect of liquid medium on global DNA methylation has not been widely reported, due to which the underlying mechanism behind the higher rate of methylation in TIB systems is far from being completely understood. However, there are reports of altered methylation pattern of oil palm regenerants from long term embryogenic suspension culture, where the global methylation rate varied from 14.85 – 25.23% (Rival

et al. 2013). The origin of methylation in this case could not be specified, if the medium type and/or duration of culture came into play to cause the change in the methylation pattern.

In the present study, not only the culture type but also externally added ZEA into the media played an important role in changed methylation pattern among the regenerants. Manipulation in the BM may also induce an altered methylation pattern. Previously it was observed that at the callus formation stage explants go through a dynamic change and cytokinins are found to be effective for genome modification during callusing phase (Ghosh et al. 2017). In carrot cell suspension culture, a stable DNA methylation pattern (16%) was observed upon the application of an exogenous cytokinin in the media (LoSchiavo et al. 1989). Another study with in vitro carrot culture showed higher methylation rate with the application of kinetin in the media of freshly inoculated cell culture in comparison to already established culture (Arnholdt-Schmitt et al. 1995). Application of another cytokinin TDZ, at a concentration of 4.5 µM in basal media gave rise to the highest number (upto 94 in 'CL3') of amplified methylated bands which were also highly polymorphic (up to 27.7%) in lowbush blueberry clones and hybrid regenerants from leaf explants (Ghosh et al. 2017).

5.5 Conclusion

In this study, I have provided evidence of the role of culture systems on global DNA methylation during micropropagation of half-high blueberry. The MSAP technique

proved to be effective to detect the type of methylation, altered methylation pattern and methylation polymorphism among the regenerants. As a result of this emerging information, they can be used as a solid foundation for further characterization and identification of the genes involved in the underlying regulatory mechanisms during the in vitro culture of blueberry.

5.6 References

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Chapter 6 - Summary

Blueberries (*Vaccinium* spp.) are of high commercial importance and are known as a "super-fruit" mainly because they have high phenolic content and in vitro antioxidant properties (Kalt et al., 2020). During the past two decades research associated with food and healthcare has unveiled that blueberries are beneficial to health in various ways. As a result of this emerging information, they are in high demand among the consumers. Blueberries are primarily commercially cultivated by conventional propagation methods in farms and also harvested from wild patches. However, propagation via conventional systems fails to meet the market demand for blueberries. Micropropagation techniques for improvement and commercial production of blueberry plants is currently being used as an alternative method to satisfy the market demand. However, our current understanding of the blueberry micropropagation is limited to organogenesis (adventitious shoot regeneration and shoot elongation) (Chen et al. 2018; Debnath 2007, 2009a, 2009b, 2011; Fan et al. 2017; Frett & Smagula 1983; Liu et al. 2010).

I have developed a protocol for somatic embryogenesis (SE) in half-high blueberry plants to obtain shoot and root meristem simultaneously, for the first time, to my knowledge. The cultivars used for this study were 'St. Cloud', 'Patriot', 'Northblue', and 'Chippewa'. I have used various concentrations of thidiazuron (TDZ) in basal medium (BM) for the purpose of developing somatic embryos and the highest percentage (99%) of somatic embryos were formed at 9 µM TDZ supplemented media. Among all the four cultivars, percent germination was highest in 'St. Cloud' (69%). After data processing using statistical tools it was clear that with the increasing concentrations of

TDZ in the medium, the number of embryos per explant and percentage embryo formation also increases in all four cultivars. After somatic embryo formation they were transferred to BM supplemented with five different concentrations of gibberellic acid (GA_3), indole-3-butyric acid (IBA), ZEA, or TDZ for maturation. Unlike embryo formation, in the case of somatic embryo maturation, the highest percentage of maturation was noticed in low concentration of TDZ (2.3 µM) followed by 4.6 µM ZEA. Mature somatic embryos were then transferred to a peat: perlite medium for acclimatization. 100% survival rate of the SE regenerated plants were achieved following greenhouse acclimatization.

After conducting the SE experiments the next step was to investigate the effect of the SE process, effect of TDZ, and physiological age of plants on antioxidant properties of the regenerated plants. It is well known that somatic cells undergoing SE go through various morphological and biochemical changes, which made me hypothesise that SE brings changes in the antioxidant properties of the tissue culture regenerants. For this experiment, leaves of eight-week-old greenhouse-grown SE regenerated plants and more than 15-year-old four donor cultivars were collected, and crude polyphenolic contents were extracted. Determination of total phenolic content (TPC) was determined using the Folin-Ciocalteu method (Singleton & Rossi, 1965), following Goyali et al. (Goyali et al., 2015), total flavonoid content (TFC) was determined according to colorimetric method (Zhishen et al., 1999) following Goyali et al. (Goyali et al., 2015) and antioxidant activity (AA) using DPPH (Goyali et al., 2015) was determined. It was found that overall, SE regenerated plants have higher content of phenolics in comparison to the donor plants

except in ‘St. Cloud’ and ‘Northblue’. In case of TFC it was seen that unlike TPC, donor plants had higher TFC than the tissue culture plants in all cultivars except ‘St. Cloud’. However, all cultivars exhibited higher AA in greenhouse grown donor plants in than those of their respective SE counterparts. These results further reconfirm the hypothesis that not only the propagation method but the physiological age also had a clear effect on the AA of the four cultivars, due to the fact that all donor plants showed significantly greater levels of AA than SE plants. Furthermore, association mapping studies can be done to show how in vitro and ex vitro environments are associated with total antioxidant activity in blueberries (Ghosh et al. unpublished result).

