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Effects of Temperature and Photoperiod on Yield and Chemical Composition of Northern and Southern Clones of Bilberry (*Vaccinium myrtillus* L.)

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ABSTRACT: After pollination outdoors, individual bilberry plants from two Northern and two Southern clones were studied for climatic effects on berry yield and quality in a controlled phytotrone experiment at 12 and 18 °C. At each temperature, the following light treatments were tested: (1) 12 h natural light, (2) 24 h natural light, and (3) 24 h natural light plus red light. The first experimental year there was no difference in yield between temperatures; however, the second experimental year the berry yields was significantly higher at 18 °C. Berry ripening was faster in the Northern than in the Southern clones at 12 °C. Northern clones also showed significantly higher contents of total anthocyanins, all measured anthocyanin derivatives, total phenolics, malic acid and sucrose. Metabolic profiling revealed higher levels of flavanols, hydroxycinnamic acids, quinic acid and carbohydrates at 12 °C.

KEYWORDS: *berry quality, carbohydrates, GC-MS, HPLC-DAD, metabolite profiling, polyphenols, wild berries, climatic effects*

INTRODUCTION

Bilberry (*Vaccinium myrtillus* L.), also called European blueberry[1] is a wild growing perennial dwarf shrub native to northern parts of Europe, Asia, and western parts of North America (USA and Canada). Both berries and leaves have been used as food and medicine in the Nordic countries for thousands of years[2] and today the berries are highly valued on both the European and Asian markets.[3,4] Berry yields vary greatly from year to year[5] and the utilization rate from wild populations reported in Finland ranges as low as 4−6%.[6,7] Attempts to commercialize the production have started in Norway[1] and Denmark.[8] In Finland and Sweden, the utilization of the wild crop is advanced and increasing.[7]

Bilberries can be distinguished from their wild and domesticated relatives in North America (*Vaccinium angustifolium*, *Vaccinium corymbosum*) by a distinct, complex and pleasant flavor,[9−11] and strong bluish fruit flesh and juice.[12,1] The domesticated blueberries are mild in taste and have a translucent juice/flesh. Giovanelli and Buratti[13] reported a 2-fold and 3-fold higher content of total polyphenols and total anthocyanins, respectively, in *V. myrtillus* than in cultivated *V. corymbosum*. Similar findings have been reported by Prior et al.[14] and Riihinen et al.[12] The importance of bioactive compounds in berries relative to human health have been reviewed by Battino et al.[15]

Growth conditions, especially day length, light intensity, and temperature, have a strong impact on the quality of plants. In earlier studies, bilberries growing at Northern latitudes have been shown to contain higher levels of phenolic compounds compared to their southern counterparts.[16−19] Reports on climate effects on quality related attributes in other berry species are numerous; for example, raspberry,[20] black currants,[21,22] strawberry,[23,24] sea buckthorn,[25] and several commercial blueberry cultivars (*Vaccinium* spp.).[26] However, controlled experiments focusing on effect of temperature and day length on quality of berries using clonal plants are still scarce. To our knowledge, such studies have only been performed on cloudberry (*Rubus chamaemorus* L.).[27,28] The aim of the present study was to examine the effect of temperature and day length on the berry production and on the composition of phenolic compounds and carbohydrates in bilberry clones from northern and southern origin.

MATERIAL AND METHODS

Plant Material. The material consisted of individual bilberry (*V. myrtillus* L.) plants from Finland representing two Southern (S1 and S2) and two Northern (N1 and N2) clones originally harvested from wild populations, propagated through tissue culture[29] and planted outside in 1997. The origin of the two Southern clones was Lapinjärvi (60°45′N, 26°05′E), the Northern clone N1 was from Oulu (65°01′N, 25°28′E) and N2 from Muhos (64°46′N, 25°55′E). These clones belong to the outdoors collection of bilberry at the Botanical Gardens of University of Oulu. For the present study, individual bushes presenting the Northern and Southern clones were transported to

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Tromsø, Norway, to be tested under controlled climatic conditions. Plants were grown in pots (30 cm in diameter, 40 cm high) with a mix of turf and sand (1:1), pH 4.8. Each clone was represented by two different individuals per treatment.

Experimental Design. All plants were kept outdoors during flowering to ensure pollination by insects. After pollination, the plants were grown under controlled conditions in a phytotrone in Tromsø, Norway (69°42′N, 18°56′E) at 12° and 18 °C. At both temperatures, 3 different light treatments were tested: (1) 12 h natural light, (2) 24 h natural light, and (3) 24 h natural light with extra red light (ca. 10 μmol cm^{-2} s^{-1}) produced with 60 W lamps (Phillips). The first experiment took place the year the plants were transported to Tromsø (2008). After harvesting was completed, the plants were kept outdoors until the experiment was repeated in 2009 using the same plants that once again were kept outdoors until after pollination. Both the 2008 and the 2009 experiments started the last week of June, when there is midnight sun. Last harvest took place August 26 and 14, for 2008 and 2009, respectively. In August, day length is gradually decreasing with 18 h and 12 min for August 14, to 16 h and 15 min for August 26. Berries were sampled when ripe, weighed, and stored at −80 °C until analyzed.

