

Epigenomic Insight of Lingonberry: Health Promoting Trait Under Micropropagation

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Abstract

Epigenetic variation plays a role in developmental gene regulation and responses to the environment. An efficient interaction of zeatin induced cytosine methylation and secondary compounds has been displayed for the first time in tissue-culture shoots of lingonberry (*Vaccinium vitis-idaea*) in vitro, in vivo and its cutting-cultivar Erntedank. Through MSAP assay, we observed highest methylated sites in leaf regenerants (LC1) from all primer combinations (108 bands), with their highest variation in secondary metabolites. We measured that four tissue-culture plants showed higher methylation bands than cutting propagated donor plants (ED) which exhibited 79 bands of methylation, which is comparatively low. On the other hand, we observed the highest total phenolic content in node culture-derived greenhouse grown plants, NC3 but leaf culture-derived greenhouse grown plants, LC1 represented low phenolic content. Our study showed more methylation in micropropagated plants (NC1, NC2, NC3, LC1) than those derived from cutting propagated ED plants, where methylation was not present. On the contrary, we observed higher secondary metabolites in ED plants but comparatively less in micropropagated shoots (NC1, NC2) and plants (NC3, LC1). Our study displayed that higher methylation sites observed in micropropagated plants possessed less amount of secondary metabolites.

Introduction

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 ¹. It is used for the production of jams, jellies and candies ². It is widely spread through Greenland, Iceland, North America, Scandinavia, Northern Asia, Europe, Asia ³, (More details in supplementary Fig.S1).

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 ⁴. This process is called micropropagation or in vitro propagation. Lingonberry contains abundant secondary metabolites, including phenolic contents, flavonoids, and proanthocyanidin ⁵. Young leaves may contain up to 1,740 mg/kg anthocyanin (fresh weight) ^{6,7} along with 58% phenolic content present in leaves, 48% in stems, and 79% in fruits ⁸. Lingonberry has been found a high amount of antioxidant and antimicrobial activity in the fruit ⁹. In contrast, flavonoid content exists between 27–42% in leaf tissues ¹⁰. It has been introduced as fruit from an ancient era and a medicinal plant and used as an ornamental plant for the landscape ecology ¹¹. 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 ^{12,13}. Lingonberry fruits can be consumed raw and used to make juices, wines, pastries, sauces, jams, jellies, ice creams, cocktails and desserts ¹⁴.

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 ¹⁵. Changes in

the DNA methylation (or hydroxymethylation), histone modification or both are the crucial factors for epigenetic changes in in vitro plants ¹⁶. 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) ¹⁷. 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 ^{18,19}. 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) ^{20,21}.

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 in-vivo cultivar ²². In this way, we can get elite individuals without transgenic line generation ²². The epigenetic variation imprints the developmental program's memory ²³.

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 ²⁴. 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 ^{25,26}. It involves digestion with methylation-sensitive restriction endonucleases proceeded by amplification of restriction fragments.

Purpose of the Study are as follows: To identify the efficient tissue culture derived health promoting plant under micropropagation. To determine the phenotypic and phytochemical changes among greenhouse-grown propagated plants and cutting cultivar in lingonberry.

Results

Morphological pattern of in vitro and in vivo of lingonberry

In this study, morphological data of in vitro-grown lingonberry shoots were compared between liquid and semi-solid medium and compared between node (NC3) and leaf culture-derived shoots (LC1) along with terminal softwood cuttings of ED lingonberry. 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). NC1 produced a greater shoot size (9.7 ± 2.31 mm) compared to NC2 (8.28 ± 1.17 mm) . The shoot vigor of NC2 ($4.8 \pm$

0.45) was better than NC1 (4.8 ± 0.84) in this study. 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). 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).

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^mCCGG-3'] (Fig.1). 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'-^mCCGG-3'], mostly observed in micropropagated lingonberry (Fig.1). 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.1). 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 (Supplementary Table S2). 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^mCCGG-3'] sites obtaining from twelve combinations of selective primer, whereas it has been produced 62 hemi-methylated [5'-^mCCGG-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.2). 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.

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. All micropropagated plants were uniquely varied compared to the cutting cultivar. The decreasing order of total phenolics of lingonberry specimens was $NC2 < NC1 < ED \leq LC1 < NC3$ (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 ($P \leq 0.05$). The total flavonoid content was highest in the cutting cultivar ED and lowest TFC observed in the NC2. 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 ($P \leq 0.05$). The decreasing order of total antioxidant contents was $ED \leq NC1 < LC1 < NC3 < NC2$ (mg GAE / flw). 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 ($P \leq 0.05$). TPrC was high in greenhouse cutting cultivar and low in LC1. 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.

