Epigenetic variation in lingonberries

By

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Abstract

Epigenetic variation plays a role in developmental gene regulation, response to the environment, and in natural variation of gene expression levels. The purpose of the study is to investigate cytosine methylation and secondary compounds of lingonberry (*Vaccinium vitis-idaea*) among cutting-propagated cultivar Erntedank (ED) and its tissue-culture plants (NC, LC). This was analyzed by using Methylation Sensitive Amplified Polymorphism (MSAP) where the primers were cleaved in cytosine residues at 5'-CCGG-3' sites in CpG-islands. In leaf regenerants (LC1), we observed highest methylated sites from all primer combinations (108 bands), with their highest variation in secondary metabolites. We measured that tissue-cultured plants showed higher methylation bands than maternal plants. For instance, we identified the mother plant ED exhibited 79 bands of methylation, which is comparatively low. On the other hand, we observed the highest total phenolic content in (NC3) but LC1 represents low phenolic content. Our study showed more methylation in micropropagated plants (NC1, NC2, NC3 and LC1) than those derived from ED cutting cultivar where methylation was not present. On the contrary, we observed higher secondary metabolites in cutting cultivar ED but comparatively less in micropropagated plants (NC1, NC2, NC3 LC1). Hence, our study confirmed that higher methylation sites observed in micropropagated plants and less amount of secondary metabolites appears.

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Chapter 1 : Introduction and Review of Literature

1.1 Lingonberry: taxonomy, geographical distribution, and importance on human health

Lingonberry (*Vaccinium vitis-idaea* L.), a small perennial shrub, belongs to the genus *Vaccinium* L. of the Ericaceae family (subfamily: Vaccinioideae), which contains about 4250 species in 124 genera (Christenhusz and Byng 2016). The plant is dwarf and rhizomatous and has a tap root system with rootlets. In lingonberry, flowers are small and light pinkish, with the inferior ovary producing dark red, globose berries. Flowering occurs at the beginning of June, and fruit develops at the end of August or September. Mature fruit is acidic (pH of 2.5), and it contains tannins [7–21 mg g^{-1} flw (fresh leaf weight)], anthocyanins (1–27 mg g^{-1} flw), and 6% total sugars content (Hall I.V. and Ludwig R.A. 1961). The entire rhizomes makes up 80% of the whole plant biomass (Holloway 1983). This evergreen perennial shrub spares from arctic to north temperate regions in Eurasia to North America. Lingonberry plants are categorized into two subspecies: ssp. *vitis-idaea* L. and ssp. *minus* (Lodd) (Arigundam et al. 2020b); Fig. 1.1. The main differences between the two subspecies of lingonberry are based on plant size; ssp. *minus* found in remote arctic, is considerably shorter in height (up to 20 cm) and leaf size (length: 1.0 cm; breadth: 0.5 cm); whereas ssp. *vitis-idaea* found in extensive lowland of Eurasia, is more prolonged in height (exceeds 30 cm) and leaf size (length: 2.5 cm; breadth: 1.0 cm). Other than that, fruiting in ssp. *minus* occurs once a year, whereas it occurs twice a year in ssp. *vitis-idaea* (Andersen 1985;

Arigundam et al. 2020b).

Figure 1-1 Different species of Lingonberry grown in greenhouse: a. *Vaccinium vitis- idaea* spp. *minus*, b. *V. vitis-idaea* spp. *vitis-idaea* (Samir C. Debnath and Arigundam 2020)

Lingonberry has been recognized by different names in different places: lingberry, lingen, lingonberry (Newfoundland), dry-ground cranberry, foxberry, grains-rouges (Quebec); lingon, and puolukka (Finland); alpine cranberry, airelle rouge (France); mirtillo rosso (Italy); moss cranberry, mountain cranberry, and partridgeberry (elsewhere in Canada); preiselbeern, shore cranberry, and tytlebaer (Germany); berris and cowberry (England); linberry, lowbush cranberry, red whortleberry, and rock cranberry (Alaska); and Kokemomo (Japan). It is used for the production of jams, jellies and candies (Pärnänen et al. 2020). It is widely spread through Greenland, Iceland, North America, Scandinavia, Northern Asia, Europe, Asia (Samir C. Debnath 2005a) Fig.1-2.

Figure 1-2 Global distribution of lingonberry

1.2 Propagation

Conventionally, lingonberries are able to replicate by seed or rhizomes. For vegetative propagation, stem cutting and rhizome are used (Samir C. Debnath and Goyali 2020a). It requires 3 to 5 cm of a plant sample that regenerates into a new independent plant, which may not be of the same type as their mother plant. On the other hand, the horticulture industry has crucial goals for the betterment of production and the increase of the fresh local market. This method of propagation is highly laborious. In contrast, plant tissue culture is the substitute way of rapid propagation at a vast scale (Samir C. Debnath and Goyali 2020a). Generally, the growing and multiplication of plants are based on solid or liquid media under an aseptic regulated environment known as tissue culture (Figure 1-1). Plant tissue culture helps in the production of the pathogen-free plant. Plant growth regulators (PGRs) induce cell development, quick duplication, valuable metabolites of lingonberry, and it also helps in germplasm preservation (Samir C. Debnath and Arigundam 2020).

1.3 Micropropagation and their importance

Micropropagation is a quick and more efficient method to propagate lingonberries in masses that makes it possible to be done all the year through axillary bud proliferation and differentiation to mature plants formed from meristematic tissues to fully grown plants (Samir C. Debnath and Arigundam 2020). This process is called micropropagation or *in vitro* propagation. Under the aseptic condition, all those media components were accumulated together to provide better explant growth (Arigundam et al. 2020b). Haberlandt (1902) first reported plant tissue culture from bract cells of red dead-nettle (*Lamium purpureum* L.), cultured with Knop's solution supplemented with 1 or 5% (w/v) sucrose (Preil 2005) in the early 19th century. Those cells were still alive for a month, without cell division. In the meantime, Skoog and Miller determined the role of an auxin-cytokinin hormone in plant morphogenesis in 1957 (Samir C. Debnath and Arigundam 2020). They used a different ratio of auxin/cytokinin to observe the phenotypic expression of roots and shoots in tobacco pith tissue culture. They found high cytokinin/auxin ratio provoked shoot initiation and high auxin/cytokinin ratios stimulate root formation. On the other hand, if the auxin/cytokinin ratios are equal, it leads to irregular tissue formation. Using the different macro and micronutrients and organic ingredients, Murashige & Skoog developed the tobacco plant's medium of tissue culture (D. R. Davies 1980). This incidence of micropropagation has happened in three ways: shoot proliferation, shoot regeneration, and somatic embryogenesis (Arigundam et al. 2020b).

1.3.1 Shoot proliferation

Shoot proliferation occurs from the nodal explants. This method is considered micropropagation because it does not produce callus. The explant has been arising from the apical or lateral short shoot tip, which contains many condensed axillary buds. This method is spontaneous until the initial explant transforms into a bunch of branches. When the shoots were transferred in a fresh medium, the multiplication cycle occurs repeatedly. In-vitro shoot proliferation was initiated from shoot tip and node explants on modified Murashige and Skoog (MS) medium. Zeatin acts as a plant growth regulator, which was more effective than 2iP [N6-(2-isopentenyl) adenine] (Arigundam et al. 2020b; Samir C. Debnath and McRae 2001). It was proven that zeatin induced proliferation of 2 to 3 times better shoots in 'Regal' as 2iP, out of four media was tested for shoot proliferation, whereas modified MS medium was more effective than the woody plant medium for shoot multiplication. For acclimatization, developed young shoots were rooted ex vitro directly on a 2 peat: 1 perlite (v/v) medium after dipping in 39.4 mM indole-3-butyric acid (Samir C. Debnath 2005a).

1.3.2 Shoot regeneration

Due to some limitations in the traditional vegetative propagation methods, botanists came up with newer approaches to supply the larger quantities of genetically superior individuals (Samir C. Debnath and Mcrae 2002). Adventitious shoot regeneration from leaf explants has been reported in *Vaccinium* species, including *V. vitis-idaea* ssp. *vitis-idaea* (Samir C. Debnath and McRae 2001; Samir C. Debnath and Mcrae 2002; Foley and Debnath 2007a) but not in *V. vitis-idaea* ssp. *minus* subspecies. Gradually, the leaf explants were cultured on semi-solid gelled media to form the shoot

regeneration method. Both zeatin (9.1 μM) and thidiazuron (TDZ, 1.8 μM) promoted adventitious shoots proliferation from leaf explants. Nodal segments of regenerated shoots were measured by stationary bioreactor (SB: shoot proliferation occurs in semi-solid medium) and temporary immersion bioreactor (TIB: shoot proliferation occurs in liquid medium). After eight weeks, the culture showed 2–3 times more shoot multiplication in a liquid medium than on a semi-solid medium (Arigundam et al. 2020a).

In lingonberry, studies have proven that the presence of indole-3-butyric acid (IBA) is helpful for shoot formation culture (Samir C. Debnath and Mcrae 2002). Tissue culture plants were produced with several stems and rhizomes *in-vitro* during the first three seasons. Therefore, it provided a larger framework for shoot production during the experiment. As well the *in-vitro* cytokinin treatments were used to induce shoot regeneration and proliferation as reported earlier for *Vaccinium* species (Labokas J. and Budriuniene D. 1988). As a result, *in-vitro* methods produce more vigourous plants from the traditional cutting plant (D. R. Davies 1980). It is possible that supernumerary branching and rhizome formation in plants resulting from *in-vitro* culture could be a symptom for their regeneration. Plants derived from reversion from mature to juvenile characteristics may be a general phenomenon in plant tissue culture (Samir C. Debnath and Mcrae 2002). Cultured micro-shoots are survive more than eight weeks on 1 or 2 M zeatin-containing BM (basal medium). This process is a great way to produce *in-vitro* roots. After 5-6 weeks of BM culture with 2-5 μM TDZ, some of these buds were transformed into shoots (S C Debnath, Bishop, and Sion 2010). Shoot regeneration through adventitious organogenesis has been a commonly used method under micropropagation to scale up the commercial yield of several *Vaccinium* species. A shoot regeneration system can also be used to produce novel somaclones and to create transgenic plants through transformed technology (Samir C. Debnath 2018). Plant growth regulators

including zeatin and (TDZ), along with physical, chemical, and biological factors, can affect organogenesis process. A shoot regeneration can help in the development of novel somaclones among lingonberry and create transgenic plants through recombinant DNA technology (Samir C. Debnath and McRae 2001). Although there are many advantages of the shoot regeneration method, the development of regenerants for different genotypes in lingonberry *minus* subspecies is still a challenging process. On the other hand, a liquid culture system is a critical step to enhance the multiplications rates of shoots that were produced *in-vitro* (Preil 2005). Bioreactors having automated control for the physical and chemical environments of the culture system (Arigundam et al. 2020b).

1.3.3 Somatic embryogenesis

In-vitro cultured plants often show a phenotypic variation known as somaclonal variation (Amrita Ghosh, Igamberdiev, and Debnath 2018; Larkin and Scowcroft 1981a; 1981b). Lingonberry plants can be propagated traditionally by stem-cutting; it is a labour-intensive and time-consuming process. On the other hand, somatic cells undergo certain morphological and biochemical transformations to produce somatic embryos (Zimmerman 1993). These include globular, oblong, heart, enlarge, torpedo, and cotyledonal-shaped structure formation (Zimmerman 1993). The steps are as follows: initiation, proliferation, maturation, and plantlet formation (Baubec et al. 2010; von Arnold et al. 2002). The somatic embryogenesis (SE) is a reknowned tool for powerful clonal propagation, and it has been explored in several important crop species (Zimmerman 1993). The process is also suitable for genetic transformation and artificial seed production (von Arnold et al. 2002). Typically, the SE success rate depends on explant types and the culture media containing an optimum plant growth regulator (PGR) regime. SE can occur directly without the intervention

of callus or indirectly as they are also developed via the callus phase (Amrita Ghosh, Igamberdiev, and Debnath 2018; von Arnold et al. 2002). Among *Vaccinium* species, somatic embryogenesis was successful only in blueberries (Amrita Ghosh, Igamberdiev, and Debnath 2018).

1.3.4 Advantages and disadvantages of micropropagation

The main importance of micropropagation is to produce disease-free superior plants within a short range of time. Micropropagation undoubtedly an effective method compares to the conventional method (Rani and Raina 2000). For extinct and perennial plant species, micropropagation is one of the most efficient plantation methods (Samir C. Debnath 2011). As it does not depend on the season, the production can be continued over the year. If the mother plant is infected with a disease, it can generate disease and pathogen-free plants by micropropagation. Furthermore, any plant parts can be propagated during a tissue culture method. Epigenetic existence can be more visible in tissue culture plants. For example, Webster and Jones investigated that two apple plants were propagated from the same mother plant with different buds (Webster and Jones 1989). Those newly-originated plants showed different expression in rooting (Webster and Jones 1989). The same observation was seen on the four lines of *Helleborus* spp. (Smulders and de Klerk 2011). Another study reported DNA methylation patterns in the same plants with different nodes; thus, the DNA methylation patterns expressed significantly different from each other . However, micropropagated plants also increase the rate of bioactive components (Chattopadhyay et al. 2002). Here, the production of micropropagated plants is not dependent on the climate and geographical conditions. A novel by-product can be found in tissue culture events, which is not possible in the conventional method. It can hold a shorter and more flexible production cycle.

There are also some drawbacks of micropropagation. In several crops, unwanted epigenetic variation caused in tissue culture is a major drawback. It requires highly skilled laborers for maintaining this method. Micropropagation has a lot of complexity and some sophisticated facilities, which ultimately require expensive machinery and chemicals (Samir C. Debnath 2007). Regarding commercial production, genetic variation among the explants is undoubtedly a foremost abstract and worthless. Lastly, rooting of micro-cuttings *in vitro* is expensive and can even double the price of the cutting (de Klerk 1990; Zimmerman 1993).