Bilberry Extraction Procedure. Frozen bilberries (3−6 berries) from the same individual were sliced with a scalpel, and 320 mg of FW (fresh weight) of each sample (n = 3) was transferred to a round-bottom shaped microtube (2 mL). Precooled (−20 °C) methanol (400 μL) (Sigma-Aldrich, Germany) containing ribitol (Fluka, Germany) as internal standard (25 μg/mL) was added to each tube and vortexed for 5 s. Sample tubes were treated for 1 h at 60 °C in an ultrasonic bath, and cooled down to room temperature before the next step. To remove lipids, 200 μL of chloroform (Sigma-Aldrich, Germany) was added, and the tubes were vortexed for 5 s. Additional 400 μL of H_2O (deionized) was added and tubes were vortexed for 10 s. Samples were centrifuged at 18 000g and 4 °C for 10 min. Two aliquots of 300 μL each from the clear supernatant were transferred into two V-shaped 1.5 mL microtubes for GC-MS analysis and to store at −20 °C for later phenol analyses, respectively. Drying of sample extracts and compound derivatization with MSTFA (2,2,2-trifluoro-*N*-methyl-*N*-(trimethylsilyl)acetamide; Fluka, Germany) followed the procedures as described in Sissener et al.[30] Samples were transferred to 1.5 mL autosampler vials with glass inserts, and stored at −20 °C prior to GC-MS analysis.

GC-MS-based Metabolite Profiling. The GC-MS analysis followed the procedure as described in Sissener et al.[30] Detected compounds such as carbohydrates (fructose, glucose and sucrose), acids (malic, citric, and ascorbic acid), polyols (quinic acid and *myo*-inositol) and phenolic structures (gallic acid, chlorogenic acid, catechin and epicatechin) were quantified based on the internal standard ribitol and expressed as milligrams per 100 grams of FW (mg/100 g FW). An Agilent 6890/5975 GC-MS (Palo Alto, CA) was used for all analyses.

High Performance Liquid Chromatography (HPLC-DAD) Analysis on Single Anthocyanins and Hydroxycinnamic Acid Derivates. Analyses have been performed as previously described by Trost et al.[31] and Laaksonen et al.[32] with small modifications for the purpose and instrumentation used. Separation and quantification of anthocyanins and hydroxycinnamic acids were performed using gradient high performance liquid chromatography with the DAD detection. Quantification was made at 520 nm for anthocyanins and at 320 nm for hydroxycinnamic acids. The samples were stable for at least 48 h. Analyses were performed at room temperature with an injection volume of 20 μL. A gradient of mobile phases was used for efficient separation. Mobile phase A was composed from water while mobile phase B was composed from acetonitrile and water 60:40 (v/v). Both mobile phases were acidified with 0.2 vol% TFA (Sigma Germany). The gradient of mobile phase B changed from 10% to 25% in 40 min. In the next minute, the percentage of mobile phase B increased from 25% to 100%. Afterward gradient was steady for 4 min. In the end, equilibration to initial concentration was established. A flow rate through the gradient of 0.7 mL/min was used. All analyses were duplicated. Analyses were made with Waters Alliance chromatographic system with 2998 Photodiode Array (PDA) detector (Waters Corporation). Individual anthocyanins were quantified as cyanidin 3-glucoside equivalents (k = 53173; R^2 = 99.94%; DL = 0.01 mg/L; QL = 0.3 mg/L) while individual hydroxycinnamic acids were quantified as chlorogenic acid equivalents (k = 67733; R^2 = 99.98%; DL = 0.1 mg/L; QL = 0.4 mg/L). Individual hydroxycinnamic acid derivates were separated on Nova-Pak Column (C 18, 3.9 × 150 mm; Waters Corporation). Analysis on single anthocyanins and hydroxycinnamic acid derivates has only been done on samples from 2009.

Total Phenolics (TPH). The analysis of total phenolics content was based on a modified Folin-Ciocalteu method.[33] Berry extracts (see Bilberry Extraction Procedure) were diluted 1:40 in methanol before incubation at ambient temperature for 2 h. Samples (200 μL) were transferred to a clear 96-well microplate, and the absorption was measured at 750 nm on a plate reader (Labsystems Multiskan MS, Finland). Total phenolics were expressed as milligrams of gallic acid equivalents (GAE) per 100 grams of FW of berries (mg GAE/100 g FW of berries).

Total Anthocyanins (ACY). Total anthocyanin content in berry samples was analyzed using a modified pH-differential method as described by Giusti and Wrolstad.[34] Buffers of pH 1 (0.025 M) and pH 4.5 (0.4 M) were based on potassium chloride (KCl) and sodium acetate ($C_2H_3NaO_2$), respectively, and pH adjusted with hydrogen chloride (HCl) (all chemicals from Sigma-Aldrich, Germany). Berry extracts (see Bilberry Extraction Procedure) were diluted 1:40 in methanol, added to 0.5 mL of each buffer, and measured spectrophotometrically at wavelengths 510 and 700 nm. Results were expressed as milligrams of cyanidin 3-glucoside per 100 grams of FW (mg cyanidin 3-glucoside/100 g FW).

Antioxidant Activity (AOX). Antioxidant activity of berries was measured using the ferric reducing ability of plasma (FRAP) method[35] with some modifications. Briefly, berry extracts (see Bilberry Extraction Procedure) were diluted 1:40 in methanol. Samples (5 μL) were added to 300 μL FRAP reagent on a clear 96-well microplate, shaken and incubated for 4 min. Absorption was measured at 595 nm on a plate reader (Labsystems Multiskan MS, Finland), and expressed as millimoles of ferric iron reduced (Fe^{2+}) per 100 grams FW (mmol Fe^{2+}/100 g FW).

