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Laura Zoratti

EFFECT OF ENVIRONMENTAL, DEVELOPMENTAL AND GENETIC FACTORS ON FLAVONOID AND CAROTENOID PROFILE OF *VACCINIUM* BERRIES

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EFFECT OF ENVIRONMENTAL, DEVELOPMENTAL AND GENETIC FACTORS ON FLAVONOID AND CAROTENOID PROFILE OF *VACCINIUM* BERRIES

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Abstract

Vaccinium berries contain high yields of antioxidant compounds, such as flavonoids and carotenoids, which are recognized to benefit human health. Therefore, commercial interest in cultivated and wild *Vaccinium* berries is increasing globally.

Flavonoids and carotenoids are important secondary metabolites, the biosynthesis of which is regulated by interaction of the genetic background of the plant and the surrounding environment. In fruits the production of secondary metabolites has also a tight linkage with developmental processes. The present thesis is focused on developmental and environmental factors affecting the biosynthesis of carotenoids and flavonoids in berries of the genus *Vaccinium*.

In bilberry altogether eight carotenoids were detected, with lutein and β-carotene being the most abundant compounds, accompanied by minor amounts of xanthophylls, such as antheraxanthin, neoxanthin, violaxanthin and zeaxanthin. During ripening the accumulation of carotenoids decreased. Simultaneously the expression of a carotenoid cleavage dioxygenase (*VmCCD1*) gene increased, indicating degradation of carotenoids during the fruit development. Simultaneously, flavonols and anthocyanins accumulated during the last stages of berry ripening.

Environmental factors had a marked effect on the anthocyanin profile of ripe bilberries. Especially lower temperatures affected more on the accumulation of the delphinidin-based anthocyanidins than on the cyanidin-based ones. However, the spectral light composition also played a role, as an increased proportion in blue wavelengths increased the delphinidin-based anthocyanins at ripe stage. The effect of light quality on other phenolic compounds and carotenoid accumulation was less pronounced. The present results can be applied to cultivation of *Vaccinium* species in order to produce berries with high nutritional value.

Keywords: altitude, anthocyanins, bilberry, blueberry, carotenoids, flavonoids, light quality, solar radiation, temperature, *Vaccinium*

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Tiivistelmä

Vaccinium-suvun marjat sisältävät runsaasti antioksidatiivisiä yhdisteitä, kuten flavonoideja ja karotenoideja, joilla on viimeaikaisissa tutkimuksissa havaittu lukuisia terveysvaikutuksia. Tämän johdosta kiinnostus sekä viljeltyjä mustikoita että metsämustikoita kohtaan on kasvanut maailmanlaajuisesti.

Flavonoidit ja karotenoidit ovat kasvien tuottamia sekundäärimetabolian yhdisteitä, joiden biosynteesin säätelyyn vaikuttavat sekä kasvin geneettinen tausta että ympäristötekijät. Hedelmissä ja marjoissa sekundääriyhdisteiden tuoton säätelyyn vaikuttaa huomattavasti myös kehityksellisten vaiheiden eteneminen. Tässä väitöskirjatyössä selvitettiin marjan kehitykseen liittyvien tekijöiden sekä ympäristötekijöiden vaikutusta flavonoidien ja karotenoidien biosynteesiin sekä viljelyillä mustikoilla että metsämustikoilla.

Metsämustikoista tunnistettiin kahdeksan erilaista karotenoidi-yhdistettä, joista luteiini ja β-karoteeni esiintyivät yleisimpinä ksantofyllin, antheraksantiinin, neoksantiinin, violaksantiinin ja zeaksantiinin ohella. Karotenoidien pitoisuudet olivat suurimmillaan marjankehityksen alkuvaiheessa ja laskivat kypsyvissä marjoissa. Vastaavasti karotenoideja pilkkovan *VmCCD1* entsyymigeenin ilmeneminen lisääntyi kohosi marjan kypysymisen loppuvaiheessa. Antosyaanien ja myrisetiini-pohjaisten flavonolien pitoisuudet sen sijaan kohosivat kypsymisen aikana.

Tässä tutkimuksessa ympäristötekijöillä osoitettiin olevan suuri vaikutus antosyaanien laadulliseen kertymiseen, sillä delfinidiini-tyypin antosyaanien pitoisuudet muuttuivat enemmän ympäristötekijöiden vaikutuksesta verrattuna syanidiini-tyypin antosyaaneihin. Alhaisempi lämpötila yhdessä spesifisten valo-olosuhteiden kanssa lisäsi eniten delfinidiini-tyypin antosyaanien tuottoa. Myös valon laadulla voitiin osoittaa olevan vaikutusta antosyaanien biosynteesiin. Sininen valo lisäsi erityisesti delfinidiini-tyypin antosyaanien tuottoa kypsissä marjoissa. Flavonolien ja karotenoidien tuottoon valon laadun vaikutus oli sen sijaan vähäisempää. Väitöskirjatyön tuloksia voidaan hyödyntää marjojen viljelyssä haluttaessa vaikuttaa marjojen ravitsemukselliseen koostumukseen.

Asiasanat: altitudi, antosyaanit, auringon säteily, flavonoidit, karotenoidit, lämpötila, mustikka, pensasmustikka, *Vaccinium*, valon laatu

To my family and dearest friends

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Oulu, October 2015 Laura Zoratti

Abbreviations

a.s.l.	above sea level
ABA	abscisic acid
AC	anthocyanin
ANR	anthocyanidin reductase
ANS	anthocyanidin synthase
Ara	arabinoside
BCH	β-carotene hydroxylase
bHLH	basic helix-loop-helix
CCD	carotenoid cleavage dioxygenase
CHS	chalcone synthase
CRTISO	carotenoid isomerase
coum	coumaryl
Cy	cyanidin
DFR	dihydro-flavonol reductase
Dp	delphinidin
DW	dry weight
ECH	ε-carotene hydroxylase
F3H	flavanone 3' hydroxylase
F3'H	flavanone 3'hydroxylase
F3'5'H	flavanone 3'5' hydroxylase
FW	fresh weight
Gal	galactoside
GGPP	geranyl geranyl diphosphate
Glu	glucoside
HPLC	High-Performance Liquid Chromatography
LAR	leucoanthocyanidin reductase

LCYB	lycopene β-cyclase
LCYE	lycopene ε-cyclase
MBW	complex formed by MYB-bHLH-WD proteins
MEP	methylerythritol phosphate
Mv	malvidin
MYB	R2R3-MYB transcription factor
PAR	Photosynthetic Active Radiation
PDS	phytoene desaturase
Pg	pelargonidin
Pn	peonidin
PSY	phytoene synthase
Pt	petunidin
TCA	tricarboxylic acid cycle
UGFT	UDP glucose-flavonoid 3-*O*-glucosyl transferase
UPLC	Ultra-Performance Liquid Chromatography
UV	ultra-violet light
WD	WD-repeat protein family
ZDS	ζ-carotene desaturase

Original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

I Karppinen K, Zoratti L, Sarala M, Carvalho E, Pukki J, Mentula H, Martens S, Häggman H & Jaakola L (2015) Carotenoid metabolism during bilberry fruit development is regulated by biosynthesis and degradation. (manuscript)

II Zoratti L, Sarala M, Carvalho E, Karppinen K, Martens S, Giongo L, Häggman H & Jaakola L (2014) Monochromatic light increases anthocyanin content during fruit development in bilberry. BMC Plant Biology 14:377.

III Zoratti L, Jaakola L, Häggman H & Giongo L (2015) Modification of sunlight radiation through colored photo-selective nets affects anthocyanin profile in *Vaccinium* spp. berries. PLOS ONE DOI: 10.1371/journal.pone.0135935

IV Zoratti L, Jaakola L, Häggman H & Giongo L (2015) Anthocyanin profile in berries of wild and cultivated *Vaccinium* spp. along altitudinal gradients in the Alps. (manuscript)

Contents

1 Introduction

1.1 Vaccinium species

Vaccinium is a genus of about 450 plant species of the family Ericaceae, which are widely distributed in the Northern Hemisphere and also in the mountains of tropical Asia and Central and South America. A few species are also found in Africa and Madagascar (Song & Hancock 2011). The species within this genus present different levels of ploidy (2x, 4x and 6x; x=12) which results in evident morphological differences.