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. a); total proanthocyanidin was directly proportional to the total flavonoid contents (Fig.3. b); phenolic content was directly proportional to entire flavonoid content (Fig.3. c); total phenolic content was inversely proportional to the total, antioxidant content (Fig.3. d).

Portraying the partnerships of cytosine methylation and 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 $NC2 < NC1 < ED \leq LC1 < NC3$. 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 $ED \leq NC1 < LC1 < NC3 < NC2$.

In this study, the heatmap was obtained from the primer combinations of EcoR2-T and 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 events. Comparatively secondary metabolites was observed, whereas NC2 appeared lowest TPC (Fig.4). 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.4). TFC was high in NC1 and NC2, where a higher methylation rate appeared. But TFC was low in LC1 and high in ED. LC1 showed the lowest TPrC where methylation rate is highest. Lastly, NC3 and NC1 appeared low level of TAC; on the other hand, LC1, NC2, and ED had a same amount of TAC. From the dendrogram, we observed that NC1, NC2 closely related than NC3, LC1 and ED. We scrutinized that the total methylated bands compared to all secondary metabolites appeared inverse response between in different propagated methods of lingonberry.

Discussion

Epigenetic variation influences complex traits in plants. Genetic sequences remain the same during different developmental stages but are phenotypically different²⁷. Many genes have been epigenetically

modified through cytosine methylation, as demonstrated through the MSAP assay²⁸. As a result, gene expression has been altered by regulatory genes. Genetic and epigenetic variations occur in tissue-culture plants due to environmental stress²⁹. MET1 is the leading cause of methylation, which was present in the regenerated plants³⁰. 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. The higher DNA methylation was monitored in liquid culture of eggplant^{31,32}. Due to a higher concentration of thidiazuron, the methylation rate has been reduced in blueberry callus^{33,34}. 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. 1, 2).

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 blueberry³⁵. 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 other⁸. This study, TFC and TPC were higher in cutting plants than tissue-cultured lingonberry. 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, 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).

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 ^{36,37}, 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 ³⁸. Conversely, micropropagated bananas indicated 23% of methylation events, whereas conventionally propagated plants showed 18.4% of methylation events ^{32,39}. This was also documented in previous work on the blueberry, where methylation rate increases in micropropagated plants compared to conventional plants ^{33,34}. Similarly, thidiazuron-induced blueberry calli exhibited an increased methylation rate compared to cutting plants ³⁴. 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 were 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. 1, 2 and Supplementary Table S2). 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. Through MSAP assay, we obtained that the highest methylation polymorphism were 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 ^{33,34}.

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 ⁴⁰. In plants, inhibited DNA methylation could increase or decrease secondary compounds, observed in *Taxus* spp., *Salvia miltiorrhiza*, and *Vitis amurensis* ^{41,42}. Our report exhibited the inverse correlation between DNA methylation with secondary metabolites (Fig. 4). 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 ²⁵. 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^{43–45}. Previous research found higher cytosine methylation occurred with high levels of native secondary metabolites in the autopolyploid *Cymbopogon sprengel*⁴⁶. 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. expression⁴². The expression of the VaSTS10 gene was significantly increased with a decrease in methylation⁴⁶. This study depicted that cytosine methylation has a converse relationship with secondary metabolites of lingonberry among all propagated plant and cultivar.

Conclusion

This study was helpful for the growers to get efficient trait/efficient traits for large-scale cultivation. Genetic evolution was estimated by a molecular marker. 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. Among all tissue culture plants, leaf-culture derived greenhouse plant represents the highest bands of cytosine methylation. 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.

Materials And Methods

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. Node culture-derived shoots were established in vitro following the protocol of ³. 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 ⁴⁷ 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 (Growtektm culture vessels, Fischer Scientific, Ottawa, Ontario, Canada) using 200 ml of the same medium but without agar and gel red (liquid medium) ¹¹. 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 ⁴⁸.

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 ³. 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 following a previous study protocol ⁴⁹. There were 5 plants in each treatment and the experiment was replicated five times.

Data collection:

Morphological data were collected from three randomly selected explants from liquid and semi-solid media, replicated three times for in vitro-grown shoot cultures and greenhouse-grown plants. 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: micropropagated and conventionally propagated plants in greenhouse were collected ¹¹. Shoots vigor and plant vigor were 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 and leaf characteristics were recorded from three 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

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.