Statistics. Main statistical analysis was conducted by the GLM procedure of the Minitab software. Main effects of origin, clone (within origin), temperature, light and year as well as their interactions were tested. Correlations between single compounds or compound groups were visualized using a distance heat map with hierarchical clustering (Pearson's correlation, average linkage) generated with MultiExperiment Viewer software v.4.8.0.[36] Log2 (n) ratio values for heat map clustering were based on the median compound level of individual components including the following data from trial year 2009: metabolites from GC-MS analysis (11 compounds), HPLC-DAD (16 compounds), and data from TPH, ACY, and AOX analyses.

RESULTS AND DISCUSSION

Berry Yield. Berries were picked when mature. In 2008, the first berries were picked on July 22, while the last berries were picked on August 26. In 2009, the harvest season lasted from July 27 to August 14. In 2008, there were no significant differences in total berry yield between plants grown at 12 °C (158 g) and plants grown at 18 °C (151 g) (Table 1). However, when the experiment was repeated in 2009, the

Table 1. Berry Yield at 12 and 18 °C[a]

	12 °C			18 °C		
	Northern	Southern	total	Northern	Southern	total
2008	71.3	87.0	158.3	102.4	49.1	151.5
2009	144.8	107.8	252.6	289.2	284.4	573.6
Total	216.1	194.8		391.6	333.5	

[a]Results are presented by each year and represent total berry production (g) of all Northern and Southern clones.

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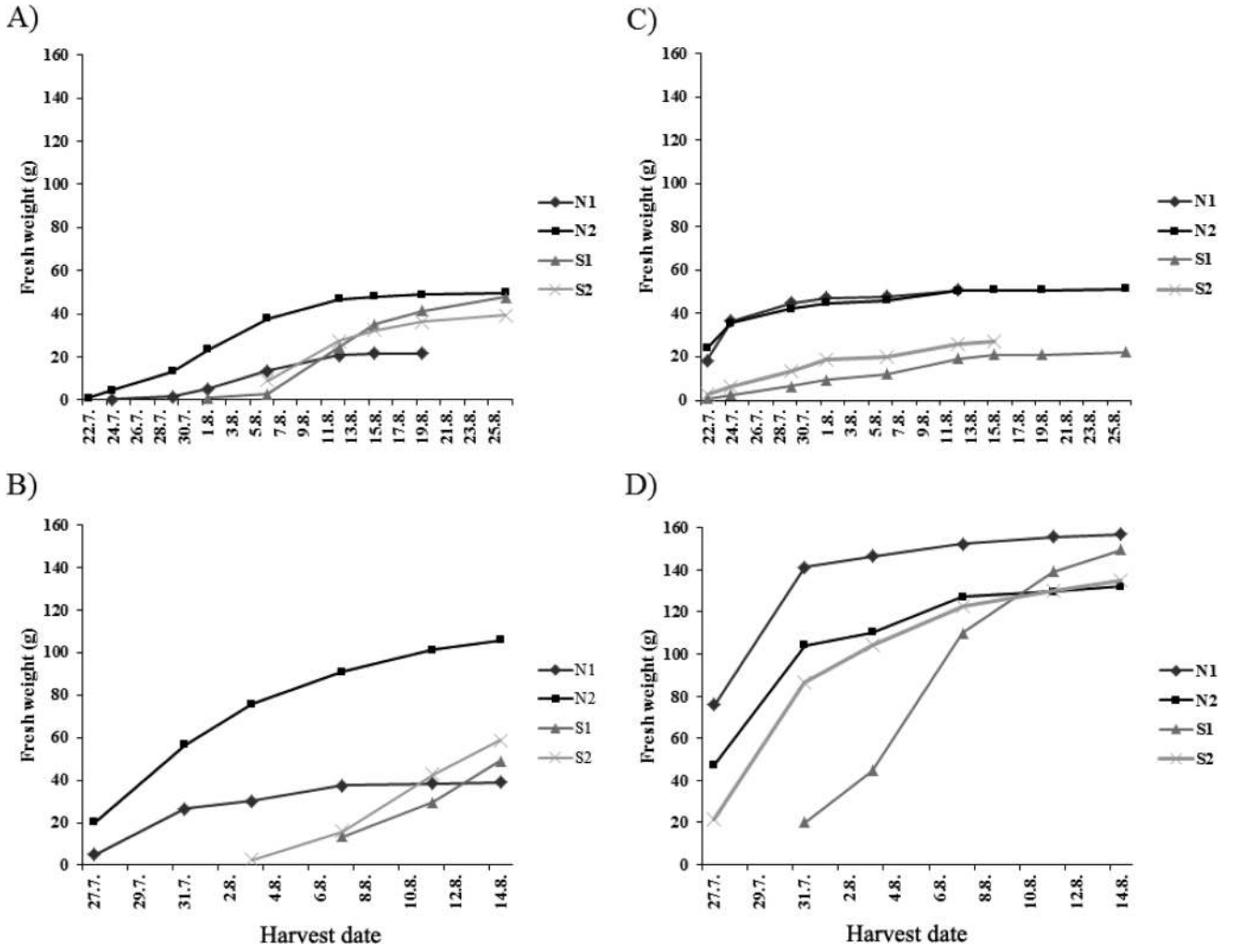

Figure 1. Berry yield in grams from the first harvest (June 22, 2008 and June 27, 2009) to the last harvest (in 2008 on August 25, and in 2009 on August 14). Results are presented for the two Northern clones (N1 and N2) and for the two Southern clones (S1 and S2). At each treatment, there were 1 or 2 individuals per clone. (A) 12 °C 2008; (B) 12 °C 2009 ; (C) 18 °C 2008; (D) 18 °C 2009.