1.4 Bioreactor on micropropagation

Functionally, plant culture bioreactors can be divided into two broad types: the submerged continuous cultures and half immersed or temporarily placed culture. Submerged bioreactors may be mechanically agitated or air-driven. Partial immersion bioreactors include gaseous phase bioreactors, liquid layer bioreactors, and temporary immersion bioreactors (Samir C. Debnath 2011) (Fig. 1-3).

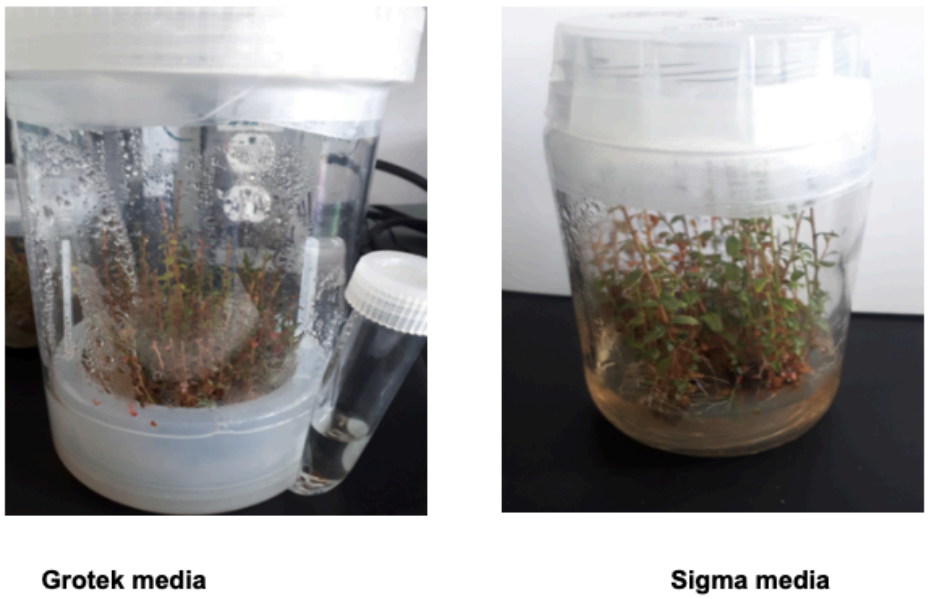

Figure 1-3 In vitro cultured lingonberry in different nutrient media

1.4.1 Types of bioreactor

There are mainly two types:

1. Temporary immersion bioreactor (TIB)
2. Stationary bioreactor (SB)

1.4.1.1 Temporary immersion bioreactor (TIB)

There are various types of TIB available in the market, where customization of the design followed the necessity of the specific plant culture sensitivity to hyperhydricity (Vinocur et al. 2000). TIB is very useful for *in vitro* propagation. It is also helpful for providing oxygen nutrients and mixing properly without contamination. These systems contain a liquid medium (Etienne and Berthouly 2002). Harris & Mason were the first ones to discover this micropropagation method, which was used for large-scale production (Etienne and Berthouly 2002). RITA† (VITROPIC, Saint-Mathieu-de-Tre´ viers, France) is a new type of TIB bioreactor that contains two compartments of cylindrical vessels of 15-cm, the top part holds the plant material, and the lower part presents the culture media. The container is set up with an automated air pump, which controls overpressure to the bottom part, pressing the medium to the top part of the container via the filter. The extra pressure was evacuated from an air vent of the lid. The air pump was regulated by a timer controlling instrument (Samir C. Debnath 2011). Arigundam et al. (2020) reported wild *V. vitis-idaea* ssp. *minus* clones produced 2-3 times more shoots than semi-solid media in the TIB.

1.4.1.2 Stationary bioreactor (SB)

Regarding low cost, SB is a potential system for *in vitro* culture of lingonberry (Arigundam et al. 2020b). This system was manufactured in such a way that the shoot proliferation rates were high.

While avoiding root injury, it helps to reduce the time for incubation and contamination. Growtek SB bioreactor was reported to produce more and vigorous shoots (1.2-23.3) (Samir C. Debnath 2011). This study reported in lingonberries where semi-solid media had produced fewer shoots than TIB or SB. SB bioreactor contains vapor/gas phase inside the vessel to grow healthier shoots (Arigundam et al. 2020b). It consists of both static and agitated conditions of liquid and semi-solid media. For the production of secondary metabolites in the hairy roots, this can be effectively used for in vitro propagation, including somatic embryogenesis (Zimmerman 1993).

1.4.2 Use of liquid media in a bioreactor system

The use of a liquid medium in bioreactor systems has a high potential for scaling-up lingonberry micropropagation, and it can also contribute towards the reduction in the cost of lingonberry propagation (Samir C. Debnath and Arigundam 2020). Improved shoot proliferation is possible using a liquid medium in bioreactors, and it allows an easy uptake of nutrients, while in a semi-solid agar medium, there is an adsorbent agent that rather complicates the nutrient movement (Samir C. Debnath and Arigundam 2020; Sandal, Bhattacharya, and Singh Ahuja 2001). Contaminations in bioreactors are low. It is possible to produce shoots in the same liquid culture container for a longer period without transferring to keep constant the container's internal atmosphere intact (J. Aitken-Christie and Jones 1987). Hyperhydricity is submerged in liquid conditions (D. P. Aitken-Christie et al. 1992). *In-vitro* TIB or SB system resulted in reduced hyperhydricity but did not completely remove it from lingonberry (Arigundam et al. 2020b). Arigundam et al (2020) found that hyperhydric lingonberry shoots, when it was transferred onto ~~a~~ peat: perlite medium, most of the shoots became normal and rooted well with 90–95% survival rate indicating that the hyperhydricity *in-vitro*-derived shoots was reversible in peat: perlite

medium and did not change the survivability of liquid culture-derived micro propagules (Arigundam et al. 2020b).

1.4.3 Advantages of bioreactor micropropagation

Bioreactor micropropagation means culture the plant with an optimal level of aeration for producing lots of shoots and rhizome (Samir C. Debnath and Goyali 2020b; Takayama and Akita 1994). For the production of better shoot growth and multiplication, processed forced aeration provides sufficient oxygen supply. It takes a shorter period of time for large scale production. It helps to eradicate apical dominance along with rapid shoot multiplication and plantlet development. Undoubtedly it reduces the labor cost. An explant can close contact with liquid media; therefore, it can easily uptake nutrients ultimately leading to rapid growth.

1.4.4 Disadvantages of bioreactor micropropagation

For an initial investment, the equipment was expensive, and its maintenance is also high. If contamination occurs to a large scale of propagules, the cost and lost time can be devastating (Leathers, Smith, and Aitken-Christie 1995). In bioreactors, embryonic propagules are more difficult to handle because the units consist of a wide variety of cell types that complicate the process's optimization level (Paek, Hahn, and Son 2001).

1.5 Phytochemicals of lingonberry

Lingonberry contains abundant secondary metabolites, including phenolic contents, flavonoids, and proanthocyanidin (Alam, Roncal, and Peña-Castillo 2018). Young leaves may contain up to 1,740 mg/kg anthocyanin (fresh weight) (Andersen 1985; Foley and Debnath 2007a) along with

58% phenolic content present in leaves, 48% in stems, and 79% in fruits (Bujor et al. 2018). Lingonberry has been found a high amount of antioxidant and antimicrobial activity in the fruit (Grace et al. 2013). In contrast, flavonoid content exists between 27%–42% in leaf tissues (Ermis et al. 2015). It has been introduced as fruit from an ancient era and a medicinal plant and used as an ornamental plant for the landscape ecology (Arigundam et al. 2020b). Furthermore, leaf and fruit parts can reduce cholesterol levels, prevent rheumatic diseases, hepatitis C, kidney, bladder infections, and have been used to treat Alzheimer's disease (Takeshita et al. 2009; Ahmed et al. 2017; Binns 1981; Meins 1983; Larkin and Scowcroft 1981a). Lingonberry fruits can be consumed raw and used to make juices, wines, pastries, sauces, jams, jellies, ice creams, cocktails and desserts (Penhallegon RH 2006).

Lingonberry has been considered as high antioxidant capacities compare to other berry fruits like blueberry, cranberry, huckleberry, soapberry (Poorva Vyas et al. 2015; Wang and Jiao 2000; Wang and Lin 2000; Wang and Stretch 2001). Some reports show that lingonberry has an anti-cancerous activity that stimulates apoptosis of human leukemia HL-60 (Bomser et al. 1995). Besides, it also has some antimicrobial effect that inhibits urinary tract infection pertaining the high arbutin levels (Poorva Vyas et al. 2015). This has been treating with sexually transmitted diseases such as gonorrhea (Dierking Jun Wilhelm, Dierking Sonja, and Dierking Beerenobst 1993). Lingonberry leaves can be used as an astringent and have diuretic activities (Samir C. Debnath and Arigundam 2020).

Antioxidant has been classified as a primary or secondary plant bioproduct. It’s a substance, which inhibits oxidation and prevents cell death. For primary antioxidants, it can react with lipid and

convert to a stable form. Primary antioxidant secretes hydrogen atom which reacts with lipids to neutralize it. Both lipid radical and hydrogen atom contain free radicals, when they lose contact with each other as they share their electrons to neutralize it. A secondary antioxidant is a compound, which acts as singlet oxygen quenchers, peroxidase decomposer metal chelators and other related enzymes (Shahidi et al. 1997; Poorva Vyas et al. 2015). Plant and antioxidant systems have defensive mechanisms to fight against the harmful effect of free radicals and plant high antioxidant activity with different kinds of enzymes and metabolites (P. Vyas, Debnath, and Igamberdiev 2013).

Phenolic components are a large group of secondary metabolites composed of an aromatic ring containing a hydroxyl group and a number of other groups (Naczk and Shahidi 2004; Poorva Vyas et al. 2015). These phenolic types are distributed in the plant as simple phenolics, phenolic acid, coumarins, flavonoids, stilbenes, proanthocyanidins, lignins, and lignans (Naczk and Shahidi 2006; 2004; Poorva Vyas et al. 2015). The phenolic compound helps regulate flavor, odour, colour, bitterness, and stability against lipid oxidation. Phenolics have two different assay types: hydroxycinnamic and hydroxybenzoic (Naczk and Shahidi 2006; Poorva Vyas et al. 2015).

Flavonoids are compounds which are responsible for pigmentation in plants; most of them were derived from benzo-gamma-pyran ring (Poorva Vyas et al. 2015; Winkel-Shirley 2002b). Flavonoids are the family of low molecular weight found everywhere in higher plants. They present every part of the plant, from roots to flowers and fruits (Poorva Vyas et al. 2015; Williams and Grayer 2004). Flavonoids are classified into fourteen groups(Havsteen 2002). Those are flavones, isoflavones, flavanones, flavanols, flavonols, flavanonols, anthocyanidin, chalcones,

xanthones (Naczk and Shahidi 2006; 2004; Shahidi et al. 1997). They have many beneficiary effects, such as pollination, plant-pathogen interaction, seed development (Winkel-Shirley 2002a; Poorva Vyas et al. 2015). But the most important property of flavonoids in plant science is antioxidants. The role of flavonoids is scavenging free radicals (Bors et al. 1990; Poorva Vyas et al. 2015) and inhibiting auto-oxidation due to metal-binding properties. They can suppress Fenton's reaction. They are involved in the metal chelation with β-ring and 4-keto and 5-hydroxy region of flavonoid (Cheng and Breen 2000).

A proanthocyanidin is a polyphenolic group of secondary metabolites, which is present in higher plants. It is classified into two groups: hydrolyzable proanthocyanidin and complex proanthocyanidin (K. Davies et al. 2018). Most of the study shows proanthocyanidin has a remarkable amount of antioxidant abilities compare to ascorbate and tocopherol (L. Shi et al. 2018). This phytochemical compound is found in seeds, leaves, fruits, and barks of many plants and provides defense for the plants. Grape seed has proanthocyanidin that helps in cardio-prevention and chemoprevention of cellular damage of humans (Bertelli and Das, n.d.; Poorva Vyas et al. 2015).

1.6 Somaclonal variation, its estimation, and its disadvantages

The termed somaclonal variation refers to the alteration of either epigenetic or genetic origin, which displays somaclones (Larkin and Scowcroft 1981b; Kaeppler, Kaeppler, and Rhee 2000a; Bairu, Aremu, and van Staden 2011; Miguel and Marum 2011). It can originate from adventitious meristems like somatic cells in callus or single-cell cultures and germ cells. The amount and type of somaclonal variation depend on genotype, explant type, plant nutrient media (growth

regulators), physical, various environmental factors, and duration of culture. First, somaclonal variations have been reported in some *Vaccinium* spp. Ploidy doubling was achieved in diploid(Norden, Lyrene, and Chaparro 2020; Yrene and Perry 1988) and tetraploid (Goldyi and Lyrene 1984) in vitro *Vaccinium* spp. Hruskoci and Read (1993) developed somaclonal cells, which were tolerant to high pH conditions with 25µM TDZ in media of *Vaccinium* spp. Unfortunately, instead of new varieties that had already been identified, those are not selected for better clones (Biswas et al. 2009; Karp 1995) because the majority of the varieties have lower quality than the mother plant. The somaclonal variation was estimated by RAPD (Random amplified polymorphic DNA) molecular marker for *in vitro* plants, where RAPD markers were an efficient tool for the early detection of somaclonal variants in tissue culture (Piccioni et al. 1997). The somaclonal variation is used to found stable genotypes that help in the trait selection for genetic engineering and propagation via somatic embryos. It allows the development of protocols that minimize variability. Thus it helps in identifying the factors that affect variability.

Disadvantages of somaclonal variation are observed on the phenotypic level. (i) The measurements made in the adult progeny of regenerated plants. Mostly, the actual measurements took longer than one year after the start of tissue culture. (ii) For many traits, the evaluation of qualitatively unusual plants depends upon vigor. (iii) Somaclonal variation has been determined on the molecular and cytological levels. Most of these determinations are laborious (González-Benito and Martín 2011). (iv) A significant correlation was unable to found because of mutations and phenotypic changes.