The most important species among the genus are *V. corymbosum* L. (highbush blueberry), *V. virgatum* Ait. (rabbitye blueberry), *V. angustifolium* Ait. (lowbush blueberry), *V. macrocarpon* Ait. (cranberry) and *V. vitis-ideae* L. (lingonberry) as the major *Vaccinium* fruit crops have been domesticated during the twentieth century, and from which comes most of the *Vaccinium* berry production. The wild *V. myrtillus* L. (bilberry) and the fruits of a number of other non-cultivated *Vaccinium* species are primarily collected from the forest but show great potential as new crops (Song & Hancock 2011).

Vaccinium fruits are perceived by the public to be a health-promoting food and the demand of berries from various *Vaccinium* species will likely continue to grow due to their antioxidant and therapeutic properties (Song & Hancock 2011). Considering this aspect, in the present study the attention was focused on two particular species. First, on the wild bilberry which is the species with the highest antioxidant properties, not only among berries but among fruits in general. The study was then extended also to the highbush blueberry, as the most important commercial crop in *Vaccinium* (Song & Hancock 2011).

1.1.1 Bilberry

Bilberry (*Vaccinium myrtillus* L.) belongs to the section *Myrtillus*, and it is a diploid species (2n=2x=24). Bilberry plants grow typically in pine and spruce heath forests and old peat bogs of Europe, Greenland, North America and northern parts of Asia, including Japan (Nestby *et al.* 2009). Bilberries are a valuable part of European nature and tradition, especially in the Northern and Eastern parts of Europe where they grow abundantly and constitute an important part of local incomes. Moreover, they are spread also within alpine environments

of Southern Europe. For instance, they cover large areas of the Alps, which are characterized by acidic soils (Barizza *et al.* 2013). Bilberry, particularly, establishes on a wide range of environmental conditions, from hilly areas to high altitudes above tree-line, although, its optimal range is between 1500 m a.s.l. and 2000 m a.s.l., where pure formations of this species may occur both in open habitats and in the understory of conifer-dominated forests (Woodward 1986).

The plant is a dwarf shrub with flowers born singly in leaf axils, a characteristic differentiating it from many other *Vaccinium* species. The flower is pink and shaped like an urn, and the fruit is a bluish-black globular berry with a flat top (Ballington 2001).

Fruit development of berries in general, is initiated by pollination and fruit set. Individual berry growth is characterized as having a double sigmoid growth curve, where each of the three portions of the curve corresponds to different stages of development and can be related to fruit ripening. There are three ripening stages, pre-maturation, maturation and senescence. The first growth stage occurs in the pre-mature berry, is initiated at fertilization and can last a time which is variable and related to the species. After fertilization, berry tissues undergo rapid cell division leading to endosperm and zygote formation. These changes are externally evidenced by increased berry diameter. The second berry growth stage also occurs in the premature berry and is characterized by the embryo growing to penetrate the endosperm plug and the initiation of seed development. In the second stage there are few outward signs of development, colour remains unchanged and berry diameter increases at a slower rate. The duration of this stage is size dependent; large berries complete growth after eight days, while small berries remain in this stage for 25 days. The third growth stage occurs in the mature berry. In this stage there is a rapid increase in pericarp tissue and water uptake as the berry grows to its full size. Changes in size are accompanied by changes in colour, from green to red to blue, and biochemical changes such as increases in pH, sugar composition and soluble solid content. Senescence is the last ripening stage and occurs after the third growth stage, which is characterized by a decrease in berry firmness (Dawn Gibson 2011).

Fig. 1. Flower and berry development of bilberry: 1, flower; 2, small-sized green berry; 3, middle-sized green fruits; 4, nearly-expanded, half-coloured fruits (purple); 5-6, fully coloured ripe berries (blue).

In bilberry (Fig. 1), five ripening stages have been identified from flower to ripe berry according to colour and berry size (stage 1: flower, stage 2: small unripe green fruit, stage 3: large unripe green fruit, stage 4: ripening purple fruit and stage 5: fully ripe blue fruit) (Jaakola *et al.* 2002). Ripening occurs usually 8 to 10 weeks following flowering with yearly variation between the years (Sjörs 1989).

1.1.2 Blueberry

The highbush blueberry (V. corymbosum L.) is a tetraploid species (2n=4x=48) of the section Cyanococcus and is native to eastern and northcentral North America. The most common cultivated species is the northern highbush, which occurs naturally in wetlands and drier upland wooded slopes from Nova Scotia, west to Wisconsin and south to Georgia and Alabama. Hybrids of this with other Vaccinium species adapted to more southern US climates (e.g. Florida and California) are collectively known as southern highbush blueberries (Song & Hancock 2011). Highbush blueberries are grown in 37 states in the US, in six Canadian provinces, in Australia, Chile, Argentina and New Zealand. They are also cultivated in Europe, where they were first introduced to Germany, Sweden and the Netherlands in the 1930s. Since then, highbush blueberry cultivation practices have been spread to other European countries (Naumann 1993).

The plant is a tree-bush, generally 120-300 cm tall, depending on the variety, which grows well in acidic soils (pH range 4-5) (Ostroluckà *et al.* 2004). The flowers are bell-shaped, white, pale pink or red, sometimes tinged greenish, and develop in racemes. The fruits are berries with a 10-16 mm diameter, a flared calyx and a small, flattened picking scar where the stem was attached. The fruit ripens 2 to 3 months after pollination, depending on the cultivar and climatic conditions. Berry ripening encounters different maturation stages, as for bilberry, but is not homogenous, as the more apical fruits in the cluster are the first to reach full ripeness, and later ripening moves towards basal berries (Song & Hancock 2011). During the initial 'expansion' phase, young fruits are hard and dark green and differ primarily by size. In the 'maturation' phase, enlarged light green fruits begin to soften and accumulate red and then blue pigments. At ripeness, berries are covered in a protective coating of powdery epicuticular wax, commonly known as the "bloom".

In cultivar Brigitta Blue (Fig. 2), the early ripening stages were identified according to the percentage of fruit formed (stage 1: 10%, stage 2: 30%, stage 3: 50%, 4-5: over 70%), in stage 6 the colour of the first fruits was advanced, in stage 7 berries were fully ripe and in stage 8 berries were slightly overripe (Giongo *et al.* 2013).

Fig. 2. Flowering and berry development of blueberry (cv Brigitta Blue): 0, flower; 1, 10% of fruit formed; 2, 30% of fruit formed; 3, 50% of fruit formed; 4-5, >70% of fruit formed; 6, colouring advanced; 7-8, ripe fruit at full size.

1.2 Nutritional and antioxidant properties of *Vaccinium* berries

Vaccinium berries are composed of the 85% water while the rest is mainly sugars (5-14%) and fibres (2-7%). Proteins (0.5-1%) and fats (0-0.8%) account for very little of the nutritional value. However, they are important natural sources of antioxidant molecules. *Vaccinium* berries contain high levels of polyphenols, including flavonols, proanthocyanidins and particularly high amounts of anthocyanins (Prior *et al.* 1999). They contain also high yields of carotenoids, especially lutein, which has been shown to provide protection to eye health (Grover & Samson 2014).

Vaccinium berries are a moderate source of vitamins. Vitamin C levels range between 8 and 44 mg/100g (Cocetta *et al.* 2012). Bilberries contain also unsaturated fatty acids (Bunea *et al.* 2012), which are mainly present in the seeds (Yang *et al.* 2011). Bilberry seed oil contains a significantly high proportion of unsaturated fatty acids like 18:1 n-9 (21.8%), 18:1 n-7 (0.6%), 18:2 n-6 (35.9%) and 18:3 n-3 (36.1%). Moreover, the oil contains α-tocotrienols (10 mg/100 g oil) and γ-tocotrienols (30 mg/100 g oil) (Yang *et al.* 2011).

1.2.1 Flavonoids

Among berry species, and fruits in general, *Vaccinium* berries are best sources of flavonoids, especially anthocyanins, which have been reported to have various health beneficial activities including antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory, antiproliferative and antimicrobial effects (Landete 2012). Flavonoids are a large group of phenolic secondary metabolites that are widespread among plants. They are involved in several aspects of plant development and defense, as they colour fruits and flowers, favour seed dispersal, and contribute to plant adaptation to cold or UV stress, and pathogen attack. In *Vaccinium* spp. several flavonoids have been described belonging to the classes of flavonols (as kaempferol, myricetin and quercetin), proanthocyanidins (catechin, epicatechin, procyanidins and prodelphinidins) and anthocyanins in ripe berries of highbush blueberry, bilberry, lingonberry, bog bilberry, lowbush blueberry, cranberry. Other phenolic compounds such coumarins and acids have also been found (Prior *et al.* 1999).