Table 2. Main Effects of Year, Origin, Temperature, and Light on the Level of Different Compounds in 2008 and 2009[a]

	effect of year			effect of origin			effect of temperature			effect of light			
	2008	2009	*p*	N	S	*p*	12 °C	18 °C	*p*	12 h	24 h	24 h + *R*	*p*
malic acid (mg/100 g FW)	312.3	658.4	***	540.9	340.6	***	380.5	484.9	***	461.8	431.8	447.2	*
citric acid (mg/100 g FW)	1285.5	1030.0	***	1245.4	1119.2		1182.5	1188.3		1172.4	1181.5	1212.4	
quinic acid (mg/100 g FW)	1578.8	2655.4	***	1713.3	2317.9	***	2321.4	1811.4	***	1911.7	2014.5	2094.0	
gallic acid (mg/100 g FW)	0.8	0.4	***	0.7	0.7		0.7	0.6		0.6	0.7	0.6	
chlorogenic acid (mg/100 g FW)	31.7	26.9	***	22.9	37.6	***	36.2	26.1	***	28.7	29.2	32.3	
ascorbic acid (mg/100 g FW)	3.0	1.3	***	2.7	2.0		1.9	2.6		2.7	2.0	2.2	
fructose (mg/100 g FW)	5004.0	6329.0	***	5477.0	5567.0		6080.0	5198.0	**	5534.0	5443.0	5608.0	
glucose (mg/100 g FW)	5041.0	4503.0	***	4754.0	4919.0		5396.0	4508.0	**	4770.0	4749.0	5039.0	
sucrose (mg/100 g FW)	525.7	923.8	***	771.7	577.4	***	909.5	549.1	***	652.2	667.0	739.8	
myo-inositol (mg/100 g FW)	216.2	325.8	***	244.5	274.9	**	288.3	241.9	***	259.1	249.4	271.9	
epicatechin (mg/100 g FW)	20.5	8.9	***	14.9	17.3	**	20.2	13.6	***	16.0	15.8	16.3	
catechin (mg/100 g FW)	5.0	2.5	***	4.2	3.8		4.6	3.6	**	4.4	3.6	4.1	
Total Phenolics (mg/100 g FW)	566.5	364.6	***	520.6	451.2	***	499.7	481.3		502.0	483.5	474.6	
Total Anthocyanins (mg/100 g FW)	143.6	269.6	***	234.8	144.8	***	179.3	200.2	**	193.8	189.4	195.4	
AOX (mmol 100 g^{-1} FW)	4.8	4.9		5.3	4.3	***	4.9	4.8		5.1	4.7	4.8	

[a] ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$

production was significantly higher at both temperatures, and this time the production was much higher at 18 °C (574 g) compared to 12 °C (253 g). All plants were stored outside the phytotrone in Tromsø covered by snow between the 2008 and

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Table 3. Main Effects of Origin, Temperature, and Light on the Level (mg/100 g FW) of Different Compounds for the Additional Analysis on Anthocyanins and Hydroxycinnamic Acid Derivates in 2009[a]

compound	N	S	*p*	12 °C	18 °C	*p*	12 h	24 h	24 h + *R*	*p*
Cyanidin 3-Arabinose	44.0	37.0	**	41.2	40.1		39.0	41.9	40.6	
Cyanidin 3-Galactose	59.5	34.2	***	42.0	49.8	***	46.2	49.2	44.4	
Cyanidin 3-Glucose	50.9	41.0	**	41.1	48.9	***	44.6	48.8	43.8	
Delphinidin 3-Arabinose	87.8	57.5	***	85.4	65.0	***	62.0	76.5	82.8	***
Delphinidin 3-Galactose	98.9	45.7	***	77.2	69.4		65.7	76.1	76.5	**
Delphinidin 3-Glu	76.4	54.6	***	70.6	62.4		57.8	70.3	69.9	***
Malvidin 3-Arabinose	9.6	2.6	***	4.2	7.3	***	4.7	6.4	7.8	***
Malvidin 3-Galactose	34.2	13.3	***	16.2	28.3	***	20.5	26.2	25.0	**
Malvidin 3-Glucose	46.8	16.6	***	25.0	35.7	***	26.4	33.9	36.4	**
Peonidin 3-Galactose	4.8	2.1	***	2.1	4.3	***	3.0	4.0	3.5	**
Peonidin 3-Glucose	17.7	9.3	***	12.7	13.9	**	11.2	14.5	15.4	***
Petunidin 3-Galactose	26.3	10.0	***	16.0	19.4	***	15.9	19.5	19.5	***
Petunidin 3-Glucose	45.3	25.9	***	33.8	36.7	**	30.8	38.6	38.3	***
SUM AC	602.2	349.8	***	467.5	481.2		427.8	505.9	503.9	***
chlorogenic acid	36.4	56.9	***	62.5	37.2	***	41.1	48.4	52.6	**
hydroxycinnamic acid derivate 1	7.4	14.2	***	12.6	10.3	*	11.3	10.8	11.2	
hydroxycinnamic acid derivate 2	21.0	31.2	***	32.4	22.4	***	25.1	26.8	26.7	
SUM HC	64.8	102.3	***	107.5	69.9	***	77.5	86.0	90.5	*

[a]***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$

2009 growth seasons. Before the first repeat in 2008, plants had overwintered in Oulu, Finland. Most importantly, the treatments given during the first year have influenced the production of the flower initials. The higher berry yield at 18 °C in the second year can be explained by a much better production of flower buds at this temperature the preceding season. Bilberry produce flower initials the year before actual flowering.[37,1] Since pollination took place outside before the pots were transferred to the different treatments in the phytotrone, availability of insects for pollination could explain difference in yield between the two years. The average temperature during pollination was 8.5 °C in 2008 and 7.9 °C in 2009.