1.7 Epigenetic variation and application of epigenetics in crop improvement

Waddington initially coined the term 'epigenetic.' A methyl group can be briefly incorporated in the fifth position of cytosine residues where plants have three apparent phases of cytosine: CG, CHG, and CHH (where H is C, A, or T) (Gruenbaum et al. 1981). However, CG islands exist as non-methylated and CHG are widely distributed throughout the whole plant genome. Thus, the substitution of methylated DNA takes place internally or externally in the transcribed regions of transposable genes elements (Dowen et al. 2012; Meyer 2015; Secco et al. 2015). CG and CHG are regulated by DNA METHYLTRANSFERASE 1 (MET1), CHROMOMETHYLASE 3 (CMT3), and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), which help in the catalyzation of de novo DNA methylation. This mechanism is called RdDM. It was induced by DDM1 and CHROMOMETHYLASE 2 (CMT2) (Cubas, Vincent, and Coen 1999; Law and Jacobsen 2010; Kapazoglou et al. 2013; Matzke and Mosher 2014; Stroud et al. 2014; Zemach et al. 2013). In this process, all genes play a significant role in controlling phenotypic expression. *Arabidopsis* is used as a DNA methylation model with cytosine methylation depletion in MET1 (E. Jean Finnegan, Peacock, and Dennis 1996). Cytosine methylation directly involves plant development during cell initiation, cell-elongation, and early flowering (Pignatta et al. 2014; Wada et al. 2004a). Molecular biologists predicted epigenetics is not entirely dependent on DNA variation (Haig 2004). From previous studies, we observed two potentially separable fields in epigenetics: a memory of gene expression that occurs during development or by environmental conditions and transgenerational memory of gene expression (Eichten et al. 2013). Long-term memory of gene expression states that fewer changes occur among cells if the gene expression is

transferred across generations. Besides, epigenetic regulation often affects transgene behavior. Molecular biologists also used epigenetic for the development of novel epialleles.

Epigenetic variation means DNA methylation and the modifications of amino acids as well as the tail of histones in the way of mitotically and/or meiotically heritable and non-heritable alterations (Bird 2002). Few generations in plants carried out changed genetic patterns when heritable epigenetic variation occurs. Non-heritable changes have been found in the grape, established by exposure to tissue culture technology and thermotherapy. Those are reverted, and plants returned to epigenetic states similar to those of maternal plants (Baránek et al. 2015). In some cases, epigenetic factors affect phenotypic expression in the *in vitro* plant, but sometimes, the phenotypic expression is not seen in morphology while the epigenetic variation has already occurred (Miguel and Marum 2011; Meijón et al. 2009; Smulders and de Klerk 2011; Valledor et al. 2007a). Changes in the DNA methylation (or hydroxymethylation), histone modification or both are the crucial factors for epigenetic changes in *in vitro* plants (Chinnusamy and Zhu 2009).

Several changes have been occurred due to the epigenetic variation in tissue culture plants, such as vitrification (hyperhydricity), recalcitrance (absence or loss of organogenic potential) and somaclonal variation (González-Benito and Martín 2011; Li et al. 2014; Rosa, Monte-Bello, and Dornelas 2016). Generally, hyperhydric plant shows plant is typically hyperhydrated, aberrant morphology, curly and undifferentiated shoot, translucent tissues and physiological dysfunction (Vinocur et al. 2000). Some studies reported leaf shape in the begonia plant differentiated between the plants regenerated from the intermediate callus phase and the plant regenerated directly from leaf *in vitro* (Smulders and de Klerk 2011; Us-Camas et al. 2014). Additionally, there was a

discrete morphological variation in strawberry, such as hyper-flowering habit, abnormal fruit setting, and unique buds' development (stipular-buds) (Boxus 1999; Arezki et al. 2000). The epigenetic variation in oil palm (*Elaeis guineensis*) ultimately leads the fruit abortion and zero yields (Kubis et al. 2003). In other ways, decreased methylation also affects the morphological and phenotypic abnormalities in *Arabidopsis thaliana,* including modified leaf size and shape, decreased apical dominance, shape, altered flowering time, diminished fertility, and reduced plant size (E J Finnegan, Peacock, and Dennis 1996). (Park et al. 2009) reported that DNA methylation changes in somaclonal variants of *Doritaenopsis* were found where it is shown deformed flowers such as complete fusion of lateral sepals with labellum, magenta pigmentation at lateral sepals and small flowers with faintly magenta petals. Due to the stress and in vitro environment, DNA methylation has been changed with phenotypic variation.

By subculturing the plants, the plant is commonly used to keep the juvenility (Norden, Lyrene, and Chaparro 2020). Cassells and Curry (2001) showed persistent juvenility is closely related to DNA methylation (Cassells and Curry 2001). In addition, juvenile potato plants derived from tissue culture were more susceptible to light than the tuber-derived plant (Cassells A. C. 1991). According to the leaf tip and bud necrosis, some changes occurs in the morphological characteristics. DNA methylation leads to prolonged flowering, loss of apical dominance, yield quality, especially the tuber's number and size, lower leaf number, and lower leaf size, as shown in the *in vitro* regenerates potato plant (Cassells A. C. 1991). Additionally, DNA methylation is responsible for some morphological abnormalities in tissue-culture plants, where wheat plants produce inferior genotypes, including fewer, lighter kernels per spike. They yielded less than

donors, but they have higher protein grain levels than the donor plants (Qureshi, Huc1, and Kartha 1992).

Epigenetics studies have a major impact on agriculture due to the food supply and the consequences of global changes. Furthermore, it is essential to analyze the basic epigenetic mechanism in *vitro* cultured plant production. As the tissue culture plant tends to a wide range of epigenetic variation, it is possible to analyze breeding programs to establish a more diverse cultivar. Several studies reported that DNA methylation pattern stability was observed between *in-vitro* and *ex-vitro* cultivar (Us-Camas et al. 2014). In this way, we can get elite individuals without transgenic line generation (Us-Camas et al. 2014). The epigenetic variation imprints the developmental program's memory (Smulders and de Klerk 2011).

1.7.1 Estimation of epigenetic status and examples

For an appropriate assessment of the epigenetic level, the main methods are MSAP, bisulphite sequencing and chip assay. There are similarities found in the DNA sequence, but main changes occur in cytosine methylation (Jaligot, Beulé, and Rival 2002). There are various methods and protocols for DNA methylation analysis, including methylation-sensitive amplification polymorphism (MSAP), bisulphite sequencing. MSAP is the advanced form of AFLP, based on the sensitivity of restriction endonucleases to site-specific methylation (Mcclelland, Nelson1, and Raschke2 1994; Vos, Hogers, Bleeker, Reijans, van de Lee, et al. 1995). It involves digestion with methylation-sensitive restriction endonucleases proceeded by amplification of restriction fragments. This is independent of the availability of prior genome sequence information; rather, it relies on than the approximate genome size. In this technique, a high number of methylation can

be characterized with a small number of primer combinations and even detect novel methylation sequences. Thus, MSAP is a suitable technique to investigate epigenetic changes at the level of DNA methylation in lingonberry plants. The tissue culture method focuses on the genome region, and it linked the changes between dedifferentiation and differentiation. For this analysis, plant material was taken from different ages through the tissue culture process. Previous studies examined hop cultures with few molecular methods: retrotransposon microsatellite amplified polymorphism (REMAP) and random amplified DNA polymorphism (RAPD). Those techniques did not detect any proper genetic variation between the original plants and tissue-cultured plants even after 12 cycles (2 years) of micropropagation. Epigenetic changes can easily be estimated of the genetic variation with methylation-sensitive amplification polymorphism (MSAP) and bisulphite sequencing. 30% of the epigenetic variation was detected among the plants of different subcultures, and 63% of changes occur between field plants and *in-vitro* plants (Peredo, Arroyo-García, and Revilla 2009). *Vaccinium* ssp. (blueberry) reported higher methylation in tissue-cultured plants than cutting cultivar (Goyali, Igamberdiev, and Debnath 2018a). Besides, the blueberry study reported higher methylation found in callus (TDZ treatment-induced methylation rate) rather than the cutting plant (A. Ghosh, Igamberdiev, and Debnath 2017). It was reported that in the palm, high DNA methylation rates occur in the soma-clonal variant (Jaligot, Beulé, and Rival 2002; Peraza-Echeverria, Herrera-Valencia, and Kay 2001).

Bisulphite treatment of DNA changes occurs in unmethylated cytosine to uracil, but no changes occur in methylated cytosine (Gonzalgo and Jones 1997; Xiong and Laird 1997). Therefore, bisulphite is the backbone m^5C (Riess et al. 2019). Bisulphite sequencing was performed in tobacco plants to detect high levels of methylation, manifesting cell-to-cell methylation diversity in callus (Peredo, Arroyo-García, and Revilla 2009). Nowadays, DNA methylation has been

investigated with next-generation sequencing (Riess et al. 2019; Zhou et al. 2019). (Flusberg et al. 2010) stated that real-time sequencing had been used for recognizing DNA methylation single-molecule.

Additionally, chromatin immunoprecipitation (ChIP) is useful to track the 5-methylcytosine for histone modifications (Matarazzo Maria Rosaria et al. 2004; Roh et al. 2004; Y. Shi et al. 2013). Some researchers use whole-genome microarray hybridization to separate methylated from non-methylated DNA (Tanurdzic et al. 2008). Few studies have been investigated with the histone modification, small interfering RNA (siRNA), leading to the variation in epigenetic plants (Miguel and Marum 2011). In the suspension culture of *Arabidopsis thaliana*, variation in Histone H3 and H4 have been detected (Berdasco et al. 2008; Tanurdzic et al. 2008; Valledor et al. 2007b), and potato reflected the levels of siRNA (Law and Jacobsen 2010). Similarly, the levels of acetylated H3 and modification of Lys9-methylated H3 in protoplast culture of *Nicotiana tabacum* were increased (Ranawaka et al. 2020). However, covalent histone modification was transcribed randomly at start sites (TSs), and gene bodies have been regulated by gene expression (Lee and Seo 2018; Pikaard and Scheid 2014). Global histone modification is identified during callus formation. H3 and H4 acetylation, H3K4me3, H3K36me3, and H2Aub are the active epigenetic marks that increased in callus tissues compared with differentiated somatic cells (Alatzas and Foundouli 2006; Lee and Seo 2018). In eukaryotes, the cellular programming is being deposed of different kinds of histone gradients such as H1A, H1B, H2A.Z, H3.2, and H3.3 (Alatzas and Foundouli 2006; Lee and Seo 2018; Jullien et al. 2012). *Arabidopsis thaliana* is used as a model plant to detect DNA methylation and histone modification (Berdasco et al. 2008; Tanurdzic et al. 2008).

1.8 Purpose of the Study

i) To identify the efficient tissue culture derived health promoting plant under micropropagation.

ii) To determine the phenotypic and phytochemical changes among greenhouse-grown propagated plants and cutting cultivar in lingonberry.

Chapter 2 : Materials and Methods

2.1 Plant material and shoot proliferation in vitro on a semi-solid medium and in a bioreactor containing liquid medium

In vitro-grown shoots and greenhouse-grown tissue culture and cutting propagated plants of lingonberry cultivar Erntedank (ED) were used for this study (Table 2-1). Node culture-derived shoots were established in vitro following the protocol of (Samir C. Debnath 2005a). Shoots proliferated from nodal explants were divided into three-node stem sections and cultured on a semi-solid medium in 175-ml jars (Sigma Chemical co., St. Louis, USA) containing 35 ml (Samir C. Debnath and McRae 2001) nutrient medium D, which contains 25 g L^{-1} sucrose, 3.5 g L^{-1} agar, 1.25 g L^{-1} Gelrite (Sigma Chemical Co.) and 1 µM zeatin. Another culture was set in Growtek stationary bioreactors (Growtek^{tm} culture vessels, Fischer Scientific, Ottawa, Ontario, Canada) using 200 ml of the same medium but without agar and gel red (liquid medium) (Arigundam et al. 2020b). The experiment was replicated three times. There were five explants on a semi-solid medium and eight in the liquid medium. Proliferated shoots were sub-cultured every 8-weeks in a fresh medium following the protocol of (Samir C. Debnath 2005a; 2005b). The culture conditions of the in vitro grown shoots have been listed in Table 2-1.

2.2 Evaluation of tissue culture derived and cutting propagated plants under greenhouse condition

Node (NC3) and leaf culture-derived shoots (LC1) along with terminal cutting cultivars of ED lingonberry were established in the greenhouse following the protocol from (Table 2-1) (Samir C. Debnath 2005a). Briefly, in node and leaf-culture derived tissue culture shoots and cutting cultivars

of Erntedank were treated with indole 3-butyric acid (IBA, 39.4mM) and transferred to 45 cell plug trays containing peat-perlite (v/v) and maintained in 95% humidity at 22 ± 2 °C, 16-hour photoperiod 55 µmol $m^{-2} s^{-1}$ for rooting. After six weeks, the survived plants were transferred to the greenhouse and grown followingly a previous study protocol (S. C. Debnath et al. 2012). There were 5 plants in each treatment and the experiment was replicated five times.

Table 2-1 Growing conditions of in vitro culture and of the greenhouse-grown lingonberry plants for cultivar Erntedank*.

Material	Growth condition	Container	Medium	PPFD	Photoperiod
Shoot culture from nodal explant (NC1)	In vitro	Growtek jar	Liquid	30 µmol $m^{-2} s^{-1}$	16 hours cool white fluorescent lamp
Shoot culture from nodal explant (NC2)	In vitro	Sigma bottle	Semi solid	30 µmol $m^{-2} s^{-1}$	16 hours cool white fluorescent lamp
Node culture-derived tissue culture plants (NC3)	Greenhouse	10.5x10.5x1 2.5 cm^3 plastic pot	3:1 peat-perlite (v/v)	maximum 90 µmol $m^{-2} s^{-1}$	Natural light source
Leaf culture-derived tissue culture plants (LC1)	Greenhouse	10.5x10.5x1 2.5 cm^3 plastic pot	3:1 peat-perlite (v/v)	maximum 90 µmol $m^{-2} s^{-1}$	Natural light source
Cutting propagated plants Erntedank (ED)	Greenhouse	10.5x10.5x1 2.5 cm^3 plastic pot	3:1 peat-perlite (v/v)	maximum 90 µmol $m^{-2} s^{-1}$	Natural light source

*All the experiments were maintained at 20 ± 2 °C. PPFD = Photosynthetic photon flux density.