The flavonoid pathway commonly found in *Vaccinium* berries (Fig. 3) begins from chalcone synthase (CHS) which condenses one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA into naringenin chalcone. Chalcone is

isomerized to a flavanone by the enzyme chalcone flavanone isomerase (CHI). From these central intermediates, the pathway diverges into several side branches, each resulting in a different class of flavonoids. Flavanone 3-hydroxylase (F3H) catalyzes the stereospecific 3β-hydroxylation of (2S)-flavanones to dihydroflavonols. For the biosynthesis of anthocyanins, dihydroflavonol reductase (DFR) catalyzes the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanins), which are converted to anthocyanidins by anthocyanidin synthase (ANS) and glycosylated by UDP glucose-flavonoid 3-*O*-glucosyl transferase (UFGT). Anthocyanidins can be diverted into proanthocyanidins via anthocyanidin reductase (ANR), which produces epicatechin-type flavan-3-ols, whereas catechin-type flavan-3-ols are produced from leucocyanidins by leucoanthocyanidin reductase (LAR) (Zifkin *et al.* 2012).

1.2.2 Anthocyanins

Anthocyanins (ACs) are the class of flavonoids, which generate the characteristic red, blue and purple pigments in leaves, flowers and fruits of several plants. There is a huge variety of ACs present throughout nature, as many differences occur between them in terms of molecular structure. Differences cover the number of hydroxylated groups, the nature and the number of bonded sugars, the aliphatic or aromatic carboxylates bonded to the sugars in the molecule and the position of these bonds (Kong *et al.* 2003). ACs consist of an anthocyanidin aglycon that is bound to one or more sugar moieties. Six anthocyanidins are classified according to the number and position of hydroxyl and methoxyl groups on the R1 and R3 groups on the flavan B-ring: cyanidin (Cy), pelargonidin (Pg), peonidin (Pn), delphinidin (Dp), petunidin (Pt) and malvidin (Mv). The key enzyme which differentiates the different classes of ACs is the flavanone 3’5’-hydroxylase (F3’5’H), which is responsible for the hydroxylation at the 3’5’ positions of the B-ring of the precursor dihydrokaempferol into Dp. Methylation of the 3′ and 5′ hydroxyl groups of Cy gives rise to Pn, whereas methylation of Dp gives arise to Mv and Pt (Fig. 3).

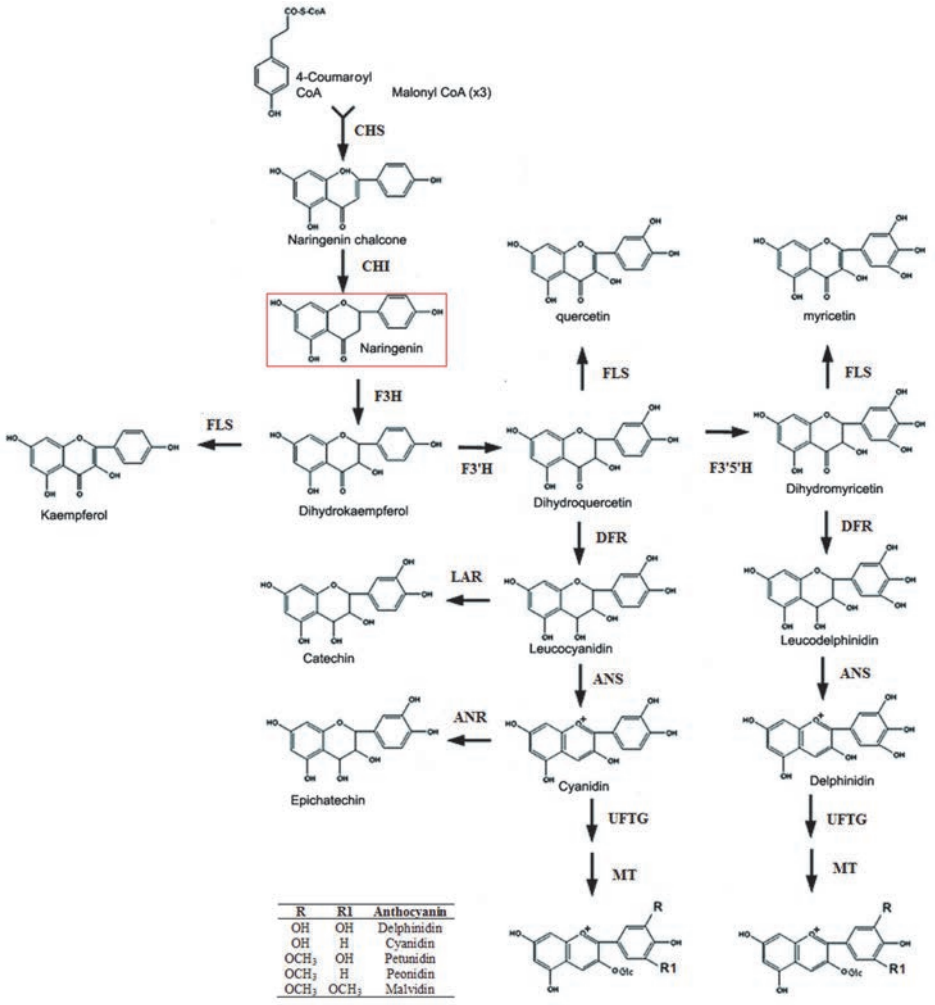

Fig. 3. Schematic presentation of the flavonoid pathway showing the compounds common in *Vaccinium* species. ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3H, flavanone 3 hydroxyase; F3'H, flavanone 3' hydroxyase; F3'5'H, flavanone 3' 5' hydroxyase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; MT, 3-*O*-methyl transferase; UFGT, UDP glucose-flavonoid 3-*O*-glucosyl transferase. The red square marks the B-flavan ring.

The 15 major anthocyanidin glycosides commonly found in *Vaccinium* berries are 3-*O*-monoglucosides, -monogalactosides or -monoarabinosides of Cy, Dp, Pn, Pt

and Mv, whereas Pg have rarely been found in *Vaccinium* berries (Kähkönen *et al.* 2001, Jaakola *et al.* 2002). Also minor amounts of other anthocyanidin glycosides have been found in bilberry, as Cy-sambubioside (Du *et al.* 2004), and acylated forms of ACs, either with acyl or coumaryl groups, were found in bilberry and blueberry (Kalt *et al.* 1999). The sugars and the acetyl groups provide greater stability of AC against degradation by change in pH, heat and light (Patras *et al.* 2010). Moreover, as recently found in grapevine, acylation appears to be essential for transportation of ACs from cytoplasm to vacuole, as ACs were carried by anthoMATE1 transporter proteins, which have been found to mediate specifically the trasport of acylated ACs and could not transport Mv 3-*O*-glucoside or Cy 3-*O*-glucoside (Gomez *et al.* 2009).

1.2.3 Carotenoids

Carotenoid pigments are essential for all plants, as they are part of the light-harvesting complexes involved in photosynthesis, prevent photo-oxidative damages to tissues as well as provide red, orange and yellow coloration to many flowers and fruits for attraction of insects, birds and animals for pollination and pollinating seed dispersal (Cazzonelli & Pogson 2010). The main carotenoids detected in berries of *Vaccinium* species are β-carotene and lutein, although also the xanthophylls zeaxanthine, violaxanthine, neoxanthine and antheraxanthine have been detected (Lashmanova *et al.* 2012). Bunea *et al.* (2012) analyzed the carotenoid composition of bilberries in comparison to cultivated blueberries. They identified lutein and β-carotene in both species although the contents of all individual as well as total carotenoids were higher in bilberry compared to different blueberry cultivars (Bunea *et al.* 2012). A study on carotenoid levels in different berry species in Komi republic, Russia, indicated the presence of high levels of lutein in bilberry compared also with lingonberry (Lashmanova *et al.* 2012).