Methylation-sensitive amplification polymorphism (MSAP) assay

MSAP assay is the modified version of the AFLP protocol³³. 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. 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 a 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 S1). 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 (Supplementary Table S1). 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. 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. For more details follow supplementary information (Fig.S2).

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 ³⁴. 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 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 ⁵⁰.

Estimation of the total flavonoid content

The flavonoid content of lingonberry samples was analyzed by the colorimetric method ⁵¹. 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. 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 ⁵².

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 ^{53,54}. 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. The scavenging activity was derived from the following formula:

$$\text{DPPH scavenging \%} = [(A_{517\text{nm}}(\text{Blank}) - A_{517\text{nm}}(\text{Extract})) / A_{517\text{nm}}(\text{Blank})] \times 100 \text{ [A=Absorbance]}$$

Estimation of the total proanthocyanidin content

Leaf extract was investigated for determining proanthocyanidin content using the modified vanillin technique ⁵⁵. 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 ⁵². Thus, the absorbance was measured at 500 nm. Total proanthocyanidin (TPrC) content was denoted by CE mg / g flw.

Statistical analysis

In the current studies, the morphological data and MSAP assay were analyzed by using GraphPad Prism 8.0.0 software⁵⁶. For statistical analysis, t and Wilcoxon test were evaluated at $\alpha = 0.05$ for all the parameters⁵⁷. In this study, all morphological data are expressed as the means $\pm$ SD of three replications. The treatment means were compared by the least significant difference (LSD) using the t and Wilcoxon test. Data of secondary metabolites was performed by one way ANOVA with a standard significance threshold of $p < 0.05$. DNA methylation events and secondary metabolites were correlated by python matplotlib⁵⁸.

Declarations

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Author contributions

S.C.D and A.S designed the experiments; A.S, U.S performed the experiments. A.S. and S.C.D conducted and analyzed the MSAP assay and analyzed MSAP profile scoring and secondary metabolites. A.S and U.S conducted the secondary metabolites experiment. A.S, U.S, R.B, A.U.I, and S.C.D prepared, revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Additional information

Supplementary information is available

Competing financial interests: The authors declares no competing financial interests.