2.3 Data collection:

2.3.1 Data collected from in vitro-grown shoot cultures

Morphological data were collected from three randomly selected explants from liquid and semi-solid media (Table 2-1), replicated three times. In vitro-grown shoots on a semi-solid medium and in a bioreactor containing liquid medium were used in this study. The morphological data of in vitro-grown propagated explants were collected based on the following characteristics:

i. Number of shoots per explant.
ii. Number of leaf per shoot.
iii. Length of shoots (mm).
iv. Shoots vigor (range: 1-8).

Shoots vigor was determined by visual assessment, ranging from scale 1 (very poor) to 8 (fully normal and healthy plants with large green leaves and excellent vigor). Shoot characteristics were recorded from five fully expanded growing mature shoots selected randomly from each explant in both liquid and semi-solid media.

Number of shoots per explant = Number of shoots / Number of explants

2.3.2 Data collected from greenhouse-grown plants

Morphological data were collected from the micropropagated and conventionally propagated plants in greenhouse (Arigundam et al. 2020b; Samir C. Debnath 2005a). The following morphological data were collected:

i. Number of shoots per plant.
ii. Number of rhizome per plant.

iii. Number of rhizome branches per plant.

iv. Number of leaves per shoot.

v. Length of shoots (cm).

vi. Length of rhizomes (cm).

vii. Length of leaf size (mm).

viii. Breadth of leaf of five shoots (mm).

ix. Plant vigor (range: 1-8).

Plant vigor was determined by visual assessment, ranging from scale 1 (very poor) to 8 (fully normal and healthy plants with large green leaves and excellent vigor). Leaf characteristics were recorded from ten fully expanded mature leaves selected randomly from each plant in a pot.

Number of shoots per plant = Number of shoots / Number of plants

2.4 DNA isolation

For both DNA isolation and biochemical components analysis like phenolics, flavonoids, proanthocyanidin etc. of young leaves were plucked and immediately frozen in liquid nitrogen and stored at -80 °C. Genomic DNA was isolated from 100-145 mg of young lingonberry leaves. DNeasy Plant Mini Kits (Qiagen GMbH, Hilden, Germany) was used and followed the manufacturer's instructions with few modifications. DNA concentration ranges from 55-150 ng μL^{-1}, and the absorbance ratios A260/A280 and A260/A230 of 1.8-1.9 and 2.1-2.4 respectively.

2.5 Methylation-sensitive amplification polymorphism (MSAP) assay

MSAP assay is the modified version of the AFLP protocol (Goyali, Igamberdiev, and Debnath 2018b; A. Ghosh, Igamberdiev, and Debnath 2017). This assay was performed the experiment

three times to detect MSAP digestions; methylation-sensitive restriction enzymes (isoschizomers) EcoRI, MspI, and HpaII (Thermo Scientific) were used in this study. MspI and HpaII are the pair of isoschizomers with different sensitivity to methylation in outside or inside cytosine (Reyna-López, Simpson, and Ruiz-Herrera 1997). MspI cleaves the internal cytosine residues ($C^{m}CGG$) but not the external ($^{m}CCGG$). Whereas, HpaII is sensitive to hemi and fully methylated cytosine and only fully methylated on external cytosine (Goyali, Igamberdiev, and Debnath 2018a). Different types of selective primers and the enzyme of the isoschizomers pair helps in the detections of all restriction sites of methylation pattern (Miguel and Marum 2011). In this way, the MSAP technique has proven as an effective method for detecting methylation sites. It cleaved the DNA double helix at specific recognition site 5'-CCGG-3' when cytosine residues were methylated. Isolated DNA samples were digested for 1.5 hours at 37 °C with the restriction enzyme of 75 µL EcoRI (#FD0274, Thermo Fisher Scientific, Waltham, MA) and then, 15-minute incubation at 65 °C where EcoRI enzyme was activated. Then digested DNA was separated into three parts: MspI, HpaII, and MspI+HpaII. After that, the total volume containing 10X Fast Digest buffer was incubated for 3 hours at 37 °C and then 15 minutes at 65 °C, where digestion was carried out The digested DNA was ligated with an combination of EcoRI adapter, MspI and HpaII adapter in a 100 µL reaction containing ligase buffer, T4 DNA ligase (#EL0014, Thermo Fisher Scientific), and P.E.G. The ligation was done for 5 h at 23 °C, and then 10 minutes at 65 °C to stop the ligation. Ligated fragments were pre-amplified using pre-selective complementary primers (Table 2-2). I assessed the pre-amplified products by 1.8% agarose gel electrophoresis, where visible smear was observed from 100 to 1000 bp. Pre-amplified products were diluted five times with 0.1X T.E. buffer. Diluted pre-amplified products were performed in selective amplifications with a combination of selective primers. After that, the total number of selective primers and their

twelve combinations were used. Selective amplifications were carried out with the combinations of two EcoRI forward primers (EcoRI 1 and EcoRI 2) and six MspI-HpaII reverse primers (MH1 to MH6), selective amplification was assembled using master mix 1X PCR buffer in 25 µL final volume (Table 2-2). Selective-amplified products were visualized using 6% denaturing polyacrylamide gel electrophoresis (PAGE). The gel was run at 55 V for 3 hours and 35 minutes. The DNA fragments were stained with PAGE GelRed™ dye and visualized to detect the molecular-sized marker compared to a 50 bp ladder. The DNA fragments showed reproducible results between replicates. The mechanism of methylation-sensitive amplification polymorphism is given below in (Figure 2-1).

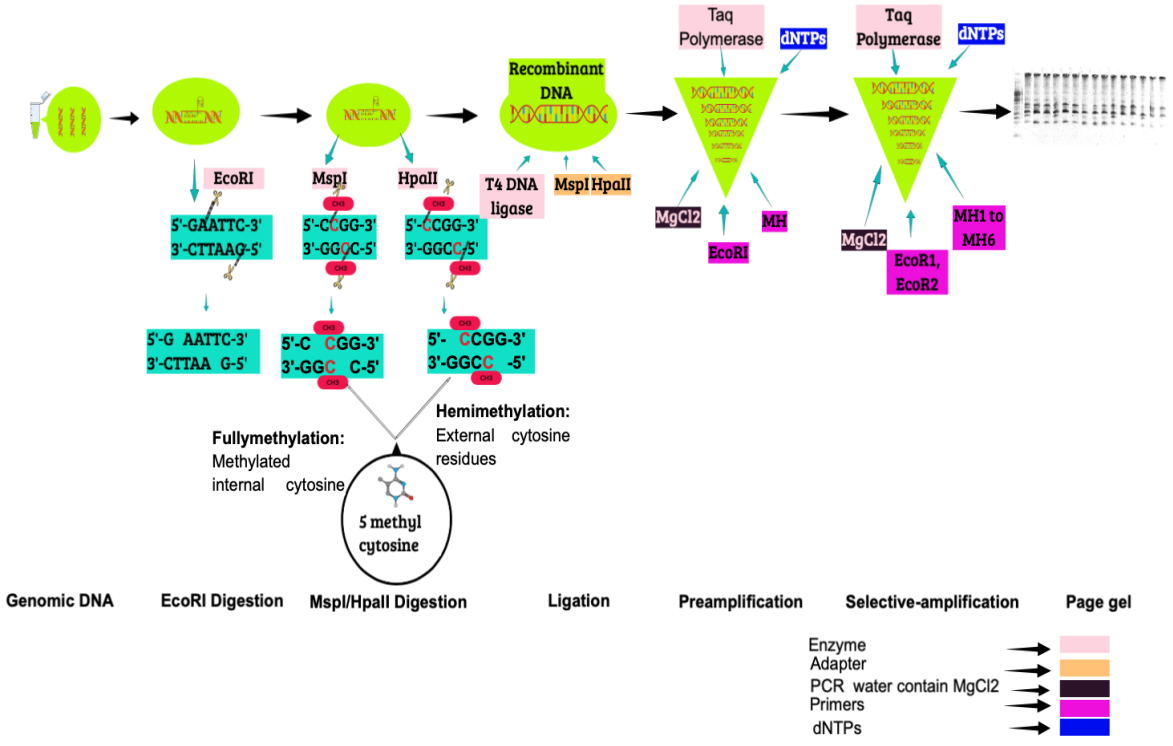

Figure 2-1 Mechanism of DNA methylation using Methylation Sensitive Amplification Polymorphism (MSAP) assay.

Table 2-2 The sequences of the adapters and primers which were used in MSAP assay:

Adapters:	
EcoRI:	5'- CT GTA GAC TGC GTA CC -3'
	3'- CA TCT GAC GCA TGG TTAA -5'
MspI-HpaII:	5'- GA TCA TGA GTC CTG CT -3'
	3'- AG TAC TCA GGA CGA GC -5'
Pre-selective primers:	
EcoRI:	5'- GAC TGC GTA CCA ATT CA -3'
MspI-HpaII (MH):	5'- ATC ATG AGT CCT GCT CGG -3'
Selective primers:	
EcoRI 1:	5'- GAC TGC GTA CCA ATT CAC G -3'
EcoRI 2:	5'- GAC TGC GTA CCA ATT CAC T -3'
MH1:	5'- ATC ATG AGT CCT GCT CGG AAT -3'
MH2:	5'- ATC ATG AGT CCT GCT CGG ACT -3'
MH3:	5'- ATC ATG AGT CCT GCT CGG TCC -3'
MH4:	5'- ATC ATG AGT CCT GCT CGG AAC C -3'
MH5:	5'- ATC ATG AGT CCT GCT CGG CGA A -3'
MH6:	5'- ATC ATG AGT CCT GCT CGG TAG C -3'

2.6 Leaf extraction for secondary metabolites

100 mg of fresh young leaves were collected from the greenhouse and growth chamber and stored at – 80 °C in liquid nitrogen. Pre-frozen extract leaves were homogenized in a homogenizer (FastPrep-24 Tissue and Cell Homogenizer M.P. Biomedicals, Irvine, CA, U.S.A.) containing 80% aqueous acetone solution and 0.2% formic acid (1:4 g/mL). Subsequently, the homogenate was kept as slow agitation at 4 °C for 30 minutes and then centrifuged at 13,000 rpm in 15 minutes at 4 °C (Allegra 64R Beckman Coulter Inc., Palo Alto, CA, U.S.A.). The final volume of the

secondary metabolic crude extract was preserved in the ultralow freezer (Thermo Scientific, Burlington, ON, CA). For further chemical analysis, three replication and mean values were used in this study.

2.7 Estimation of the total phenolic content

Total phenolic contents were measured using Folin-Ciocalteu reagent, an acidic phosphomolybdotungstate solution where oxidized phenolates blue color were formed (A. Ghosh, Igamberdiev, and Debnath 2017; Goyali, Igamberdiev, and Debnath 2013a). Diluted extract samples were treated with 100 mL of Folin-Ciocalteu reagent and 200 mL of saturated sodium carbonate and then mixed gently by adding 1.5 mL distilled water. The treated samples were kept in the dark for 35 minutes at room temperature, followed by centrifuging at 13,000 rpm for 10 minutes. The reading of absorbance was taken at 725 nm against the blank. Total phenolic content (TPC) was detected by Gallic acid equivalents mg/g fresh leaf weight. In our study, we used Gallic acid equivalents as a standard. It is comparatively a good standard because it is relatively cheap, and the pure form is stable in the dry form (Waterhouse AL 2002).

2.8 Estimation of the total flavonoid content

The flavonoid content of lingonberry samples was analyzed by the colorimetric method (Zhishen, Mengcheng, and Jianming 1999; Murakami 1970). Extracted samples and standard solution of catechin were added with 2 mL of distilled water, 150 mL of 5% (w/v) sodium nitrite and 150 microlitres of 10% (w/v) aluminum chloride. 1 mL of 1 M sodium hydroxide (NaOH) solution was added previously with aluminum chloride. Then, the mixture was diluted with 1.2 mL of dH_2O, and the absorbance of the crude mixture turns into pink color. It was measured at 510 nm

against the blank. The total flavonoid content (TFC) of leaves was expressed with as catechin equivalent (CE) as standard, and the unit is mg / g flw. Catechin has a range of 50 to 500 µm concentration, which is the standard for expressing flavonoids (Pękal and Pyrzynska 2014).

2.9 Estimation of the total antioxidant content

2,2-diphenyl-1-picrylhydrazyl (DPPH) having the scavenging effect was performed for the estimation of antioxidant activity of leaf extracts, and gallic acid equivalent (GAE) was used as a standard for the expression of the total antioxidant assay (Arab, Alemzadeh, and Maghsoudi 2011; Foley and Debnath 2007b; Amrita Ghosh, Igamberdiev, and Debnath 2018; Goyali, Igamberdiev, and Debnath 2013b; Grace et al. 2013; Yuan 2011). It is comparatively a good standard because it is relatively cheap, and the pure form is stable in the dry form (Waterhouse AL 2002). 100 mL of diluted extract solution and the standard solution was mixed gently with 1.7 mL of methanol, 0.06 mM DPPH solution, and 80% aqueous acetone as blank. Extracted leaf samples, standard GAE and blank solutions were incubated at room temperature and kept in the dark for 45 minutes; the absorbance was measured at 517 nm. Gallic acid (5 mg / mL, ≥ 98% purity) was used to measure the standard curve. Thus, the linearity of the gallic acid standard curve was carried out between 20–80 mg / mL and the results of TAC (Total antioxidant content) were expressed as GAE mg / g flw (fresh leaf weight). The scavenging activity was derived from the following formula [Foley & Debnath 2007]:

DPPH scavenging % = [(A517nm(Blank) – A517nm(Extract)) / A517nm(Blank)] × 100 [A=Absorbance]

2.10 Estimation of the total proanthocyanidin content

Leaf extract was investigated for determining proanthocyanidin content using the modified vanillin technique (Takeshita et al. 2009; Yuan 2011). 0.5 mL of diluted extracts and standard catechin equivalents (CE) was added in 0.5% vanillin-HCL reagent (2.5 mL). Then, the solutions were mixed and incubated in the dark for 20 minutes. Catechin has a range of 50 to 500 µm concentration, which is the standard for proanthocyanidin expression (Pękal and Pyrzynska 2014). Thus, the absorbance was measured at 500 nm. Total proanthocyanidin (TPrC) content was denoted by CE mg / g flw.