The biosynthesis of carotenoids in plants occurs by nuclear-encoded enzymes in chloroplasts, where they have key roles in photosynthesis, and in chromoplasts, where they act as secondary metabolites. The immediate precursor of carotenoids is geranyl geranyl diphosphate (GGPP) that is mostly derived from the plastidial methylerythritol phosphate (MEP) pathway. The condensation of two molecules of GGPP catalyzed by phytoene synthase (PSY) leads to colorless phytoene. In many species, the PSY step has been reported to have a major influence on the extent of carotenoid biosynthesis (Cazzonelli & Pogson 2010). The red carotenoid

lycopene is further biosynthesized from phytoene by a series of reactions involving the action of phytoene desaturase (PDS), ζ-carotene desaturase (ZDS), and at least two isomerases (Cazzonelli & Pogson 2010). The cyclization of lycopene by lycopene cyclases forms a branching point in the carotenoid pathway

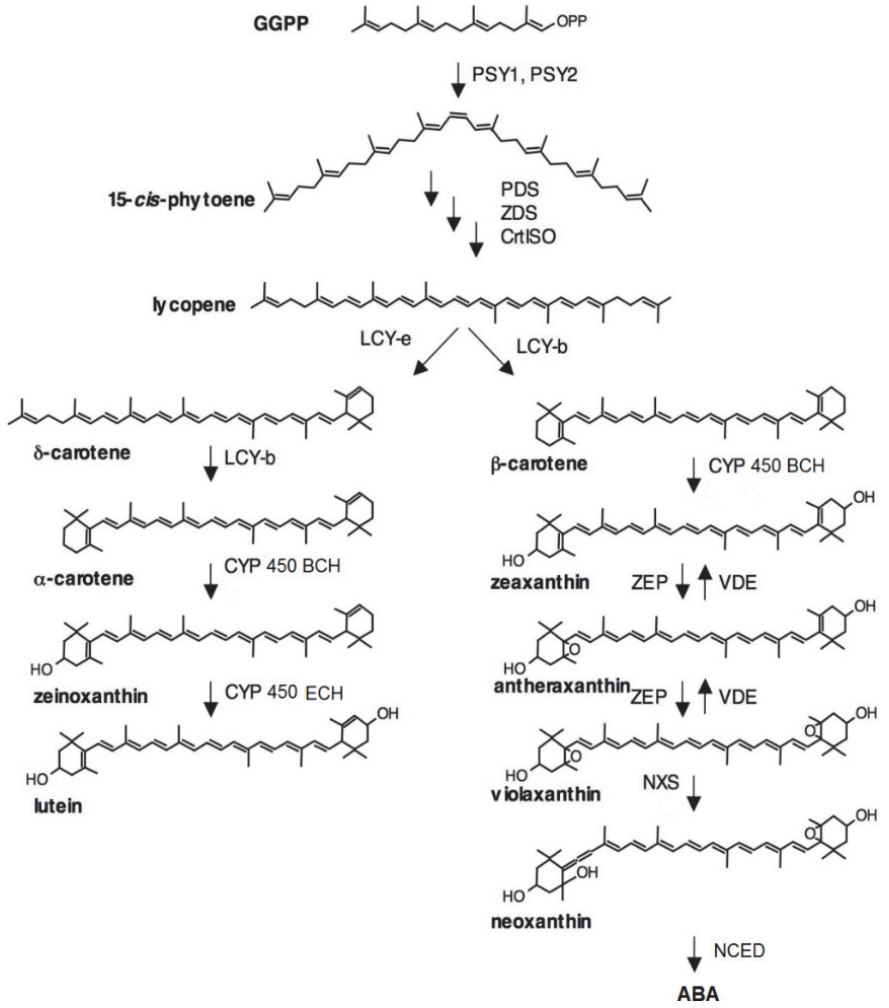

Fig. 4. Schematic representation of the carotenoid pathway. ABA, abscisic acid; BCH, *β*-carotene hydroxylase; CRITSO, carotenoid isomerase; CYP 450, cytochrome P450; GGPP, geranil geranil diphosphate; LCYB, lycopene *β*-cyclase; LCYE, lycopene *ε*-cyclase; NCED, 9-cis-epoxycarotenoid dioxygenase; NXS, neoxathin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; ZEP, zeaxanthin epoxidase; ZDS, *ζ*-carotene desaturase.

leading to α-carotene and β-carotene. The formation of α-carotene requires enzymatic activities of both lycopene β-cyclase (LCYB) and lycopene ε-cyclase (LCYE) whereas LCYB alone converts lycopene to β-carotene. The α-carotene is further hydroxylated by the enzymes ε-carotene hydroxylase (ECH) and β-carotene hydroxylase (BCH) to lutein, while hydroxylation of β-carotene by BCH results in the formation of zeaxanthin via β-cryptoxanthin. Epoxycarotenoids violaxanthin and neoxanthin are produced from zeaxanthin in abscisic acid (ABA) biosynthetic pathway and further metabolized to plant hormone ABA.

1.3 Regulation of berry ripening process

The fruit ripening is a developmental process, genetically regulated, involving the activation of a high number of primary and secondary metabolic pathways. This process involves the expression of ripening-related genes, which encode enzymes involved in the various physiological changes during ripening (e.g. softening, colour development). In berries, as in many fleshy fruits, a multitude of physiological and biochemical changes take place during fruit development and ripening, resulting in cell enlargement, softening, sweetening, changes in colour, sugar, acidity, texture and aroma volatiles, which are important to determine the nutritional properties and the sensory quality of fruits at maturation.

The developmental changes are coordinated by the interplay of thousands of genes. In blueberry cultivar Bluecrop, cDNA libraries from fruits collected at various stages of ripening (green, white, pink and blue) resulted in ~1900-2300 contigs for each berry stage, for a total of 6726 contigs for all berry sequences assembled together (Rowland *et al.* 2012). Many of the highly abundant transcripts appeared to have a complex, differential expression pattern during fruit development. Levels of metallothionein-like, lipid transfer, dehydrin, ribulose-bisphosphate carboxylase oxygenase small subunit, and light harvesting complex II proteins appeared to decline during fruit development. Transcripts for pectate lyase, cytochrome b5, cysteine protease-like protein, cyclophilin, glutathione peroxidase, and chalcone synthase appeared to rise, peaking at the pink fruit stage, and then to decline afterwards at the blue/ripe fruit stage. Transcripts for aspartic proteinase, flavonoid 3'-hydroxylase, and 1-aminocyclopropane-1-carboxylate oxidase appeared also to rise during fruit development, and peak at the blue/ripe fruit stage (Rowland *et al.* 2012).

The whole ripening process is under the control of hormonal signals, and fruits can be divided into two groups according to the hormonal regulatory mechanisms

underlying the ripening process. In climacteric fruits, such as tomato (*Solanum lycopersicum* L.), apple (*Malus x domestica* L.) and pear (*Pyrus communis* L.) the ripening process is initiated concomitantly with the burst of plant hormone ethylene. By contrast, in non-climacteric fruits, ripening lacks of the ethylene-associated respiratory peak, which seems to be instead regulated by the hormone abscisic acid (ABA) which is released by developing seeds (Mondher *et al.* 2010). Many berry species are non-climacteric, such as grapevine (*Vitis vinifera* L.) (Wheeler *et al.* 2009), strawberry (*Fragaria* spp.) (Jia *et al.* 2011), and also bilberry and blueberry (*Vaccinium* spp.) (Zifkin *et al.* 2012, Karppinen *et al.* 2013). Blueberries are considered weak climacteric fruits as they show a peak in respiration rate which corresponds to the onset of maturity, but only a few clones respond to the application of commercial ethylene generating agrochemicals. Moreover, in lowbush blueberry, the application of an ethylene inhibitor did not result in increased berry yield or weight, further suggesting that lowbush blueberry is not climacteric fruit (Dawn Gibson 2011). Indeed, in highbush blueberry, the physiologically active *cis*-ABA increased markedly at ripening initiation, increasing by nearly 6 times between stage 5 and 6, and peaked in fruits at stage 7 (Zifkin *et al.* 2012). Similarly in bilberry, a high increase in ABA content was found just at the onset of the bilberry fruit ripening (stage 3) increasing by nearly 7 times between stage 3 and 4, when ABA levels were highest. In ripe bilberries (stage 5), the ABA levels were decreased by half compared to stage 4, but were equally distributed in berry pulp and skin (Karppinen *et al.* 2013).