When the clonal origin was considered at the two different cultivation temperatures, berry ripening turned out to be faster at 12 °C in the Northern clones than in the Southern ones (Figure 1). The Northern clones produced ripe berries more than a week earlier at 12 °C than the Southern clones while there were small differences between the clones at 18 °C. This indicates that the Northern clones are better adapted to low temperatures. In 2008, the Southern clones produced slightly higher yields than the Northern at 12 °C, while in 2009, the Northern clones produced the highest yields. At 18 °C, the Northern clones yielded best in 2008, while the production was equal in 2009 (Table 1). The differences in yields between years and clones are not consistent and therefore difficult to explain, but the results indicate that Northern and Southern clones have unequal climate requirements for flower bud formation.

Phenolic Compounds. *Anthocyanins.* Total anthocyanin content was significantly higher in Northern clones (Table 2) as also previously reported by Lätti et al.[17] They analyzed anthocyanins from 20 different populations on a south-north axis in Finland and found significantly higher levels in berries produced in Northern regions. Similar trend with increasing anthocyanidin levels toward north was detected in bilberries growing in Sweden.[19] Moreover, a common garden trial with bilberry clones from different origins showed that the Northern clones had the highest yields of anthocyanidins even when growing in the same site as the Southern clones.[19] These results are consistent with our observation, and suggest the existence of latitude related genetic adaptation in anthocyanin production of berries.

In the present study, the anthocyanin levels were significantly higher at 18 °C than at 12 °C and higher in 2009 than in 2008 (Table 2). The higher anthocyanin content at 18 °C was due to the Northern clone;, the Southern clones produced equal amounts of anthocyanins at both temperatures ($p = 0.002$). There was also an interaction between light and origin. The Northern clones produced highest levels of anthocyanins at 24 h with addition of red light and lowest at 24 h light, while the Southern clones showed opposite results ($p = 0.032$). It is possible that the Northern clones are more responsive to additional red light, which has been detected in *Arabidopsis thaliana* populations of different origins.[38] Also the ratio of red to far-red light can affect the anthocyanin biosynthesis differently in plants of the same species but with different origin, as has been shown in *Stellaria longipes*.[39]

In Table 3, additional analyses on anthocyanin- and hydroxycinnamic acid derivatives levels from berries harvested in year 2009 are presented. In accordance to the results on total anthocyanin levels, levels of all measured anthocyanin derivatives were significantly higher in Northern clones than in Southern clones. Except Del 3-Ara that was significantly highest in berries grown at 12 °C, berries produced at 18 °C had significantly higher levels of most anthocyanin derivatives. Both temperature and origin had different effects on the levels of the different anthocyanin derivatives. The Southern clones produced quite equal levels of anthocyanin derivates at both temperatures, except of Del 3-Glu, Del 3-Ara and Del 3-Gal, which had the highest levels at 12 °C. The Northern clones produced higher levels at 18 °C, again with the exception of Del 3-Glu, Del 3-Ara and Del 3-Gal. For Del 3-Gal and Del 3-Glu the production was equal at both temperatures, while for Del 3-Ara, the levels were highest at 12 °C. Lätti et al.[17] found that delphinidin glycosides dominated in berries from northern

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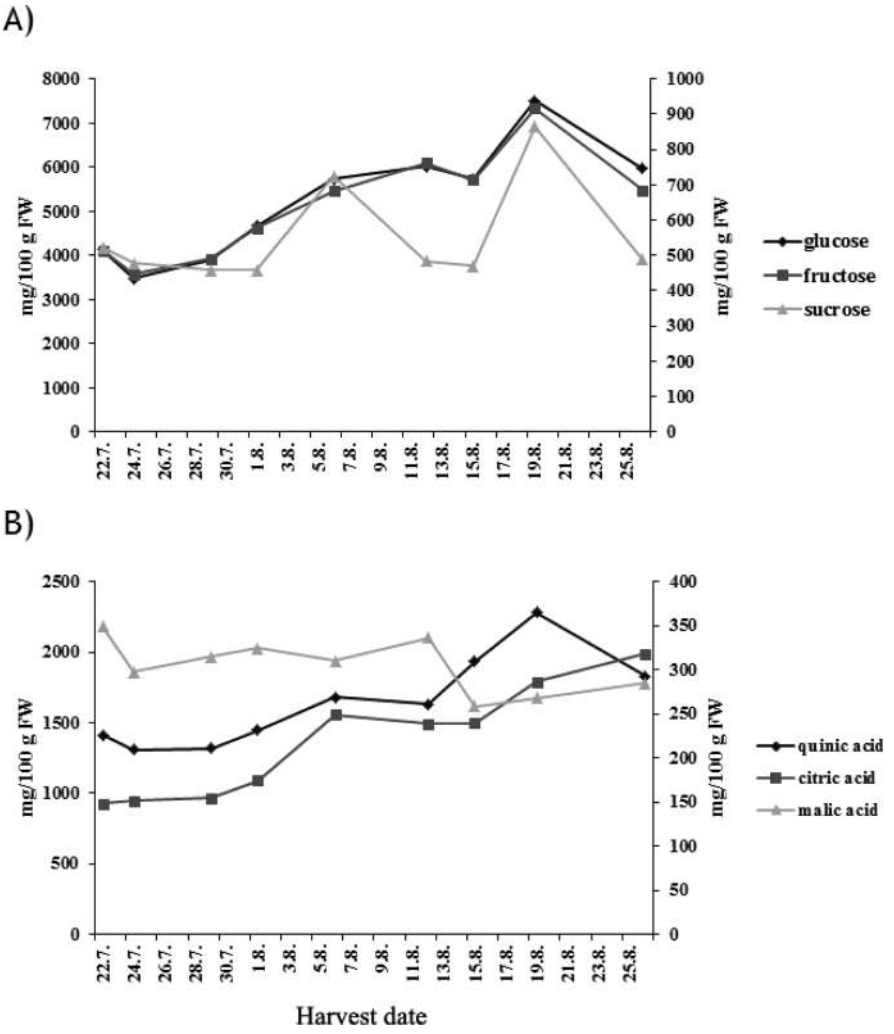