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Figures

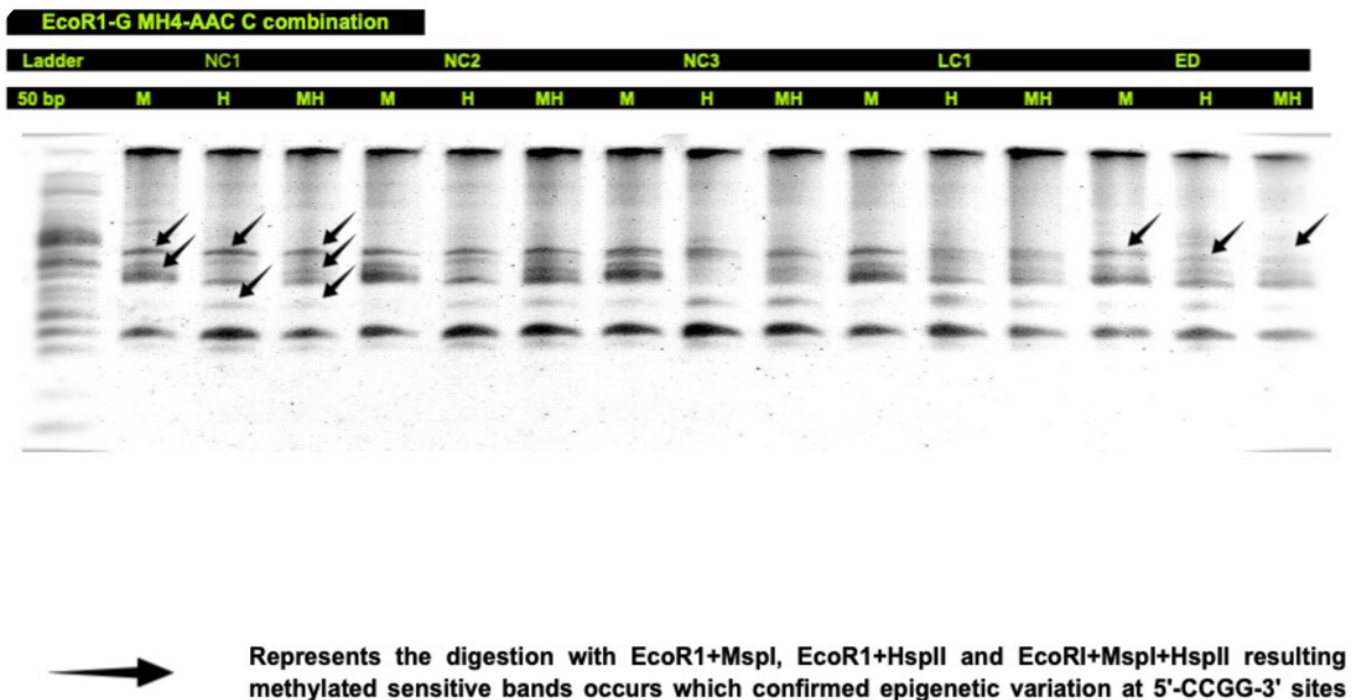


Figure 1

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+HspII, and EcoR1+MspI+HspII, 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'-CmCCGG-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.

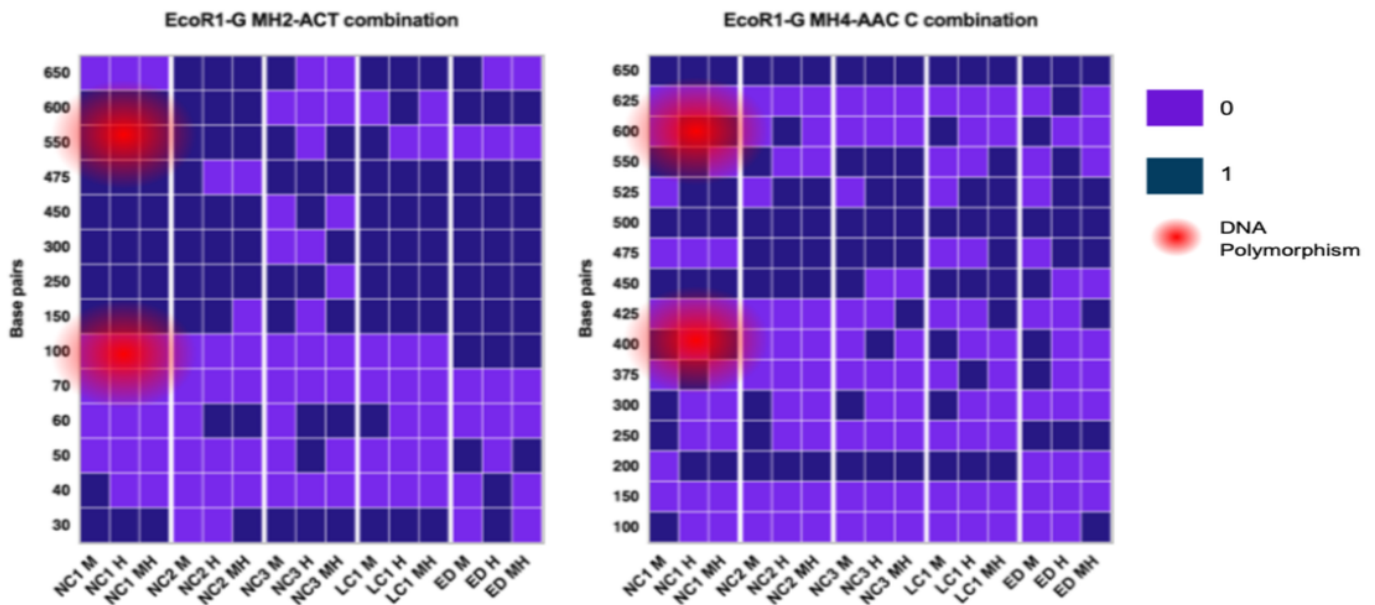


Figure 2

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.