2.11 Statistical analysis

In the current studies, the morphological data were analyzed by using GraphPad Prism 8.0.0 software (Motulsky 2007). For statistical analysis, t and Wilcoxon test was calculated to investigate the effect of greenhouse-grown and in vitro-grown plant (Blair and Higgins 1980). All morphological data are expressed as the means ± SD of three replications to investigate number of shoots, number of leaves per shoot, length of shoots and shoot vigor (range 1-8). Statistical t and Wilcoxon test were evaluated at $\alpha = 0.05$ for all the parameters. The treatment means were compared by the least significant difference (LSD) using the t and Wilcoxon test.

MSAP assay scoring was analyzed by comparing the DNA bands of specific restriction sites (5'-CCGG-3') and the different combinations of EcoR1 + MspI, EcoR1 + HpaII, and EcoR1 + MspI + HpaII, which determines the cytosine methylation. Three replicates were performed for each experiment with the randomly designed model. The data were analyzed by using GraphPad Prism

8.0.0 software where fully-methylated, hemi-methylated, and non-methylated DNA banding patterns present and its polymorphism were visualized through Heatmap see Fig. 3-4 (Motulsky 2007; Wilkinson and Friendly 2009).

Data of secondary metabolites was performed by one way ANOVA with a standard significance threshold of $p < 0.05$ in GraphPad Prism 8.0.0. For the analysis of secondary metabolites data, means ± SD were used where two factors being studied in cultivar and propagated plants. To control the error rate while conducting the ANOVA, a Bonferroni correction was applied to correct the p-value given the number of tests presented, and only significant results are shown. The one way ANOVA compared the means between the groups. I was interested to determines whether any of those means are statistically significantly different from each other. The relationship among all the secondary metabolites was correlated by simple linear regression at a 95% confidence interval. I conducted a one way ANOVA and t and Wilcoxon test between cytosine methylation and secondary metabolites to test the hypothesis that cytosine methylation positively or negatively influenced the secondary metabolites of leaves collected in the in vitro and greenhouse with a standard significance threshold of $p < 0.05$ and $\alpha = 0.05$.

Chapter 3 : Results

3.1 Morphological pattern of shoot proliferation in in vitro on a semi-solid and in a bioreactor containing liquid medium

In this study, morphological data of in vitro-grown lingonberry shoots were compared between liquid and semi-solid medium (Fig. 3-1). Among all node culture explant, NC2 produced a highest vigorous shoot number per explant (55.2 ± 2.049) and leaf number per shoot (16.2 ± 1.30) compared to NC1 shoot number per explant (42.8 ± 6.760) and leaf number per shoot (10.4 ± 7.127) (Fig. 3-1). NC1 produced a greater shoot size (9.7 ± 2.31 mm) compared to NC2 (8.28 ± 1.17 mm) . The shoot vigor of NC2 (4.8 ± 0.45) was better than NC1 (4.8 ± 0.84) in this study.

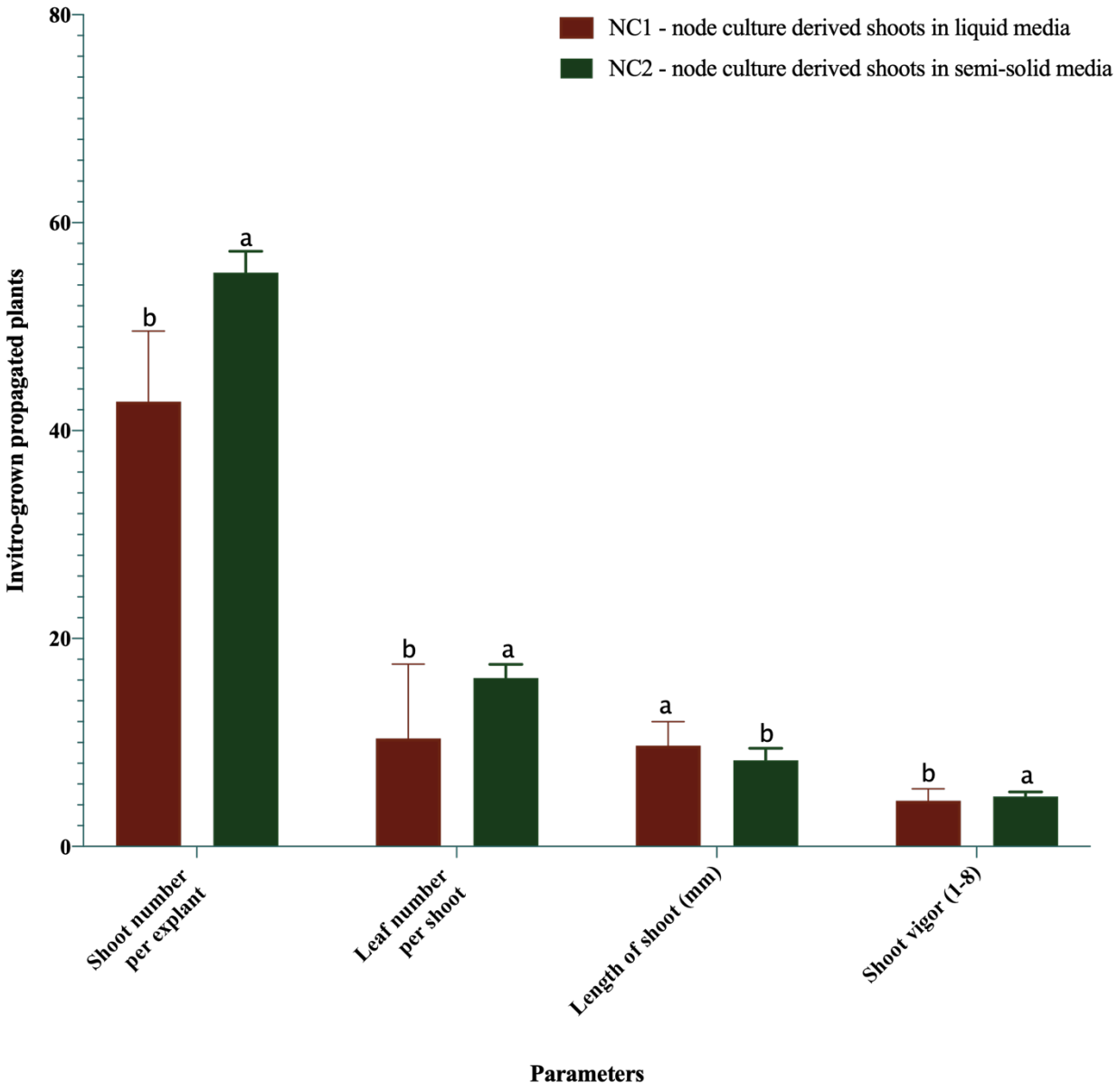

Figure 3-1 Effects of liquid and semi-solid media on morphological features of in vitro-grown lingonberry.

Data are expressed as the means ± SD, Bars followed by different letters indicating significantly differences at $\alpha = 0.05$ by t & Wilcoxon test. NC1 node culture derived propagated explants from liquid media and NC2 node culture derived propagated explants from semi-solid media were used in this study.

3.2 Evaluation of tissue culture derived and cutting propagated plants under greenhouse conditions

Morphological data were compared between node (NC3) and leaf culture-derived shoots (LC1) along with terminal softwood cuttings of ED lingonberry (Fig. 3-2). Softwood cutting (ED) yielded a more vigorous plant with fewer shoots and leaf per plant compared to NC and LC (NC - node culture, LC - leaf culture) (Fig. 3-2). Leaf culture derived shoot LC1 produced the highest number of rhizomes per plant (71.6 ± 4.28) and shoot number per plant (74.6 ± 4.28), compared to node culture derived plant NC3 produced a comparatively low number of rhizomes per plant (40.4 ± 4.1) and shoot number per plant (42.2 ± 2.17) and ED produced (8.2 ± 0.84) number of rhizomes per plant and (11.2 ± 1.30) shoot number per plant respectively. LC1 was also appears highest length of the rhizome (10.8 ± 3.12 cm). In the criteria of the number of leaf per shoot, softwood cutting ED was best (24.4 ± 12.28) in comparison with NC3 (12.4 ± 2.07) and LC1 (14.6 ± 2.509). Additionally, the length of the shoot in LC1 was high (10.38 ± 4.52 cm), NC3 (10.1 ± 2.484 cm) whereas ED represents the lowest length of the shoot (8.1 ± 3.38 cm). The length and breadth of the leaf appear high in softwood cutting ED (2.34 ± 0.18 mm), (1.2 ± 0.24 mm) compared to NC3 (1.36 ± 0.114 mm) (0.74 ± 0.114 mm); LC1 (1.5 ± 0.406 mm) (0.88 ± 0.192 mm) respectively. Also, the plant vigor appears highest in leaf culture derived plant LC1 (8 ± 0).

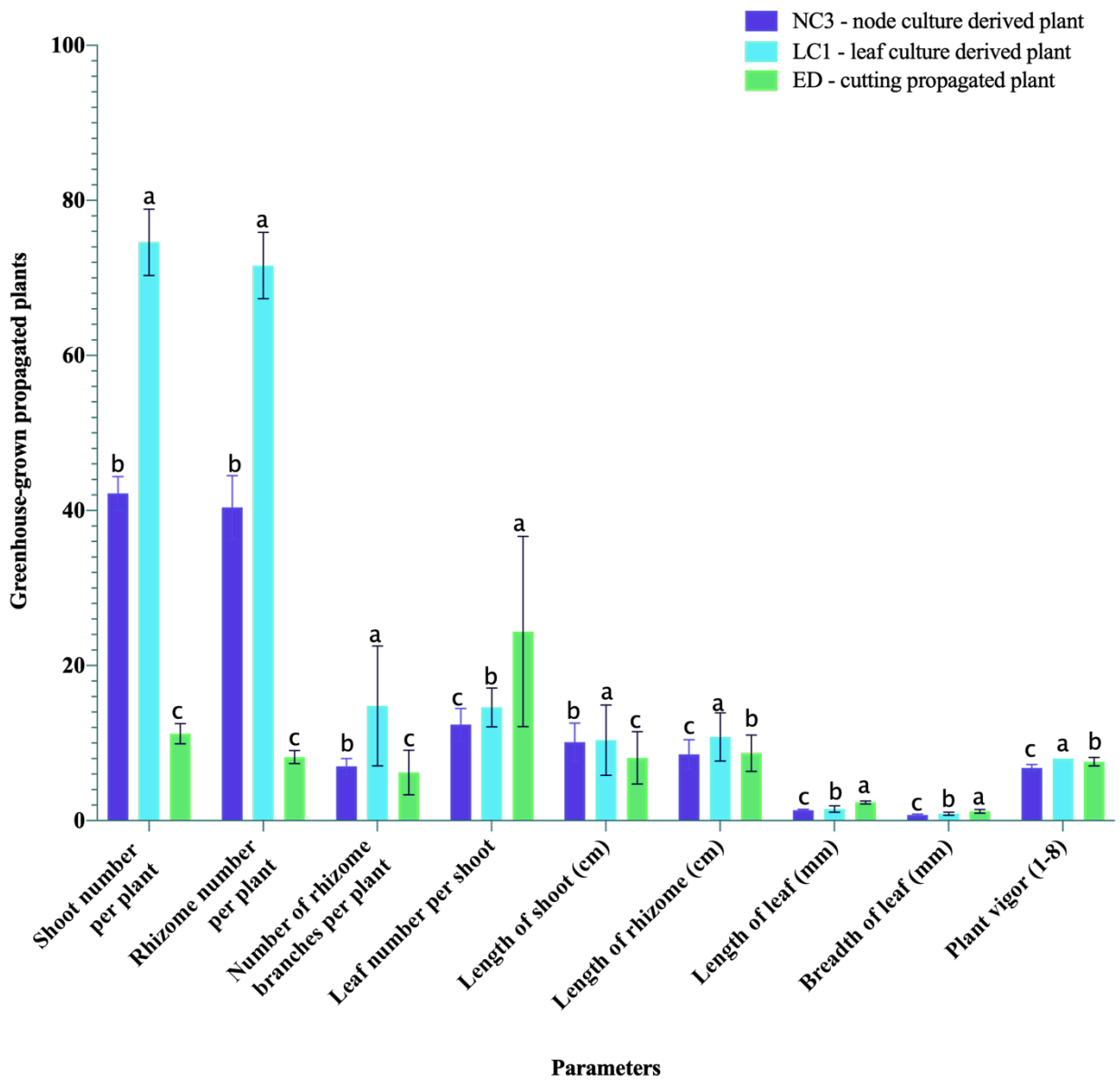

Figure 3-2 Effects of greenhouse-grown lingonberry were observed based on morphological features.

Data are expressed as the means ± SD, Bars followed by different letters indicating significantly differences at $\alpha = 0.05$ by t & Wilcoxon test. NC3 node culture derived propagated plants, LC1 leaf culture derived propagated plants and ED cultivar was used in this study.

3.3 Recognition of cytosine methylation and its polymorphism using Methylation-sensitive amplification polymorphism (MSAP) assay

For the detection of methylation-sensitive DNA bands, twelve combinations of selective primers [EcoR1 and EcoR2, MspI, and HpaII] were used. The methylation-sensitive DNA bands of lingonberry in vitro-derived nodal explants, leaf culture-derived, shoot culture-derived and cutting cultivar were observed in polyacrylamide gel electrophoresis [PAGE].