Figure 2. Content of the carbohydrates glucose, fructose (*y*-axis on the left) and sucrose (*y*-axis on the right), and quinic acid, citric acid (*y*-axis on the left) and malic acid (*y*-axis on the right) in berries picked in 2008 expressed as mg/100 g FW. All berries were picked at maturity, the first ones on June 22 and the last ones on August 26. Results are mean of all clones harvested at respective dates.

regions whereas cyanidine glycosides were most common in southern regions. The results of the present study also indicate a positive effect of low temperatures on levels of delphinidin glycosides. In addition, the results show that long days (24 h light and/or 24 h light with additional red light) significantly increased levels of all measured anthocyanin derivatives except Cy 3-Ara, Cy 3-Gal and Cy 3-Glu (Table 3). This result can also explain earlier findings[17,19] that cyanidin glycosides are most common in bilberries from Southern regions. Higher levels of delphinidin glycosides were also detected in bog bilberries growing in North Finland.[18] Similarly, in black currant, the varieties from Scandinavia had more delphinidin glycosides while British varieties were dominated by cyanidin glycosides.[40] Contradictory results have been reported by Martinelli et al.[16] who found higher contents of cyanidin glycosides in bilberries from Norway and Sweden than in berries from Italy and Romania, while delphinidin glycosides were higher in Italian and Romanian bilberries.

Flavanols. The concentration of flavan-3-ols, (−)-epicatechin and (+)-catechin, the monomeric units of proanthocyanidins, were significantly higher in berries growing at 12 °C. The earlier reports on the effect of temperature on flavanol contents are scarce. In tea (*Camellia sinensis*) leaves, increase in (+)-catechin levels has been detected along decreasing temperatures.[41,42] Berries from Southern clones had significantly more epicatechin. For catechin conten,t we did not find any effect of origin, but the clonal effect was obvious in the case of one southern clone having significantly higher levels of catechins than all the other clones studied.

Simple Phenolics and Polyphenols. Northern clones had significantly higher levels of both total phenolics and total anthocyanins (Table 2) and this was reflected in a significantly higher level of antioxidant activity as well. Level of antioxidant activity did not differ between years, but there was an interaction between year and origin where the Northern clones showed highest levels in 2009, while the Southern clones had highest levels in 2008 (p = 0.005). There was also an interaction between temperature and light where at 12 °C the levels were highest at long days, whereas at 18 °C short days gave the highest levels (p = 0.025). A study on blackberry cultivars in North America concluded that antioxidant activity mainly depended on the genotype and not on the climate or the season,[43] while Jousuttis et al.[44] found that antioxidant capacity in three different genotypes of strawberry was generally increased with higher latitudes. Interactions between genotype and response to environmental stress have been demonstrated