1.4 Regulation of flavonoid and carotenoid accumulation during berry development

Secondary metabolite composition changes along the berry ripening process as a part of the fruit's genetically regulated developmental program. At the early stage of blueberry maturation, when fruit is green, chlorophylls, carotenoids and flavonols are the predominantly accumulated fruit pigments. During maturation the berries turn from green to pink, as chlorophylls are degraded and AC pigments start to accumulate, together with flavour compounds and aromatic volatiles (Eicholz *et al.* 2015). In *Vaccinium* species, the hormone ABA appears to regulate also the accumulation of ACs. In blueberry, ABA content was maximal at the beginning of colour accumulation (stage 6), indicating that ABA synthesis immediately preceded the rapid increase in AC synthesis (Zifkin *et al.* 2012). In bilberry, as well, the level of ABA highly increased at the onset of bilberry fruit ripening, at the stage when the

expression of AC biosynthetic genes *VmCHS* and *VmANS* also increased (Karppinen *et al.* 2013).

The synthesis of flavonoids in the berries of both wild and cultivated *Vaccinium* species correlates with the expression of the flavonoid pathway genes during fruit ripening in e.g. bilberry (Jaakola *et al.* 2002) and blueberry (Rowland *et al.* 2012). Flavonoid biosynthesis in plants is controlled by the tissue-specific expression of transcription factors belonging to the R2R3 MYB, basic helix-loop-helix (bHLH), and WD-repeat protein families. These physically interact and the MBW formed complexes have been shown to be responsible for the regulation of AC, proanthocyanidins, and flavonol biosynthesis in a variety of species and tissues including flowers and fruit. MBW complexes are involved in both types of developmental and environmental regulation at the transcriptional level, mainly through the activation of late biosynthetic genes of the pathway (Xu *et al.* 2015). In bilberry, the transcription factor *VmMYB2* gene is highly expressed from the turning colour stage until berry ripening, showing the same trend of *VmANS*. Moreover, the expression is down-regulated in white and pink bilberry mutants, indicating the involvement of the *VmMYB2* transcription factor in the regulation of AC biosynthesis in bilberry fruit (Jaakola *et al.* 2010).

Recently, also a SQUAMOSA-class MADS box transcription factor (*VmTDR4*) was found to be associated with AC biosynthesis in bilberry fruits (Jaakola *et al.* 2010). Levels of *VmTDR4* expression were spatially and temporally linked with colour development and AC-related gene expression. When the *VmTDR4* expression was suppressed by virus-induced gene silencing, a substantial reduction in AC levels in fully ripe fruits was observed and also the expression of *VmMYB2* was suppressed, indicating that *VmTDR4* plays an important role in the accumulation of ACs during normal ripening in bilberry, probably through direct or indirect control of transcription factors belonging to the R2R3 MYB family (Jaakola *et al.* 2010).

A tight temporal control of gene transcription in blueberry suggested that flavonoids might also be under precise tissue-specific control in *Vaccinium* species (Zifkin *et al.* 2012). Differences in AC accumulation are found among berry tissues, as in blueberry, ACs are mainly present in the berry skin and outer layer of the pulp, while in bilberries ACs accumulate also in the fruit flesh (Riihinen *et al.* 2008). In ripe blueberry fruits (stage 7), the levels of *VcANR* and *VcLAR* transcripts were low in all examined tissues and *VcUFGT*, *VcF3'H*, *VcF3'5'H* and *VcMYBPA1* transcripts were nearly exclusive to the skin tissue, which is the site of AC-based coloration in ripening fruit (Zifkin *et al.* 2012).

Plants have developed also complex regulatory mechanism controlling carotenoid biosynthesis and accumulation. While the composition and relative abundance of various carotenoids is conserved in green tissues, it broadly varies in non-green tissues and organs. During fruit ripening, transcriptional regulation of carotenoid gene expression appears to be the major mechanism by which the biosynthesis and accumulation of carotenoids are regulated (Cazzonelli & Pogson 2010). The metabolic turnover of carotenoids by carotenoid cleavage dioxygenases (CCD) produces important signaling and accessory apocarotenoid molecules, as the carotenoid pathway links with plant hormone gibberrellin and biosynthesis, but also helps to maintain the steady level of carotenoids in plants (Bramley 2002; Lu & Li 2008) So far, the accumulation of carotenoids during the ripening, has been less studied in *Vaccinium* species. In a study on ABA biosynthesis in bilberry, the expression of 9-cis-epoxycarotenoid dioxygenase (*VmNCED1*) and putative neoxanthin synthase (*VmNSY*) was high in berry tissues and increased markedly at the onset of berry ripening along with the accumulation of ABA. In contrast, the expression of zeaxanthin epoxidase (*VmZEP*) was mostly associated with bilberry leaf tissues with no obvious relation to ABA content during berry development (Karppinen *et al.* 2013).

1.5 Environmental effect on accumulation of flavonoids and carotenoids

In *Vaccinium* species the extent of changes in composition of secondary metabolites in berries has been shown to be modulated both by the plant genotype (Prior *et al.* 1999) and environmental factors (Lätti *et al.* 2008, 2010, Åkerstrom *et al.* 2010). Among these, light environment and temperature have been reported to influence the biosynthesis of flavonoids in bilberry (Uleberg *et al.* 2012) as well as in many other berry species (grapevine, strawberry, cranberry and raspberry - *Rubus ideaus* L.) (Jaakola & Hohtola 2010, Zoratti *et al.* 2014).

1.5.1 Solar radiation and temperature

Solar radiation is the source of energy for photosynthesis and it is fundamental for physiological processes such as sugar accumulation and activation of secondary metabolism during berry maturation. The balance between pathways of primary carbohydrate metabolism, such as sucrose synthesis and degradation, glycolysis, and the tricarboxylic acid (TCA) cycle, plays a central role in the final

composition of berries. Sugars, especially sucrose, glucose, and fructose, determine the sweetness of the fruit. Respiration of sugars via glycolysis, the oxidative pentose phosphate pathway, and the TCA cycle provides energy (ATP), reducing power [NAD(P)H] and precursors for the synthesis of organic acids, amino acids, ACs and many other secondary metabolites, including defense and aroma compounds. In addition to generating ATP and NADH, the TCA cycle also plays an important role in regulating fruit acidity, and some of the intermediates of the TCA cycle may act as signal compounds that regulate metabolic fluxes and gene expression (Dai *et al.* 2013).

Several studies have demonstrated that solar radiation is directly involved in the accumulation of secondary metabolites in the fruits (Zoratti *et al.* 2014). In both climacteric and non-climacteric fruits, light stimulates the expression of an array of both early (*CHS*, *CHI*, *F3H*) and late flavonoid biosynthetic genes (*F3'5'H*, *DFR*, *ANS, ANR*), which leads to the increased content of ACs, proanthocyanidins as well as flavonols (Zoratti *et al.* 2014). Recently, also some R2R3 MYB transcription factors associated with flavonoid biosynthesis have been found to respond to light. Light-inducible R2R3 MYB transcription factors controlling flavonoid biosynthesis in fruits have been identified in apple, pear, nectarine, Chinese bayberry, strawberry, litchi and grapevine, where they have been shown to be positive regulators of general flavonoid pathway, as well as those specifically responsible for AC, flavonol and proanthocyanin biosynthesis (Zoratti et al. 2014). Interestingly, the expression of flavonoid pathway genes and the accumulation of flavonoid compounds in response to light have been shown to continue in post-harvest fruits, indicating that the physiological response to the light stimulus is located in the fruit (Zoratti et al. 2014).