Fully-methylated (Fmet), hemi-methylated (Hmet), and non-methylated (Nmet) sets of bands were identified at 5'-CCGG-3' sites in micropropagated plant and its cutting cultivar ED. In micropropagated lingonberry plant, the DNA bands were present in both lanes [EcoR1+MspI (M) and EcoR1+MspI+HpaII (MH)] but absent in [EcoR1+HpaII (H)], which indicates that the internal cytosine was fully-methylated [5'-C^{m}CGG-3'] (Fig. 3-3). Similarly, the existence of methylated bands of DNA in both lanes [EcoR1+HpaII (H) and EcoR1+MspI+HpaII (MH)] and their absence in [EcoR1+MspI, (M)] demonstrate that the external cytosine was hemi-methylated [5'-^{m}CCGG-3'], mostly observed in micropropagated lingonberry (Fig. 3-3). Some DNA bands was visualized in three lanes [EcoR1+MspI (M), EcoR1+HpaII (H) and EcoR1+MspI+HpaII (MH)] that represents non-methylation in lingonberry, mostly observed in cutting cultivar (ED) (Fig. 3-3). The total number of methylated and non-methylated bands in micropropagated plant and cutting cultivar were observed in NC1=139, NC2=144, NC3=148, LC1=162, ED=136 (Appendix Table A-1). The rate of methylation in all lingonberry samples were LC1>NC3>NC2>NC1>ED, where LC1 produced the highest level of methylation. On the other hand, nodal explant NC1 produced a low methylation level which was obtained from liquid medium. In this study, LC1 has produced 46 fully methylated [5'-C^{m}CGG-3'] sites obtaining from twelve combinations of selective primer,

whereas it has been produced 62 hemi-methylated [5'-^{m}CCGG-3'] sites obtaining from twelve combinations of selective primer. A heatmap based on PAGE provides a visualization of the banding patterns and DNA polymorphisms in vitro-grown shoots, micropropagated greenhouse plant and cutting cultivar of ED (Fig. 3-4). In this study, the primer combination EcoR1-G/MH2-ACT and EcoR1-G/MH4-AAC C were used to observe DNA polymorphism through a heatmap. M, H, and MH refers to the digestion with combinations of EcoR1+MspI (M), EcoR1+HpaII (H), and EcoR1+MspI+HpaII (MH), respectively. In NC1, DNA bands were marked by a red spot, present in M and MH digestion lanes but not present in H lanes, which indicate internal cytosine methylation (fmet) in EcoR1-G/MH4-AAC C combination. Whereas, in NC1 methylated DNA was marked by a red spot that absent or present in M, H and MH lane altogether found in EcoR1-G/MH2-ACT primer combination.

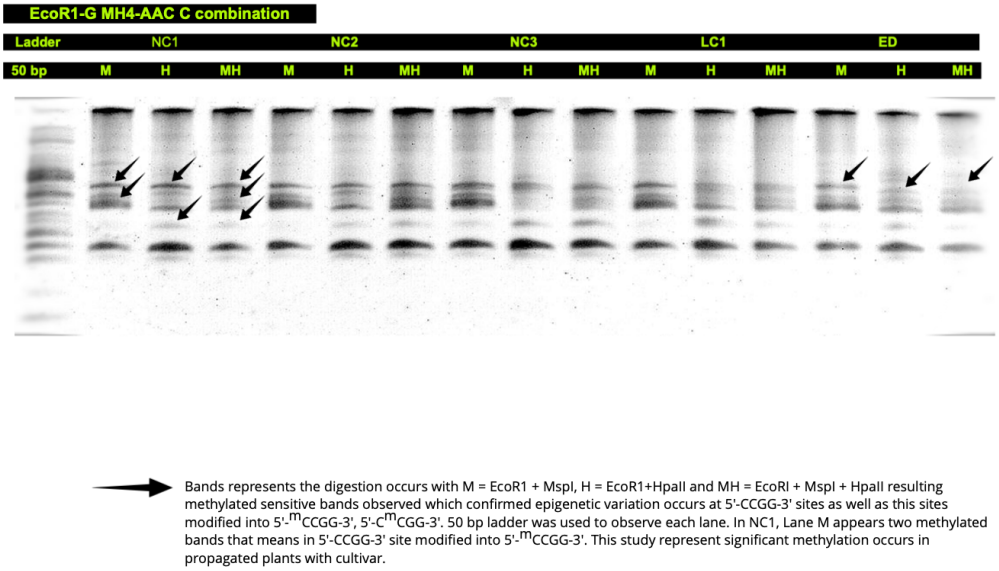

Figure 3-3 DNA methylation patterns observed in NC1, NC2, NC3, LC1 and ED propagated plants by tissue culture and cutting cultivar.

Selective amplification was carried out using an EcoR1-G/MH4-AAC C primer combination. M, H, and MH refer to DNA fragments originating from digestion with combinations of EcoR1+MspI, EcoR1+HpaII, and EcoR1+MspI+HpaII, respectively. DNA bands (marked arrows) present in H digestion lanes but not in M lanes indicate hemi-methylated external cytosine (5'-mCCGG-3') at 5'-CCGG-3' sites, whereas DNA bands (marked arrows) present in M digestion lanes but not in H lanes indicate fully methylated internal cytosine (5'-CmCGG-3') at 5'-CCGG-3' sites in genomic DNA. Ladder: 50 bp (New England Biolabs Ltd., Whitby, ON). NC1 = node culture explant from liquid media, NC2 = node culture explant from semi-solid media, NC3 = node culture plant from Greenhouse, LC1 = leaf culture plant from Greenhouse, ED = cutting cultivar from Greenhouse.

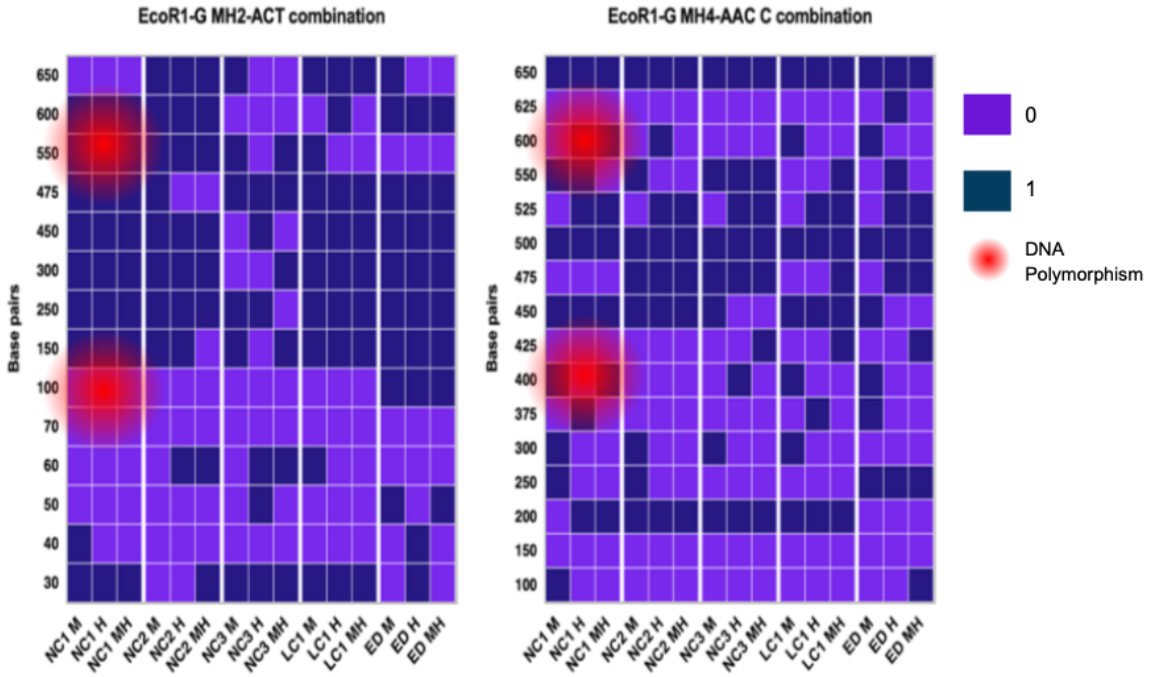

Figure 3-4 Heatmaps represents the example of methylation sensitive amplification polymorphism (MSAP) profiles in micropropagated lingonberry plants of ED obtained by using the primer combination EcoR1-G/MH2-ACT and EcoR1-G/MH4-AAC C.

M, H, and MH refer to the digestion with combinations of EcoR1+MspI (M), EcoR1+HpaII (H), and EcoR1+MspI+HpaII (MH), respectively. "0" refers to the absence of methylated DNA band, and "1" refers to the presence of methylated DNA band. In NC1, DNA bands (marked by red spot) present in M digestion lanes but not in H lanes indicate internal cytosine methylation in EcoR1-G/MH4-AAC C combination. NC1 banding pattern (marked by red spot) absent in M lane in EcoR1-G/MH2-ACT combination indicates DNA methylation polymorphisms. Likely, in NC1 banding pattern (marked by red spot) present in H digestion lane but not present in M lanes indicates external cytosine methylation in EcoR1-G/MH4-AAC C combination. On the other hand, in NC1 banding pattern (marked by red spot) present in both M and H lane of EcoR1-G/MH2-AAT combination indicates non-methylation. NC1, NC2, NC3 and LC1 are the micropropagated plants of ED. Ladder: 50 bp (New England Biolabs Ltd., Whitby, ON). NC1 = node culture explant from liquid media, NC2 = node culture explant from semi-solid media, NC3 = node culture plant from Greenhouse, LC1 = leaf culture plant from Greenhouse, ED = Erntedank cultivar from greenhouse.

3.4 Analysis of secondary metabolites and their comparative study

The total phenolic content (TPC) of lingonberry node culture, leaf culture and cultivar were dependent on various cofactors like environment and different growing conditions. This experiment of lingonberry was notably varied by one-way ANOVA ($P \leq 0.05$). The greenhouse plants exhibited high phenolic activity compared to the in vitro-grown node culture derived explants of lingonberry (Fig. 3-5). All micropropagated plants were uniquely varied compared to the cutting cultivar. The decreasing order of total phenolics of lingonberry specimens was

NC2<NC1<ED≤ LC1<NC3 (mg GAE / flw), respectively. In this study, TPC was highest in NC3 (7.585 ± 0.0 mg GAE / flw) followed by the greenhouse propagated plant where NC3 represents the highest TPC along with ED (7.584 ± 0.0004 mg GAE / flw) and LC1 (7.584 ± 0.0004 mg GAE / flw) appeared the same amount of TPC. On the other hand, NC2 represented lowest TPC (2.483 ± 0.982 mg GAE / flw) and low TPC was observed in NC1 (3.791 ± 0.732 mg GAE / flw).

Different growing conditions had several effects on total flavonoid content (TFC). The data were analyzed by one-way ANOVA (P≤0.05). The total flavonoid content was highest in the cutting cultivar ED and lowest TFC observed in the NC2; more details are in Fig. 3-5. The decreasing order of total flavonoid content was NC2< LC1< NC1< NC3< ED (mg CE / flw). NC2 represents the lowest amount of TFC (3.264 ± 1.138 mg / flw), LC1 had (4.490 ± 0.303 mg CE / flw), NC1 (6.240 ± 0.422 mg CE / flw). On the other hand, NC3 has a high TFC (7.260 ± 1.575 mg CE / flw) as well as cutting cultivar ED appears the highest TFC (7.917 ± 0.384 mg CE / flw).

Total antioxidant content (TAC) of micropropagated plant and cutting cultivars were analyzed using DPPH assay, where lowest amount of TAC were observed compare to all other berry plant. The data were analyzed by one-way ANOVA (P≤0.05). The decreasing order of total antioxidant contents was ED≤NC1< LC1<NC3<NC2 (mg GAE / flw) (Fig. 3-5). The TAC of cutting cultivar and propagated plant are presented. TAC was high in NC1 (0.035 ± 0.0012 mg GAE / flw) among all of the propagated plants.

Propagated plant from the greenhouse and growth chamber were significantly different for total proanthocyanidin content (TPrC). The data of this experiment were analyzed by one-way ANOVA

(P≤0.05). TPrC was high in greenhouse cutting cultivar and low in LC1 (Fig. 3-5). The order of TPrC of lingonberry leaves was LC1<NC2<NC3<NC1< ED (mg CE / flw). Besides, we observed LC1 has highest TPrC (0.0013 ± 0.0004 mg CE / flw) but ED produced lowest TPrC (0.0049 ± 0.0011 mg CE / flw).

Consequently, almost all propagated plants and cultivar were produced the same amount of TPC except NC2, which produces the lowest amount of TPC. The cultivar was produced the highest amount of TPrC than other propagated plants. In TFC, the highest quantity was observed in cutting cultivar rather than other propagated plants. NC2 displays the highest production of TAC than cultivar and other propagated plants.