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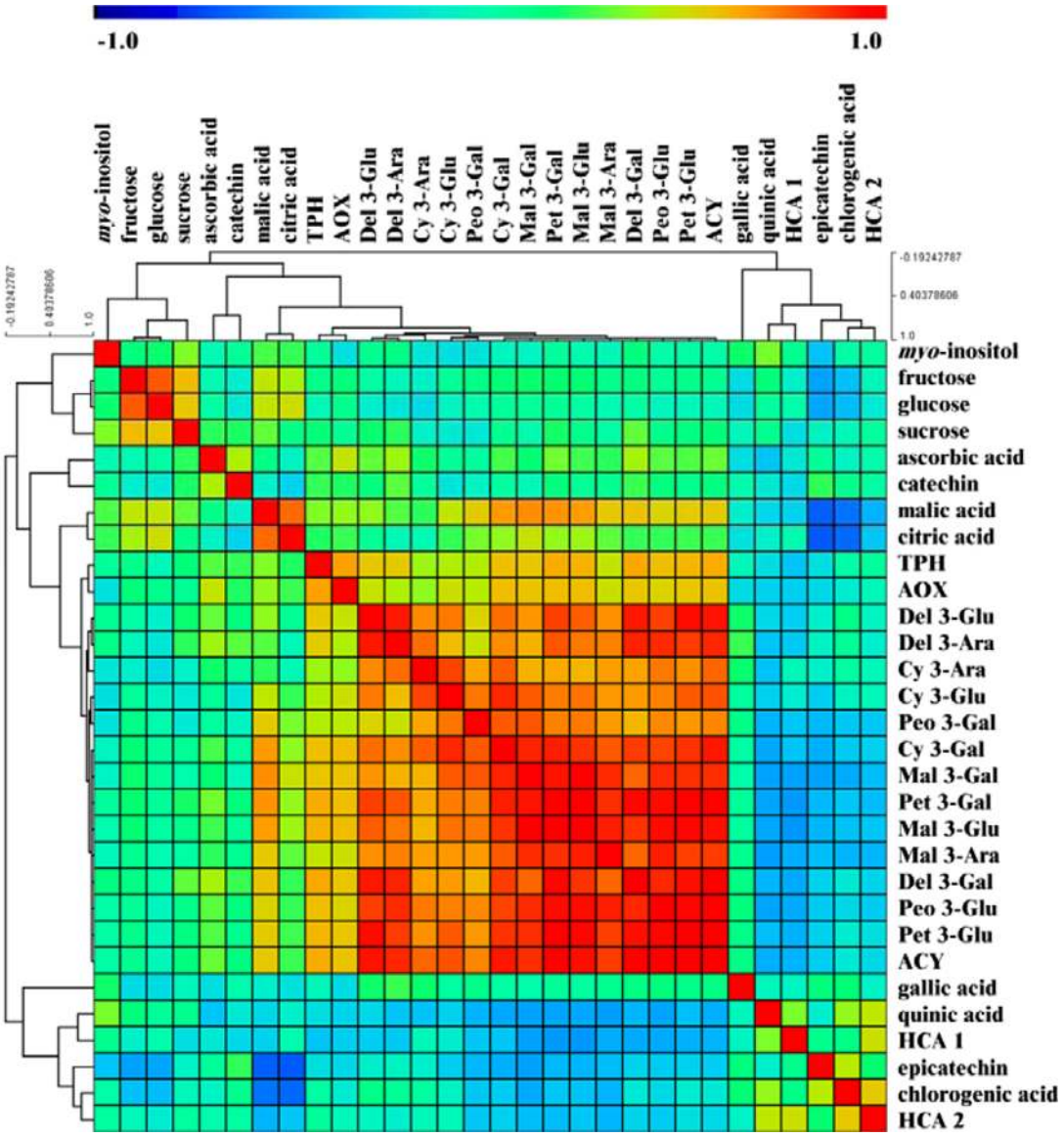

Figure 3. Distance heat map showing correlations and clustering of metabolites from GC-MS analysis (11 compounds), HPLC-DAD (16 compounds), and data from TPH (total phenols), ACY (total anthocyanins), and AOX (antioxidant activity). Abbreviations: HCA1 (hydroxycinnamic derivate 1) and HCA 2 (hydroxycinnamic derivate 2).

in strawberry by Tulipani et al.,[45] and some of the genotypes were clearly more affected by stress than others.

The additional analysis on hydroxycinnamic acids (Table 3) showed that the concentration of chlorogenic acid and the hydroxycinnamic acid derivatives were significantly higher in berries growing at 12 °C. Hydroxycinnamic acid derivatives and chlorogenic acids were also significantly higher in berries from the Southern clones. This is in consistence with the earlier results on bilberry leaves. Martzt et al.[46] analyzed the phenolic compounds in bilberry leaves from 116 growth sites from south to north (60°00′N to 69° 60′N) in Finland. The results indicated higher yields of all phenolic compounds toward north, except chlorogenic acid and hydroxycinnamic acid derivatives, which were higher in the leaves of Southern bilberry clones. Long photoperiod, compared to 12 h photoperiod, enhanced the levels of chlorogenic acid.

Acids. Malic acid was highest in berries produced at 18 °C. On the contrary, levels of quinic acid were higher in berries produced at 12 °C (Table 2). Temperature did not affect levels of the other analyzed acids (citric acid, ascorbic acid and gallic acid), but for citric acid there was an interaction between origin and temperature where the Northern and Southern clones produced equally at 12 °C, but the production of Northern clones was higher than that of the Southern ones at 18 °C (p = 0.045). Berries from Northern clones had significantly more malic acid, while berries from Southern clones had significantly more quinic acid. On the contrary, Zheng et al.[47] reported that the content of malic acid was higher in *Ribes* sp. cultivars grown in southern part of Finland than in North Finland. The only significant effect of light treatment was that berries produced under short days (12 h) had significantly higher levels of malic acid than berries produced under long days. For quinic acid, there was an interaction between temperature and light treatments; at 12 °C, there was no differences between the light treatments, but at 18 °C, long days gave higher contents (p = 0.000).

Contents of quinic acid and citric acid increased throughout the season (2008), while the levels of malic acid were quite

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stable (Figure 2). All berries were picked at mature stage; however, it is likely that the berries picked in the beginning of the season were less mature than berries picked later. Differences in acid content throughout the season have also been reported before indicating lower content of most acids in overripe berries than in unripe.[48,49]

Carbohydrates. Levels of the carbohydrates *myo*-inositol, fructose, glucose and sucrose were significantly higher at 12 °C than at 18 °C (Table 2). A positive correlation between low temperatures and levels of carbohydrates has been reported in strawberry,[23] while a negative correlation has been reported in *Ribes*.[47] Berries from Southern clones had significantly more *myo*-inositol while berries from Northern clones had significantly higher levels of sucrose. On the contrary, there were no effect of origin on levels of fructose and glucose. There was an interaction between temperature and light treatment for *myo*-inositol. At 12 °C, contents were highest at short days, whereas at 18 °C, the levels were highest at long days with additional red light ($p = 0.000$).