Different classes of phenolic compounds have been shown to react differently also to specific portions of the light spectrum. The composition of the visible light spectrum regulates many physiological responses in plants, including flavonoid and carotenoid biosynthesis. Especially predominance of shorter wavelengths in the light spectrum, in the range of blue and UV light wavelengths, generally increase the amount of flavonoids, and in particular the class of ACs, during ripening, harvesting and post-harvesting of fruits. UVB has been shown to induce specifically the accumulation of flavonols in grapevine (Koyama *et al.* 2012), whereas blue light increased the accumulation of ACs, especially the class of Mv (Kondo *et al.* 2014) and the same was detected in strawberry (Kadomura-Ishikawa *et al.* 2013). Few studies have been conducted among *Vaccinium* berries. Wavebands towards red and far red light increased the accumulation of ACs in

cranberry (Zhou & Singh 2002). Wang *et al.* (2009) studied the effect of UV-C dosages from 0.43 to 6.45 kJm^{-2} (1–15 minutes treatments) on flavonoid contents and antioxidant activity in post-harvest blueberries. They found substantial increase in flavonols, ACs and antioxidant activities instantly after the UV-C treatments. However, the contents decreased to the same level with the untreated control berries 19 hours after the treatments. Because carotenoids act as photo-protectants whose synthesis is regulated by phytochrome (Cazzonelli & Pogson 2010) their production is also induced by light exposure. In addition, quantity and quality of light, and especially red light, have been demonstrated to affect to accumulation of carotenoids in fruits (Liu *et al.* 2009, Ma *et al.* 2012, Kondo *et al.* 2014).Solar radiation also heats plant tissues, influencing enzymatic reactions, which determines the final composition of the berries. Studies conducted in grapevine showed that high temperatures (above 25 °C) accelerate plant development and are associated with a more rapid succession of phenological stages, including earlier maturation. In cool regions, low temperatures often limit photosynthesis and sugar production in the leaves, although growth and sink activity of the fruit decrease more than photosynthesis (Keller 2015). However, temperature has been shown to affect also the composition of the secondary metabolite profile. For example, AC accumulation was preferred in grape berries grown at temperatures lower than 20 °C compared to 30 °C, as the expression of *CHS*, *F3H*, *DFR* and *UFTG* was inhibited in presence of high temperatures (30 °C) (Mori *et al.* 2005). Among *Vaccinium* berries, a study by Uleberg *et al.* (2012), showed that in bilberries temperatures of 12 °C favoured the accumulation of the ACs belonging to the class of Dp, compared to temperatures of 18 °C.

1.5.2 Latitude and altitude

Fruiting and ripening times are affected by the season and by local climate conditions. These may vary markedly with altitude and latitude. For example, the peak of *Vaccinium* crop harvesting can vary from June to August (in the northern hemisphere) depending upon the climate conditions (Prior *et al.* 1999).

Latitude appears to influence the accumulation of ACs in *Vaccinium* berries, as a clear increasing trend in AC production towards north has been reported in high latitudes for bilberry and bog bilberry (*V. uliginosum* L.) (Lätti *et al.* 2010). Higher anthocyanin contents were reported in northern (63-70°N) latitudes of Northern Europe, compared to more southern latitudes (54-62°N) (Lätti *et al.*

2008, 2010, Åkerstrom *et al.* 2010). The berries of the northernmost clones have been shown to contain higher total yields of ACs and a higher proportion of the more hydroxylated anthocyanidins, Dps, whereas Cys accumulated more in the more southern latitudes (54-62°N) of North European populations.

AC accumulation is not only responsive to light intensity, but also to day length. Longer days seem, in general, to be associated with more intense AC production than shorter days (Jaakola & Hohtola 2010). In bilberry, this photoperiod effect may be one reason, in addition to the cooler temperatures, for more rapid AC accumulation and for higher concentrations of ACs at higher latitudes compared to lower (Uleberg *et al.* 2012).

Studies have been performed also in relation to different altitudes, although they have given contradictory results. Areas of the Alps from 200 m a.s.l. up to 1000 m a.s.l. of altitude are well suited for soft berry fruit cultivation (e.g. blueberry and raspberry, *Rubus* spp.), which fits well to alpine climate and soil conditions. Spinardi *et al.* (2009) reported higher levels of ACs and ascorbic acid in blueberries grown at 600 m a.s.l. compared with the same cultivar grown at 450 m a.s.l. in Valtellina (Northern Italy). In a two year study in Austria (Rieger *et al.* 2009), decreasing AC contents were found along with increasing altitude (between 800 m a.s.l. and 1500 m a.s.l.) in bilberry fruits. In studies performed in the areas of Northern Europe, where altitudinal differences are less pronounced, no clear relationship with elevation and AC concentration have been reported (Åkerstrom *et al.* 2010).

2 Aim

The aim of the work was to investigate the factors affecting the accumulation of flavonoids and carotenoids in *Vaccinium* berries. The studies were focused on the wild species of bilberry, which is one of the richest sources of such compounds. The accumulation of flavonoids and carotenoids was initially investigated during the bilberry fruit development, observing the gene expression of flavonoid and carotenoid pathway genes and the metabolic profiles of berries during the berry ripening process.

The studies were further oriented to investigate the accumulation of carotenoids and flavonoids in response to environmental factors, as light and temperature. Particular attention was given the anthocyanidin profile of bilberries grown in different environmental conditions, in order to explain recent findings showing that concentrations of the more hydroxylated AC forms, i.e. delphinidin-, petunidin- and malvidin-glycosides, were markedly higher in populations growing above 65°N latitude. Indeed, there is evidence that this phenomenon is under strong genetic control, but it is also influenced by climatic factors as differences in temperature, day length and spectral composition of sunlight are closely correlated with the latitude. The same environmental conditions were studied on another *Vaccinium* species, the cultivated highbush blueberry, to investigate the effect of the environment on secondary metabolite accumulation at species level.

The specific aims of the studies I-IV were:

1. to characterize the flavonoid and carotenoid composition and related gene expression during the development and ripening of bilberries (I);
2. to study the effect of different light conditions on the accumulation of flavonoids and carotenoids both in the controlled and field conditions in wild and cultivated *Vaccinium* berries (I, II, III, IV);
3. to study the altitudinal effect on the accumulation of ACs in bilberry populations (IV).

3 Materials and methods

3.1 Plant material and experimental fields

Bilberry (*V. myrtillus* L.) plants from Northern (Oulu, 65°N, Finland; I, II) and Southern Europe (Alps in the Trentino region, 46°N, Italy; III, IV) were used as research material in the study. The study was initiated with the analysis on the metabolic composition of berries during fruit development. Berries were collected in natural forest stands in the Oulu area, at the developmental stages identified by Jaakola *et al.* (2002) (Fig. 1). The carotenoid composition was analyzed for the developmental stages from 1 to 5 (I), whereas a detailed analysis of phenolic composition was performed only on stages 2 and 5-6 (Table 1), which were considered for light experiments to be conducted later (II, III).

The experiments with modified light conditions were conducted on plants from forest stands of Finland and Italy, which were harvested with the root system, potted in peat soil and fertigated through the ripening of berries (I-III). Environmental studies were conducted also on plants from six natural bilberry populations, growing at different altitudes, between 1166 m a.s.l. and 1829 m a.s.l. in the Italian Alps (IV). On each site, 10-25 bilberry plants were chosen in an area of about 20 m^2 and bilberries from each plant were analyzed for the AC profile.

Also, blueberry plants (*V. corymbosum* L., cv. Brigitta Blue) were used in the study to compare the effect of different growing environments on wild and cultivated Vaccinium species. Blueberry plants were cultivated at different altitudes (between 485 m a.s.l. and 1034 m a.s.l.) in the Trentino region (Italy) according to local farming methods (III, IV).

The experiments were set at predetermined phenological stages during the ripening, which were identified according to Jaakola *et al.* (2002) for bilberry (Fig. 1) and according to Giongo *et al.* (2013) for blueberry (Fig. 2).

3.2 Light experiments

Controlled light experiments were conducted on bilberry plants by the use of VP3411 M16 LED lamps (Valopaa Ltd., Oulu, Finland) (I), Ecoline R7s halogen lamps (Osram,Vantaa, Finland) (I) or Selador LED lamps from PALETTA$_{TM}$ (BMI supply, NY, USA) (II).

The accumulation of carotenoids was analyzed in response to different photoperiods (I). The light experiment was performed on plants holding berries at stage 2 and also later in the season, when plants were holding berries at stage 5. Plants were placed for 5 days under continuous white light or circadian light conditions, i.e. 16 hours day under white light or white light added with red wavelengths (600-800 nm) and 8 hours night (I).

Plants holding berries at the developmental stage 2 were treated with portions of the visible light spectrum, in the range of white (400-800 nm), blue (400-500 nm), red (600-700 nm) and far red (700-800 nm) wavelengths continuously for 48 hours and later were moved to greenhouse under natural solar light until berries were ripe (stage 6). The developmental stage 2 was chosen because the preliminary experiments indicated stage 2 to be the most reactive stage in the accumulation of flavonoids in response to light (II). In both experiments (i.e. berries at stage 2 and stage 5), a set of plants was also kept in total darkness and was considered as a negative control (I, II).