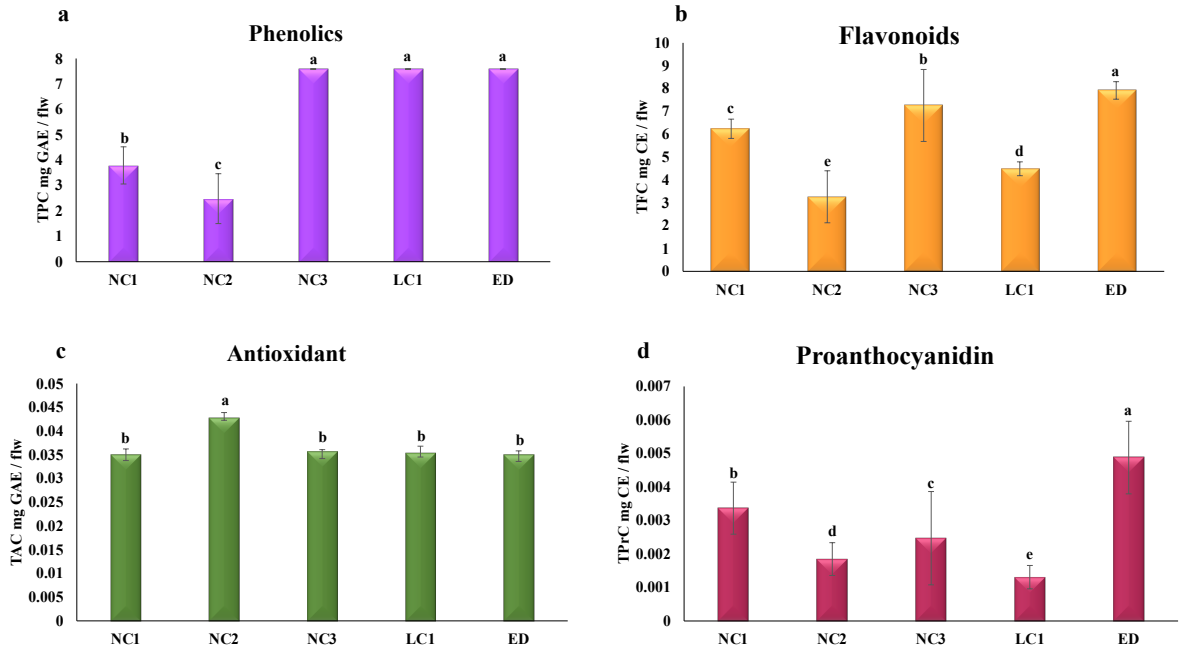

Figure 3-5 Total phenolic, flavonoid, antioxidant and proanthocyanidin contents in leaves of cultivar and propagated lingonberry.

Data are expressed as the means ± SD. Different letters indicate significantly differences occurs at $P \leq 0.05$ by oneway ANOVA. GAE = gallic acid equivalent, flw = fresh leaf weight, CE = catechin equivalent. TPC total phenolic contents, TPrC total proanthocyanidin, TFC total flavonoid content & TAC total antioxidant activity. NC1, NC2, NC3 and LC1 are the micropropagated plants of ED. NC1 = node culture explant derived from liquid media, NC2 = node culture explant derived from semi-solid media, NC3 = node culture plant derived from Greenhouse, LC1 = leaf culture plant derived from Greenhouse, ED = Erntedank cultivar from greenhouse.

3.5 Correlations of secondary metabolites on lingonberry propagated plant and cultivar

All secondary metabolites (TPC, TPrC, TAC, TFC) of propagated lingonberry had a significant relationship. These data were analyzed by linear regression in [GraphPad Prism 8.0.0 software]. Total phenolic content directly proportional to the total proanthocyanidin content (Fig. 3-6. a); total proanthocyanidin was directly proportional to the total flavonoid contents (Fig. 3-6. b); phenolic content was directly proportional to entire flavonoid content (Fig. 3-6. c); total phenolic content was inversely proportional to the total, antioxidant content (Fig. 3-6. d).

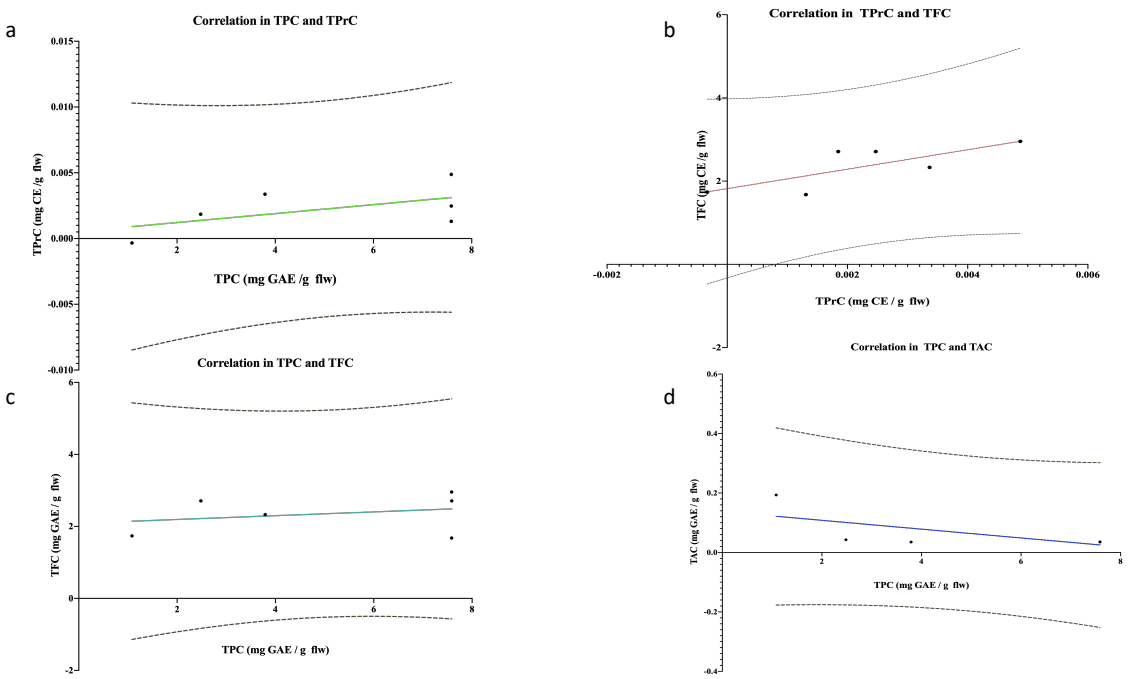

Figure 3-6 Linear regression in secondary metabolites of cultivar and propagated lingonberry.

Data were analyzed based on means ± SD, n = 6. Significant differences not present at $\alpha = 0.05$ by Spearman test. a. Correlation between TPC and TPrC. b. Correlation between TPrC and TFC. c. Correlation between TPC and TFC. d. Correlation between TPC and TAC.

3.6 Cytosine methylation affect secondary metabolites

DNA methylation plays a critical role in the regulation of secondary metabolites. Data were analyzed by t and Wilcoxon tests, and it appears significant difference ($\alpha = 0.05$). On the other hand, the same data was performed by one-way ANOVA; it also produced the same results as the t and Wilcoxon tests. One-way ANOVA represents a significantly difference ($P \leq 0.05$). The order of methylation percentage for Fmet, Hmet, and Nmet were NC3<NC2<NC1<ED<LC1; NC3<NC2<NC1<ED<LC1; LC1<ED<NC1<NC2<NC3, respectively, whereas the following order of total phenolic content was NC2<NC1<ED≤ LC1<NC3. The order of total flavonoids was NC2<LC1<NC1<NC3<ED, the order of total proanthocyanidin content was

LC1<NC2<NC3<NC1<ED, and the order of total antioxidant contents was ED≤NC1<LC1<NC3<NC2.

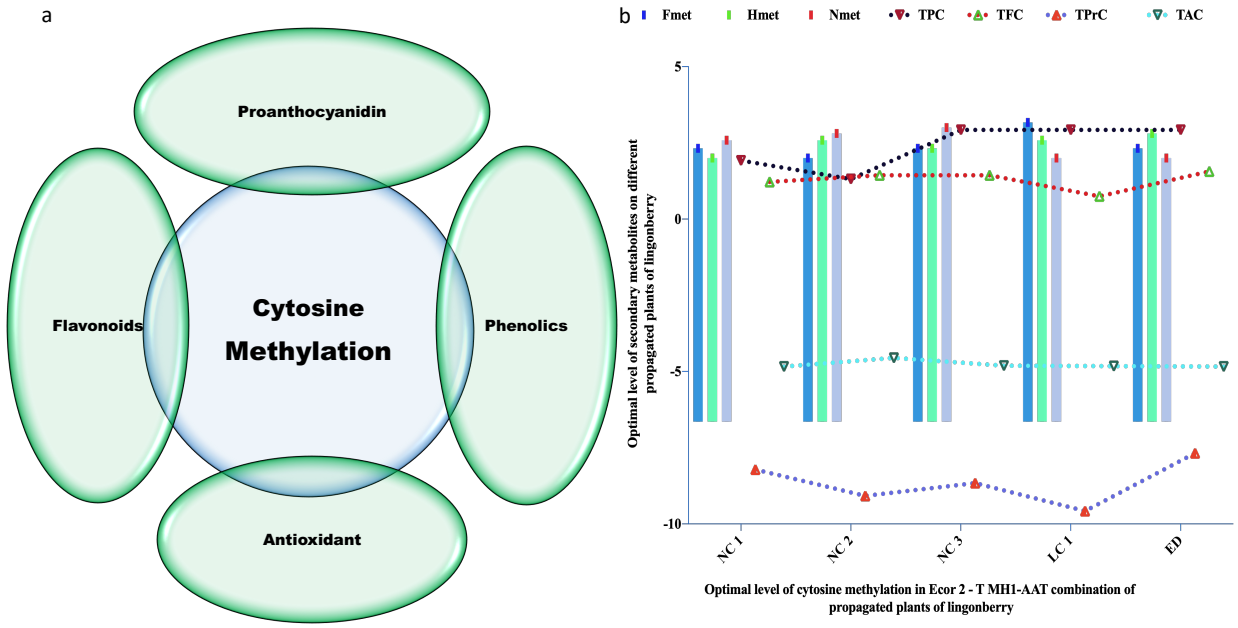

Figure 3-7 a. Schematic diagram represents that the more cytosine methylation responsible for the decreasing of secondary metabolites in lingonberry. b. The graph was obtained from the primer combinations of EcoR2-T/MH1-AAT, where more methylation and decreasing secondary metabolites were observed.

Data are expressed as mean ± SD. Fmet refers to full-methylation occurs, means internal cytosine methylation was found at 5'-CCGG-3' sites in genomic DNA. Hmet refers to hemi-methylation occurs, means external cytosine methylation was found at 5'-CCGG-3' sites in genomic DNA. Nmet refers to non-methylation occurs, means methylation was not found at 5'-CCGG-3' sites in genomic DNA. TPC total phenolic content, TFC total flavonoid content, TPrC total proanthocyanidin content and TAC total antioxidant activity. NC1, NC2, NC3 and LC1 are the micropropagated plants derived from ED. NC1 =

node culture explant from liquid media, NC2 = node culture explant from semi-solid media, NC3 = node culture plant from Greenhouse, LC1 = leaf culture plant from Greenhouse, ED = Erntedank cultivar from greenhouse.

In this study, the graph was obtained from the primer combinations of EcoR2-T/MH1-AAT; more methylation and decreasing secondary metabolites were observed based on environmental factors such as in vitro (nodal explants were regulated in the growth chambers; finally, numerous shoot formation occurs). NC1, NC2 in vitro grown lingonberry shoots showed a higher methylation rate but comparatively secondary metabolites was observed, whereas NC2 appeared lowest TPC (Fig.3-7). Consequently, LC1 has a low Nmet but a higher rate of methylation occurs whereas more amount of TPC was observed. Followingly, NC3 displayed high Nmet, but we estimated an equal amount of TPC like LC1 was observed (Fig. 3-7). TFC was high in NC1 and NC2, where a higher methylation rate appeared. But TFC was low in LC1 and high in ED (Fig. 3-7). LC1 showed the lowest TPrC where methylation rate is highest, but Nmet was lowest (Fig. 3-7). Lastly, NC3 and NC1 appeared low level of TAC; on the other hand, LC1, NC2, and ED had a same amount of TAC (Fig. 3-7). We scrutinized that the total methylated bands compared to all secondary metabolites appeared inverse response between in different propagated methods of lingonberry.

Chapter 4 : Discussion

Epigenetic variation influences complex traits in plants. Genetic sequences remain the same during different developmental stages but are phenotypically different (Rathore and Jha 2016). Many genes have been epigenetically modified through cytosine methylation, as demonstrated through the MSAP assay. Because of developmental stages and environmental conditions, DNA methylation has unique dynamics, and it is not a static process (Valledor et al. 2007a; Li et al. 2014). As a result, gene expression has been altered by regulatory genes. Genetic and epigenetic variations (DNA methylation, transposable element activations, chromosome breakages, and single-gene mutations) occur in tissue-culture plants due to environmental stress (Kaeppler, Kaeppler, and Rhee 2000b). MET1 is the leading cause of methylation, which was present in the regenerated plants (Taskin et al. 2015). Our investigation showed that the growth regulator zeatin and indole-3-butyric acid affected in-vitro plants, indicating both hyper- and hypo-methylation, which promotes apical dominance, encoded by the Auxin Response Factor-3 (ARF3) gene. Generally, the shoot apex promotes the formation of auxiliary buds through the suppression of cytokinin. The higher DNA methylation was monitored in liquid culture of eggplant (Cline, Wessel, and Iwamura 1997; Plant, Bucherna, and Szabo 2013; Baurens et al. 2003; Baubec et al. 2010; Fu et al. 2012). Due to a higher concentration of thidiazuron, the methylation rate has been reduced in blueberry callus (Goyali, Igamberdiev, and Debnath 2018b; A. Ghosh, Igamberdiev, and Debnath 2017). Our results agree with the blueberry cultivar Fundy that expressed higher DNA methylation in the clone QB9C than in the tissue-culture one. In this study, cultivar Erntedank was used as control; thus the cytosine methylation was observed in node-culture explants in liquid culture, node-culture explants in semi-solid media, node-culture plants and leaf-

culture plants in greenhouse, whereas highest methylation was expressed in leaf-culture derived plants in greenhouse (Fig. 3-3, 3-4). This is the first molecular insight in lingonberry using MSAP.