The next step of this research was to investigate the effect of TDZ on shoot regeneration and the effect of tissue culture process on DNA methylation status of one hybrid (‘H1’) and three lowbush blueberry clones (‘CL1’, ‘CL2’, and ‘CL3’). Prior research done in our group indicated that blueberry plants regenerated via tissue culture process showed significant morphological differences compared to the donor plants grown in greenhouse (Goyali et al. 2013). Further studies on this topic with molecular markers showed no difference at the genetic level of tissue culture and conventionally propagated donor plants (Goyali et al. 2015). This made me hypothesise that there is an involvement of epigenetic marking such as DNA methylation during the process of in vitro propagation which causes morphological changes. In this experiment the highest percentage of callus formation (‘H1’ – 98%) was observed in BM containing 4.5 µM TDZ. However, in lowbush blueberry clones the highest percentage of callus formation was observed at 2.3 µM TDZ. In this experiment, the MSAP technique was used to study

the altered methylation profile of blueberry plants and compared to the methylation pattern of the callus stage with their donor plants from the greenhouse. A total of 949 methylated bands were detected in the callus across all the genotypes among which 144 (15.17%) were polymorphic. The total number of methylated bands (360) and number of polymorphic bands 37 (10.27%) were comparatively less in donor plants. It was also observed that at the moderate concentration of TDZ (2.3 µM), all four genotypes displayed the highest percentage of polymorphism. Further analysis showed that methylation patterns were different not only among the tissues (callus and leaf) but also due to the various concentrations of TDZ. These results support the hypothesis that tissue culture process affects the methylation pattern.

The next step was to find out the effect of culture media type on global DNA methylation. From the earlier study it was already clear that various concentrations of PGRs and tissue culture processes have effects on altering DNA methylation patterns. This led to the idea that type of culture media may also have differential effect on DNA methylation during in vitro culture process. For this experiment, I have used TDZ and ZEA for shoot regeneration of two half-high blueberry cultivars ‘Patriot’ and ‘Chippewa’ in semi-solid medium (SSM) and then ZEA supplemented SSM and liquid medium in temporary immersion bioreactor (TIB) system for shoot elongation. The highest percentage of callus formation was observed at 2.3 µM TDZ (91 ± 0.6 % - 93 ± 0.9 %) irrespective of culture media type in both cultivars. However, during shoot elongation the highest levels of shoot formation was at 9.2 µM ZEA, the highest shoot height and number of leaves per shoot at 2.3 µM ZEA, and shoot vigour was best at 2.3 – 4.6 µM

ZEA irrespective of culture type and cultivar. After the acclimatization process 80 – 90% of the plantlets from SSM system survived while 70 – 80% of the plantlets survived from the TIB system. MSAP analyses were done with the leaves collected from the hardened off plants regenerated from both the systems. The methylation pattern of plantlets revealed that 1556 total fragments were detected in SSM, out of which 455 (225) were methylated and 2% (38) were polymorphic while in the TIB system, out of 1728 total bands 824 (32%) were methylated and 3% (83) were polymorphic in nature. Further analysis demonstrated that altered methylation patterns exist even within the same genotype depending on the culture system they regenerated from.

Global DNA methylation analysis using the MSAP technique gave the overall picture of the differential DNA methylation profile and showed the effects of various in vitro propagation techniques and their components on altered methylation pattern in in vitro and ex vitro grown plants. In plants, the level of DNA methylation is also related to gene regulation, and this epigenetic mechanism is connected to growth and development including in vitro processes such as organogenesis and SE in an incredibly complex manner. It is important to further explore the epigenomes of blueberry plants to see if within the epigenome of regenerated plants, an epigenetic footprint was left due to different culture environments. This can be done by identifying the genes subjected to differential methylation, identifying the regions of differential methylation, and by identifying the hypo/hypermethylated gene sets involved in various biological functions and molecular functions under in vitro and ex vitro conditions using advanced

methylation detection techniques such as whole-genome bisulfite modification (WGBS) (Ghosh et al. unpublished result).

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Appendix 1 Supplementary Tables for Chapter 4 and Chapter 5

Table S1: List of different adapters and primers used in methylation-sensitive amplification polymorphism analysis

Adapter/primer		**Nucleotide sequences**
EcoRI adapter (*E + 0)*		5′-CTC GTAGACTGCGTACC-3′
		3′-CATCTGACGCATGGTTAA-5′
MspI-HpaII adapters *(MH + 0)*		5′-GA TCATGAGTCCTGCT-3′
		3′-AGTACTCAGGACGAGC-5′
Pre-amplification primers		
EcoRI		5′-GACTGCGTACCAATTCA-3′
MspI-HpaII		5′-ATCATGAGTCCTGCTCGG-3′
Selective amplification primers		
E + 2	TT	5′-GACTGCGTACCAATTCATT-3′
	TG	5′-GACTGCGTACCAATTCATG-3′
MH + 3	AAC	5′-ATCATGAGTCCTGCTCGGAAC-3′
	ATG	5′-ATCATGAGTCCTGCTCGGATG-3′
	AAG	5′-ATCATGAGTCCTGCTCGGAAG-3′
	ACA	5′-ATCATGAGTCCTGCTCGGACA-3′