Photo-selective nets of blue, red, white and black colour (IRIDIUM Agritenax, Eboli, Salerno, Italy) were used to induce modifications in the intensity and/or the spectral composition of sunlight radiation over the plants in field conditions. The nets showed also the ability to affect temperature and relative humidity of the canopy during day and night, creating therefore a particular microclimate, which was a specific combination of light intensity and spectrum, temperature and relative humidity, characteristic for each net tested (Table 1). The effect of the coloured nets was tested specifically for the accumulation of ACs in berries and compared with plants growing under sunlight without any screen (III). Moreover, the nets were applied contemporarily to bilberry and blueberry plants, for comparison of the species in the accumulation of ACs in response to the light treatments. The cultivar Brigitta Blue was chosen as the development of fruits was contemporary with the fruit ripening of bilberry (III).

Table 1. Photo-selective nets create a particular microclimate over the canopy, which is a specific combination of light intensity and spectrum, temperature and relative humidity, characteristic for each net tested. PAR, temperature and relative humidity are compared to conditions under sunlight (open-field), whereas light spectrum modifications (*) under blue and red net is compared to conditions under white net which have similar shading percentage.

	Net / Light treatment				
	Sunlight (open-field)	Blue	Red	White	Black
Solar radiation intensity (PAR)	100%	75%	75%	75%	10%
Light spectrum*		+11% blue bands -5% red bands	-23% blue bands -1% red bands		
Temperature		+1.5°C 10am-2pm -1°C 3pm-9pm -0.2°C 10pm-9am	-0.5°C 9am-2pm +0°C 3pm-8am	+1°C 8am-2pm -1°C 3pm-8pm +0.5°C 9pm-7am	-2.5°C 10am-1pm +1°C 5pm-8pm -0.5°C 10pm-8am
Relative humidity		+10%	+10%		+10%

Natural variations of sunlight radiation and temperature fluctuations were also investigated along altitudinal gradients, and correlated with the accumulation of ACs in bilberry and blueberry plants growing at different altitudes (IV).

The intensity and spectral composition of light under lamps (I, II) and of solar radiation (III, IV) was measured using an USB RAD+ or USB2000 spectroradiometer (Ocean Optics Inc., Dunedin, FL, USA).

3.3 Isolation of the genes

The amplification of sequences of the key carotenoid biosynthetic genes phytoene synthase (*VmPSY*), phytoene desaturase (*VmPDS*), ζ-carotene desaturase (*VmZDS*), carotenoid isomerase (*VmCRTISO*), lycopene *β*-cyclase (*VmLCYB*), lycopene *ε*-cyclase (*VmLCYE*), *β*-carotene hydroxylase (*VmBCH*) and cytochrome P450-*β*-carotene hydroxylase (*VmCYP450-BCH*), and also of a carotenoid cleavage dioxygenase (*VmCCD1*) was achieved from bilberry cDNA with gene specific primers that were designed based on sequences of the highbush blueberry

transcriptome database (Rowland *et al.* 2012). The PCR was performed with DyNazyme^{TM} II DNA polymerase (Finnzymes, Espoo, Finland). The amplified PCR products were gel-purified using the Montage® DNA Gel Extraction Kit (Millipore, Bedford, MA, USA). The purified PCR products were ligated into a pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). All the obtained sequences of carotenoid biosynthetic genes from bilberry were deposited into the GenBank database with the following accession numbers: *VmPSY* (KR706538), *VmPDS* (KR706539), *VmZDS* (KR706540), *VmCRTISO* (KR706541), *VmLCYE* (KR706543), *VmLCYB* (KR706542), *VmBCH* (KR706544), *VmCYP450-BCH* (KR706545) and *VmCCD1* (KR706546).

3.4 Gene expression analyses

Total bilberry fruit RNA was isolated according to the method of Jaakola *et al.* (2001) except that the phenol-chloroform extraction was substituted with the RNA purification protocol in the E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek, GA, USA). The cDNA was synthetized from total RNA with RevertAid Premium Reverse Transcriptase (Thermo Scientific, USA) or with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using anchored-oligo(dT) primers, according to the manufacturer's instructions. The cDNA was purified from contaminating genomic DNA by using the method described by Jaakola *et al.* (2004) or using the RNAse (Thermo Scientific, USA).

The relative expression of the target genes: the flavonoid pathway genes and the *VmMYB2* transcription factor (II) and the carotenoid pathway genes (I) were analyzed by real-time quantitative PCR using the LightCycler 480 plate instrument (Roche, Basel, Switzerland). The quantification of transcripts was done by using actin and glyceraldehyde-3-phosphate dehydrogenase as control genes and calibration curves, specific for every gene of interest. Both biological and technical replicates (a minimum of 3 per each) were analyzed and the primer efficiencies were calculated for each primer.

3.5 Metabolite analyses

The phenolic compounds were extracted from the fresh or freeze-dried berries as described in paper II and analyzed with the UPLC-MS/MS (Ultra Performance

Liquid Chromatography) method (Arapitsas *et al.* 2012, Vrhovsek *et al.* 2012). Phenolic compounds were detected by multiple reaction monitoring (MRM) by screening the MS/MS transitions and using the parameters described in II. The single compounds were quantified with external calibration curves, which were prepared by injecting authentic standards of each compound at different concentrations. In case the authentic standard was not available, the compounds were quantified relative to other quantified compounds.

The carotenoids were extracted from the dried berries as described in paper I and analyzed with HPLC-DAD method (Carvalho *et al.* 2013).

3.6 Statistical analyses

Statistical comparisons among data were performed using STATISTICA version 9 (StatSoft Inc., Tulsa, USA). The analyses are described in detail in the original publications.

4 Results

4.1 Flavonoid accumulation during bilberry fruit development

The phenolic composition of bilberry fruits at two different developmental stages (unripe green -stage 2- and ripe bilberries -stage 5-6; Fig. 1) was analyzed in berries collected from natural forests in Northern Finland with two methods which allowed the screening of a total of 225 phenolic compounds (Arapitsas *et al.* 2012, Vrhovsek *et al.* 2012). The methods provided a detailed analysis of the bilberry metabolites quantifying 68 phenolic compounds, out of which new compounds could be identified in bilberry (Table 2, unpublished results). The phenolic compounds detected in bilberries included benzoic derivative acids, hydroxycinnamic acids, flavanones, flavonols, flavan-3-ols (proanthocyanidins) and ACs. Among phenolic acids, chlorogenic, neochlorogenic, ellagic, caffeic and ferulic acids were detected and quantified. The coumarins fraxin and scopoletin, the stilbenes t-piceide and (-)-astringin and the flavone luteolin 7-*O*-glucoside were found only in trace amounts.

Naringenin (the precursor of flavonoid compounds) was present in low concentrations in both early and late stages of ripening, whereas it was found in much higher concentration in the glycosylated form (naringenin 7-*O*-glucoside) at ripeness.

Flavonols included kaempferol 3-*O*-rutinoside, the quercetin derivatives (quercetin 3-*O*-glucose, quercetin 3-*O*-galactose, quercetin 3-*O*-glucuronide) and the myricetin derivatives (syringetin 3-*O*-glucose, syringetin 3-*O*-galactose and myricetin hexoses) in amounts comparable with earlier reports for bilberry (Mikulic-Petkovsek *et al.* 2012). Proanthocyanidins included monomers of catechin, epicatechin, epigallocatechin and gallocatechin, and polymers of procyanidin A2, B1, B2 and/or B4 and B3.

ACs were the most abundant class of flavonoids present in ripe bilberry fruits. The total average amount of ACs in ripe berries from Northern Europe, Finland, was about 2600 mg/100 g DW, which is comparable with the amounts earlier reported for bilberries originated at the latitude 65°N (Jaakola *et al.* 2002, Lätti *et al.* 2008). The major constituents of the AC profile were the 15 known anthocyanidins; Dp's, Cy's, Pt's, Pn's and Mv's combined with the sugars glucose, galactose and arabinose (Lätti *et al.* 2008). In addition, acetylated and *p*-

coumaroyl-binded forms of ACs, Pg's and Cy 3-*O*-sambubioside compounds were found.

The bilberry populations analyzed in Southern Europe (Alps of Italy; III, IV), showed similar anthocyanidin profile and content as bilberries from Northern Europe (II). In these samples, three additional acetylated compounds were detected: Dp acetyl 3 gal, Pn 3 acetyl 3 gal and Pt acetyl 3 gal, as the analyses was performed on 1 g of fresh material (III, IV). In total, 37 AC forms have been detected in ripe bilberry fruits with the analysis method used.