The secondary metabolites TPC, TFC, TAC, and TPrC were synergistically and antagonistically affected by various environmental factors and ages of lingonberry propagated plant and cultivar. Significant interactions among all propagated plant in different growth conditions was reported, TPC, TFC, TAC, and TPrC, were higher in cutting plants and comparably lower in the tissue-cultured plant in blueberry (Butler, Price, and Brotherton 1982). In lingonberry, our study showed high TPC, TFC, TPrC content but low antioxidant activity. A similar study in lingonberry reported that total phenolic content was high and antioxidant content was low, so these compounds are highly correlated with each other (Bujor et al. 2018). Similarly, it was reported that TPC and TFC were higher in cutting plants than tissue-cultured blueberry (Meins 1983). Conversely, in our study, TFC and TPC were higher in cutting plants than tissue-cultured lingonberry. In the current study, the multiple comparisons in propagated and cultivar of lingonberry was displayed number of secondary metabolites (Fig. 3-5). Compared to the cutting cultivar ED, the decreasing order of TPC was followed: LC1<NC3. According to all the secondary compounds, total phenolic activity was the highest in lingonberry. The highest TPC was occupied by NC3 (7.5850 GAE mg / g flw). Cutting cultivar ED, NC1, NC2 represents high TFC. Here, the highest performance of TFC conveyed by NC3 2.7103 mg C.E. / g flw, and the lowest LC1 was 1.6760 CE mg / g flw. In TAC, the following ratios were observed ED<NC1<LC1<NC3<NC2. All propagated plants are display a significant effect in the total antioxidant activity, where in vitro NC2 has the highest one. For the proanthocyanidin activity, we observed the following sequences ED<NC1<LC1<NC3<NC2 in lingonberry. Although it is depicted in Fig. 3-5, the TPrC is comparatively low in all propagated

plant, but NC3 performed the more significant TPrC, which was 1.1610 CE mg / g flw. As a result, we observed higher secondary metabolite content present in greenhouse samples than in vitro-grown of lingonberry. Greenhouse samples were thirteen years old, which may be one of the reasons for being rich in secondary metabolites, whereas growth-chambered samples that were low in secondary metabolites were less than a year old. We analyzed using linear regression and predicted a relationship between secondary metabolites and each propagated plants based on our data. TPC was directly proportional to TPrC, exhibiting a positive relationship. TPrC correlated to TFC was found in our study. TPC is proportional to TFC was also observed in cultivar and propagated plants of lingonberry. TPC is inversely proportional to TAC, where TAC decreased, but TPC increased (Fig. 3-6).

Epigenetic variation was studied by analyzing changes in methylation patterns between cutting plants with propagated plant. Micropropagated plants have higher epigenetic variation and affect positively in nutritional value than mother plants while decreasing with the generations (Akomeah et al. 2019; Baránek et al. 2015; 2016). In addition, there was more variance in DNA methylation among propagated plant were present. In a study on Rhododendron, tissue-cultured plants displayed 12.17 % nonmethylation at 5'-CCGG-3' sites compared to mother plants (Klerk 1990), whereas in lingonberry tissue-culture LC1 showed 8.58% more cytosine methylation than maternal genotype ED, but NC1, NC2, and NC3 had low methylation rates than LC1 (4.85%, 6.7%, 12.14% respectively). Among 24,794 bands in PAGE, 26.61% methylated bands were observed in Rhododendron. It has been reported that DNA methylation increased in floral buds while decreased in vegetative buds (Meijón et al. 2009). Conversely, micropropagated bananas indicated 23% of methylation events, whereas conventionally propagated plants showed 18.4% of

methylation events (Baurens et al. 2003; Díaz-Martínez et al. 2012; Peraza-Echeverria, Herrera-Valencia, and Kay 2001). In blue agave (*Agave tequilana* F.A.C. Weber), 40 more bands were reported for in vitro than in vivo plants (Díaz-Martínez et al. 2012). This was also documented in previous work on the blueberry, where methylation rate increases in micropropagated plants compared to conventional plants (Goyali, Igamberdiev, and Debnath 2018b; A. Ghosh, Igamberdiev, and Debnath 2017). Similarly, thidiazuron-induced blueberry calli exhibited an increased methylation rate compared to cutting plants (A. Ghosh, Igamberdiev, and Debnath 2017). In the present study, we compared each micropropagated plants with a cultivar using the MSAP assay, and the methylation rate increased from in vitro regenerates to acclimatized tissue-cultured greenhouse propagated plant. In leaf regenerants (LC1), we obtained 14 more methylation bands than shoot regenerants (NC3), where the fully-methylated and hemi-methylated rate was the highest among all propagated plants. Therefore, we can summarize the total methylated segments present in each clone where the most elevated amount was exhibited by LC1 (162 bands) and lowest present by ED (136 bands) (Fig. 3-3, 3-4 and Supplementary Table 1). The fully-methylation level was high, and the hemi-methylated level was low for the lingonberry genome, which is compatible with previous studies in banana, blueberry, and blue agave. Reciprocally, in micropropagated banana plants, the low rate of fully-methylation and high rate of hemi-methylated DNA found at 5′-CCGG-3′ sites. DNA methylation polymorphism was noticed in different degrees and patterns among in-vitro and in-vivo conditions. Through MSAP assay, we determined that the highest methylation polymorphism was observed in leaf regenerants among all propagated plants. Our results were comparable with micropropagated banana, where 3% of DNA methylation were polymorphic, whereas conventionally propagated banana plant was not. However, it has been reported that the in-vivo genotype shows low methylated polymorphism compared with in-vitro

callus in blueberry (Goyali, Igamberdiev, and Debnath 2018b; A. Ghosh, Igamberdiev, and Debnath 2017).

Due to several environmental factors, DNA methylation regulates gene expression. Altered DNA methylation leads to improved plant disease resistance and drought stress tolerance by recruiting chromatin remodelers histone deacetylases and histone methyltransferases to repress transcription (Lang et al. 2015; Lavania et al. 2012; Wada et al. 2004b; Wang and Lin 2000). In plants, inhibited DNA methylation could increase or decrease secondary compounds, observed in *Taxus spp*., *Salvia miltiorrhiza*, and *Vitis amurensis* (Fu et al. 2012; Kiselev et al. 2011; Yang et al. 2018). Our report exhibited the inverse correlation between DNA methylation with secondary metabolites (Fig. 3-7). The cytosine analog 5-azacytosine dramatically increased phenolic acid accumulation and expressions of key genes involved in the phenolic acid biosynthesis pathway. However, decreased methylation levels of CG and CHG sites were found. CHH methylation helps in the synthesis of the rosmarinic acid synthase gene (RAS) as a promoter (Mcclelland, Nelson1, and Raschke2 1994). This study suggested that when the methylation rate increased, the efficacy of phenolics was decreased. Greenhouse leaf regenerants (LC1) exhibited high amounts of methylation bands along with low phenolic contents. In addition, shoot regenerants (NC3) had the greatest number of the phenolic compound with the lowest efficacy of methylation bands.

Similarly, we observed flavonoids and proanthocyanidin also represented an inverse relationship with cytosine methylation. On the other hand, the formation of 'double lock' cooperation was observed between DNA methylation and histone modification (Baubec and Schübeler 2014; Baubec et al. 2010; 2014). Previous research found higher cytosine methylation occurred with high levels of native secondary metabolites in the autopolyploid *Cymbopogon sprengel* (Lavania et al.

2012). We speculated the same trend in antioxidant content, where 50% methylation was inversely proportional to total antioxidant contents among all environmental factors. Fully-methylated DNA compared with TPC, we found that both in vivo and in vitro specimen exhibited high methylation with low phenolic content. P.A.L., 4CL, C4H, T.A.T., HPPR, CYP98A14, and R.A.S. genes were identified as phenolic acid biosynthesis by DNA methylation in *S. miltiorrhiza* hairy roots. Followingly, increased DNA methylation reduces the expression of P.A.L. and CYP98A14 at the level of 16.7% and 45.5%, respectively. However, decreased methylation enhanced R.A.S. expression (Yang et al. 2018). The expression of the VaSTS10 gene was significantly increased with a decrease in methylation (Lavania et al. 2012). This study depicted that cytosine methylation has a converse relationship with secondary metabolites of lingonberry among all propagated plant and cultivar.

Chapter 5 : Conclusions and Future directions

Lingonberry (*Vaccinium vitis-idaea* L.) are popular perennial plants in North America for their nutritional compounds as well as important phytochemical compounds which are effective in the prevention of various chronic diseases such as reduce cholesterol levels, prevent rheumatic diseases, hepatitis C, kidney, bladder infections, and have also been used to treat Alzheimer's disease. Both in vitro and in vivo conditions, lingonberry is considering a medicinal fruit. But the process of commercial lingonberry production is not fulfilling the market demands. Conventionally propagated production of plants is time consuming and laborious process. On the other hand, tissue culture regenerated plants produce multiple plants in a short range of time. Micropropagation of lingonberry is well established, which can easily fulfill the market demands of lingonberry. Besides, tissue culture plants have enhanced morphological potentiality in lingonberry. This study was conducted to understand the variation in epigenetic change and secondary metabolites among cultivar of lingonberry and their propagated plants. It was helpful for the growers to get efficient trait/efficient traits for large-scale cultivation. For the analysis of secondary metabolite production and DNA methylation, Erntedank, node-culture regenerated plants, and leaf-culture regenerated plants were grown in the greenhouse as well, as node-culture explants were grown in Growtek and Sigma bottle in the Growth chamber. Genetic evolution was estimated by a molecular marker. Epigenetic variation, especially the DNA methylation, was detected in the propagated plants and cutting cultivar. The ultimate goal of the present study was to isolate the potential propagated plant through DNA methylation with their respective metabolite production. DNA methylation helps in plant development, where the biochemical compounds

resemble the final product. This process aids in the selection of significant cultivar. This will ultimately lead to correcting the errors of propagated plants at the genic level to enhance the rate of secondary metabolite concentration in lingonberry.

In the present study, micropropagation enhances the rate of secondary metabolite concentration in lingonberry. However, those effects were genotype-specific. Overall, leaf culture regenerated plant in the greenhouse was highest phytochemical content. This study proved that in vitro propagated greenhouse plants had tissue-specific effects from phytochemical characteristics and phenotypic expression in lingonberry. The highest level of phenolic content was observed in greenhouse plants, and the highest level of antioxidant was observed in node-culture explant grown in Sigma bottle. The highest proanthocyanidin was observed in the cutting cultivar. Greenhouse-grown cutting cultivar represents the highest flavonoid content.

The epigenetic analysis of the micropropagated and greenhouse-grown lingonberry plants using MSAP assay. So present investigation of DNA methylation reflected that in vitro plants had a sustainable effect on cytosine methylation in lingonberry. Among all tissue culture plants, leaf-culture derived greenhouse plant represents the highest bands of cytosine methylation. MSAP technique detects the global cytosine methylation pattern in lingonberry based on the recognition sites of isoschizomers' pair. In the methylation analysis, if more bands present in cytosine methylation, that represents more phenotypic changes.

A large body of data library in lingonberry epigenetic study will be used as new efficient tools for understanding the origin of lingonberry, evolution, taxonomy. It will be very much useful for

genome reprogramming, gene identification, gene characterization, transcriptome analyses may reveal different heat/cold stress responses, gene editing by CRISPER, protoplast isolation and transfection during cell differentiation, plant regeneration, and reproduction. Finally, these data will help to characterize various types of epigenomic changes for epimutation. They are ultimately identifying the potential tissue culture-derived health-promoting lingonberry plant. This is undoubtedly to say that DNA methylation will serve as the important biotechnological tool to cover our current increasing food demand in the sense of quality and quantity of commercial lingonberry production. For the future, the proteomic study of lingonberry should be more effective for plant breeding methods.

Appendix

Table A-1 Summary of total number of bands, number and percentage (%) of DNA methylation events detected by methylation sensitive amplification polymorphism (MSAP) technique using twelve selective primer combinations in five samples of lingonberry.

Those samples were derived from cutting cultivar Erntedank (ED) and micropropagation methods (NC node culture and LC leaf culture). NC1 node culture derived in vitro-grown shoots from liquid media, NC2 node culture derived in vitro-grown shoots from semi-solid media, NC3 node culture derived greenhouse-grown plant and LC1 leaf culture derived greenhouse-grown plant.

Combinations of Selective Primers	NC1			NC2			NC3			LC1			ED		
	fmet	hmet	nmet	fmet	hmet	nmet	fmet	hmet	nmet	fmet	hmet	nmet	fmet	hmet	nmet
Ecor1-G MH1-AAT	3	3	6	3	2	6	2	1	7	3	3	7	3	3	8
Ecor2-T MH1-AAT	5	4	6	4	6	7	5	5	8	9	6	4	5	7	4
Ecor1-G MH2-ACT	0	1	5	2	2	4	4	4	4	1	2	4	2	2	3
Ecor2-T MH2-ACT	4	3	9	4	2	10	8	4	6	3	9	1	5	1	9
Ecor1-G MH3-TCC	3	4	7	2	4	8	3	3	7	4	4	7	3	3	4
Ecor2-T MH3-TCC	2	2	4	4	2	3	3	2	5	3	8	2	2	4	2
Ecor1-G MH4-AAC C	4	4	4	2	3	6	3	3	6	5	6	4	6	6	3
Ecor2-T MH4-AAC C	4	2	7	4	3	5	3	2	8	5	7	7	3	2	6
Ecor1-G MH5-CGA A	1	2	9	2	3	7	0	1	9	2	3	6	4	2	6
Ecor2-T MH5-CGA A	3	4	2	4	4	4	2	4	6	6	7	2	6	4	2
Ecor1-G MH6-TAG C	3	3	4	3	4	4	0	0	7	3	3	4	3	3	4
Ecor2-T MH6-TAG C	5	5	1	2	3	6	3	3	7	2	4	6	0	0	6
Total bands of 3 set of methylated sites (fmet, hmet and nmet)	37	37	65	36	38	70	36	32	80	46	62	54	42	37	57
Total bands of in vitro-grown shoots and greenhouse-grown plant	139			144			148			162			136		
Total methylation (%) of in vitro-grown shoots and greenhouse-grown plant	56.92	56.92	46.76	51.42	54.28	48.61	45.00	40.00	54.05	85.18	114.81	33.33	73.68	64.91	41.91
Total methylated polymorphisms of in vitro-grown shoots and greenhouse-grown plant	51			52			46			80			64		

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