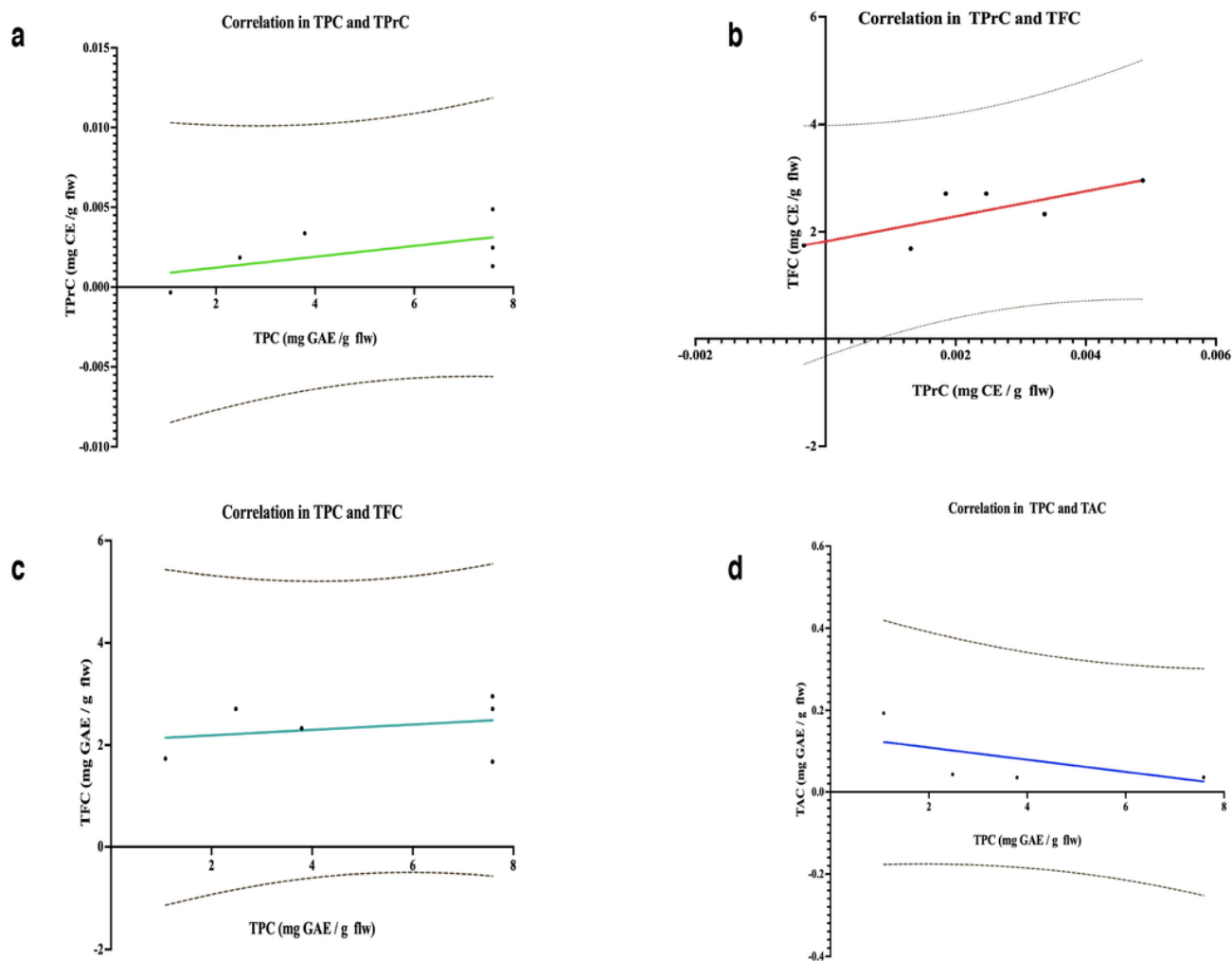


Figure 3

Linear regression in secondary metabolites of cultivar and propagated lingonberry. Data were analyzed based on means $\pm$ 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.