Contents of the carbohydrates glucose and fructose increased throughout the harvesting period and dropped at the very last harvesting day in late August while the sucrose content was fluctuating more throughout the season (Figure 2). In 2008, time to mature berries varied from 28 to 63 days after the plants were transferred from outdoors to the phytotron. Results in Figure 2 showing an increase in fructose and glucose throughout the season might indicate that the first berries picked were not fully ripen and/or that the sugar content increases along the ripening process. An early study by Uhe[50] concluded that the largest blueberries are the sweetest. There was a strong positive relation between size and sugar content and the content increased between the first and second picking, followed by a decrease in sugars between the second and third picking. However, Davik et al.[23] reported that total sugar content appeared to be stable throughout the harvesting seasons of strawberries picked at different geographical origins in Norway. Howard et al.[51] found that fruit weight of five commercial cultivars of blueberry correlated negatively with antioxidant activity and all measured phenolics. Additionally, the fluctuating levels of sucrose measured could be explained by the fact that the berries harvested at some time points could be from a few clones and that the fluctuations could be explained by clonal differences in sugar content.

Correlations. Figure 3 shows clustering and correlations between the analyzed compounds. Carbohydrates, hydroxycinnamic acids and anthocyanins together with total phenolics and antioxidants group nicely, while other phenolic compounds and acids show more variation in their clustering. Acids partly cluster together with the group of anthocyanins, phenols and antioxidants together with catechin and partly together with the hydroxycinnamic acids and epicatechin. This clustering is reflected in the correlations, where the anthocyanin derivatives were positively correlated with values ranging from 0.40 to 0.97 with the mean correlation between the derivatives as high as 0.77. Likewise, correlations between total anthocyanins and the different anthocyanin derivates were also highly positive, ranging from 0.46 to 0.89 with a mean of 0.76. There were also quite strong correlations between anthocyanins and total phenolics, antioxidant capacity, malic and citric acid. Anthocyanins showed negative correlation with quinic acid and the hydroxycinnamic acids. The carbohydrates glucose, fructose and sucrose showed high positive correlation, while *myo*-inositol showed more moderate values. Levels of carbohydrates correlated slightly with levels of phenolic compounds except for epicatechin where there was a negative correlation. Carbohydrates were on the other hand positively correlated with malic and citric acids, underscoring the close relationship between central metabolites of the glycolysis/gluconeogenesis pathway and the citric acid cycle.

Evaluation of the Main Factors. All analyzed compounds (Tables 2 and 3) were significantly affected by the year of the repeat, with the exception of antioxidant activity. The experiment was conducted under natural light conditions and therefore light intensity varied between the two growing seasons. Average number of hours with sun per day was 7.8 and 8.1 for the duration of the experiment in 2008 and 2009, respectively. The difference is rather minimal and we do not expect this to contribute to the observed difference between the years. The plants were also one year older, and as shown by the yields, affected by the first season's treatment.

Significant effect of light was found on levels of malic acid as well as most of the individual anthocyanin derivatives and chlorogenic acid. The production was higher on long days for all of these compounds except for malic acid where short days gave the highest levels. In addition to these direct effects, there were several interactions between light and other factors.

All carbohydrates showed higher levels at 12 °C than 18 °C. Likewise, the contents of flavonols and hydroxycinnamic acids were also higher at 12 °C. The acids with significant effect of temperature showed opposite effects, where malic acid was highest at 18 °C and quinic acid was highest at 12 °C. Total anthocyanins as well as most anthocyanin derivatives had highest levels at 18 °C. The exception here was Del 3-Ara, which was higher at 12 °C and Cy 3-Ara, Del 3-Gal and Del 3-Glu which were not significantly affected.

Effects of origin showed that the content of all anthocyanin derivatives, as well as levels of antioxidants and total phenolics, were highest in the Northern clones. Hydroxycinnamic acid contents were highest in the Southern clones. Northern clones had more malic acid and sucrose, while higher levels of quinic acid, *myo*-inositol and epicatechin were found in Southern clones.

Number of clones were restricted to four clones: two from north and two from south of Finland. The two Southern clones were from the same geographical area. With this small number of clones representing north and south, it might be difficult to distinguish the effect of origin from the clonal effects. However, previous studies (e.g., Åkerström et al.[19]) strongly support our findings on the effects of origin.

The presented results indicate that bilberries from Northern areas are sweeter in taste than bilberries from Southern areas, and that this could be explained both by cool temperatures and genetic factors.

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Notes

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■ ABBREVIATIONS

AOX, antioxidant activity; Cy 3-Ara, cyanidin 3-arabinose; Cy 3-Gal, cyanidin 3-galactose; Cy 3-Glu, cyanidin 3-glucose; Del 3-Ara, delphinidin 3-arabinose; Del 3-Gal, delphinidin 3-galactose; Del 3-Glu, delphinidin 3-glucose; Mal 3-Ara, malvidin 3-arabinose; Mal 3-Gal, malvidin 3-galactose; Mal 3-Glu, malvidin 3-glucose; Peo 3-Gal, peonidin 3-galactose; Peo 3-Glu, peonidin 3-glucose; Pet 3-Gal, petunidin 3-galactose; Pet 3-Glu, petunidin 3-glucose

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