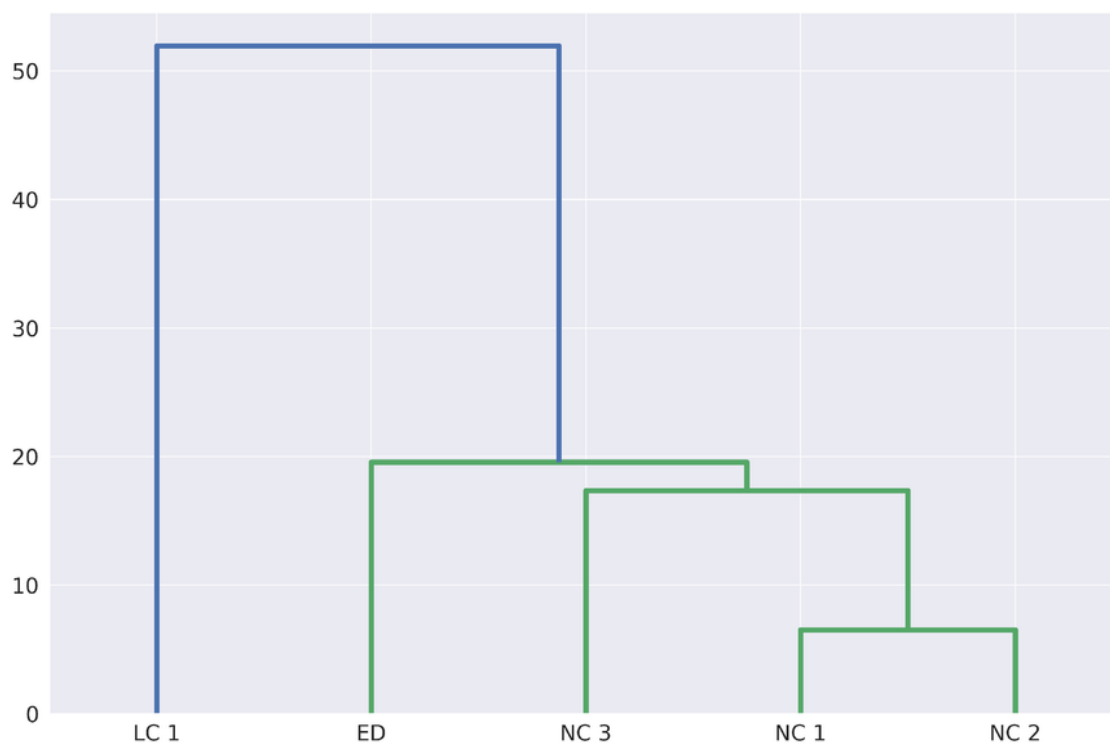
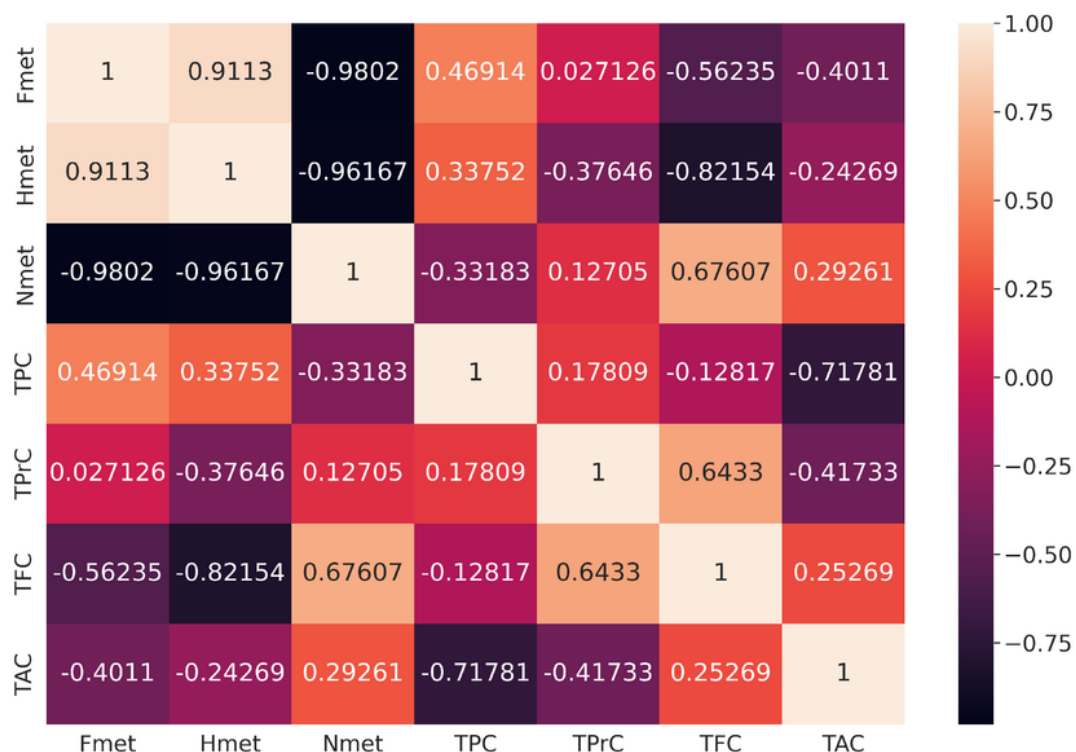


Figure 4

Heatmap represents the partnership between cytosine methylation and secondary metabolites in lingonberry. Dendrogram portraying that invitro propagated plants NC1, NC2, were closely related than NC3, LC1 and ED Fmet refers to full-methylation, Hmet refers to hemi-methylation and Nmet refers to non-methylation. 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 (More details: Supplementary Table S3).

Supplementary Files

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