Table 2. Phenolic acid (mg/100g) profiles in dry bilberries collected in the Oulu area (Northern Finland) at green unripe (stage 2) and ripe stages (stage 5-6). Compounds detected for the first time in bilberry are marked with an asterisk (*).

	Unripe bilberries (stage 2)	Ripe bilberries (stage 6)
Benzoic acid derivatives		
2,4-Dihydroxybenzoic acid *	1,6 ± 0,5	0,0
2,5-Dihydroxybenzoic acid *	2,3 ± 0,1	0,0
2,6-Dihydroxybenzoic acid *	0,5 ± 0,3	0,0
3,5-Dihydroxybenzoic acid *	1,8 ± 0,4	0,0
Vanillic acid	0,9 ± 0,1	0,0
Ellagic acid	7,8 ± 2,5	5,8 ± 1,6
Hydroxycinnamic acids		
p-Coumaric acid	2,7 ± 0,7	0,1 ± 0,1
Caffeic acid	5,5 ± 4,4	2,0 ± 1,8
Ferulic acid	0,9 ± 0,7	0,0
Neochlorogenic acid	9,1 ± 2,7	2,7 ± 1,3
Cryptochlorogenic acid *	1,4 ± 0,4	0,0
Chlorogenic acid	700,8 ± 167,6	116,8 ± 48,9
Flavanones		
Naringenin	0,5 ± 0,1	0,2 ± 0,1
Naringenin-7-*O*-glu *	0,0	50,0 ± 17,0
Flavonols		
Quercetin	0,7 ± 0,7	0,7 ± 0,3
Quercetin-3-*O*-glu	2,3 ± 1,7	7,8 ± 6,8
Quercetin-3-*O*-gal	9,5 ± 4,0	11,8 ± 7,1
Quercetin-3-*O*-glucuronide	76,0 ± 9,3	40,7 ± 6,0
Quercetin pentose	1,1 ± 0,5	1,2 ± 0,8
Syringetin-3-*O*-glu + gal	0,0	1,7 ± 0,2

	Unripe bilberries (stage 2)	Ripe bilberries (stage 6)
Kaempferol-3-rutinoside	0,0	1,4 ± 1,1
Myricetin	0,0	1,4 ± 0,8
Myricetin hexose	0,0	1,9 ± 0,3
Flavan-3-ols (Proanthocyanidins)		
Catechin	3,4 ± 0,9	1,1 ± 0,1
Epicatechin	155,0 ± 11,2	42,1 ± 6,8
Gallocatechin	1,1 ± 1,0	1,0 ± 1,0
Epigallocatechin	13,1 ± 2,1	12,6 ± 5,3
Procyanidin A2	0,3 ± 0,2	0,3 ± 0,1
Procyanidin B1	3,9 ± 1,4	0,8 ± 0,6
Procyanidin B2+B4	0,5 ± 0,4	0,2 ± 0,0
Procyanidin B3	150,4 ± 34,0	39,2 ± 5,4
Anthocyanins		
Cy 3 gal	0,0	211,7 ± 43,6
Cy 3 glu	0,0	497,1 ± 105,8
Cy acetyl 3 gal *	0,0	0,3 ± 0,2
Cy acetyl 3 glu	0,0	0,5 ± 0,4
Cy coum 3 gal	0,0	4,9 ± 1,9
Cy coum 3 glu	0,0	9,4 ± 5,5
Cy sambubioside	0,0	1,9 ± 1,2
Dp 3 ara	0,0	90,5 ± 17,5
Dp 3 gal	0,0	61,1 ± 11,6
Dp 3 glu	0,0	665,2 ± 164,2
Dp acetyl 3 glu *	0,0	0,0
Dp acetyl 3 gal *	0,0	0,0
Dp coum 3 gal	0,0	0,6 ± 0,1
Dp coum 3 glu *	0,0	0,7 ± 0,1
Mv 3 ara	0,0	25,0 ± 2,4
Mv 3 gal	0,0	16,4 ± 4,0
Mv 3 glu	0,0	40,6 ± 13,8
Mv acetyl 3 gal *	0,0	0,3 ± 0,1
Mv acetyl 3 glu	0,0	0,7 ± 0,2
Mv coum 3 gal *	0,0	0,5 ± 0,4
Mv coum 3 glu *	0,0	23,9 ± 2,3
Pl 3 gal	0,0	0,1 ± 0,0
Pl 3 glu	0,0	0,5 ± 0,5
Pn 3 ara	0,0	13,1 ± 4,8
Pn 3 gal	0,0	52,7 ± 17,4
Pn 3 glu	0,0	12,2 ± 2,3

	Unripe bilberries (stage 2)	Ripe bilberries (stage 6)
Pn acetyl 3 glu *	0,0	0,4 ± 0,2
Pn acetyl 3 glu	0,0	0,0
Pn coum 3 gal	0,0	3,1 ± 2,1
Pn coum 3 glu	0,0	27,6 ± 13,2
Pt 3 ara	0,0	34,8 ± 5,8
Pt 3 gal	0,0	25,3 ± 6,3
Pt 3 glu	0,0	14,6 ± 2,5
Pt acetyl 3 glu	0,0	0,1 ± 0,1
Pt acetyl 3 glu *	0,0	0,0
Pt coum 3 gal *	0,0	0,0
Pt coum 3 glu *	0,0	0,0

4.2 Carotenoid accumulation during bilberry fruit development (I)

This study provides the first reported documentation of carotenoid biosynthesis in bilberry during fruit development and ripening. Bilberry fruits were collected from natural forests in Oulu at five different developmental stages, from flower to fully ripe berries (Fig. 1), and were analyzed for transcript levels of carotenoid biosynthetic genes and carotenoid profile. Partial sequences of genes phytoene synthase (*VmPSY*), phytoene desaturase (*VmPDS*), ζ-carotene desaturase (*VmZDS*), carotenoid isomerase (*VmCRTISO*), lycopene β-cyclase (*VmLCYB*), lycopene ε-cyclase (*VmLCYE*), β-carotene hydroxylase (*VmBCH*), carotenoid β-ring hydroxylase of the cytochrome P450 family (*VmCYP450-BCH*) and (*VmCCD1*) carotenoid cleavage dioxygenase were isolated from bilberry. All the isolated sequences showed a high identity at the amino acid level to the corresponding sequences reported previously in fruit carotenoid biosynthesis.

All the eight examined genes were expressed at detectable levels throughout berry development but with variable expression levels. The expression of the early biosynthetic genes *VmPSY*, *VmPDS* and *VmCRTISO* and *VmLCYB* was relatively low at the early stages of fruit development but increased 4- to 5-fold at the onset of ripening (stage 4). The expression of *VmBCH* was also relatively low at the early stages of fruit development but its expression increased already at green berry stage (stage 3). The expression of the late biosynthetic genes *VmZDS*, *VmLCYE* and *VmCYP450-BCH* was quite high at the stage of flowering (stage 1) and at green stage (stage 3) but dropped in the late ripening stages. The expression of *VmLCYE* varied greatly during fruit development and its expression

was again at a relatively high level in ripe berries (stage 5). The expression of *VmCCD1* increased gradually from small green fruit (stage 2) to ripe fruit (stage 5).

Eight carotenoids were identified by HPLC-DAD: antheraxanthin, cryptoxanthin, neoxanthin, violaxanthin, zeaxanthin, lutein, β-carotene and 9z-β-carotene. The results indicate that carotenoid content varies markedly during berry development. The highest carotenoid concentrations were found from the unripe berries (stage 2) at the beginning of berry development but levels decreased sharply thereafter reaching the total carotenoid levels of 2872 ± 471 μg/100 g DW.

Lutein was the most abundant carotenoid in bilberry fruits with gradually decreasing levels from unripe to ripe fruit, final concentration in ripe berries being 1476 ± 314 μg/100 g DW. Also concentrations of *β*-carotene and its isoform 9z-*β*-carotene showed a gradual decrease from unripe to ripe fruit, being 380 ± 81 and 142 ± 12 μg/100 g DW, respectively. The whole group of xanthophylls, except cryptoxanthin which was found in traces at ripeness, followed the same decreasing trend during berry ripening.

4.3 Flavonoid accumulation in bilberries under modified light conditions in controlled room temperature (II)

Bilberry plants were treated with selected wavelengths of the visible light spectrum (blue, red, far-red or white light) during early-stage fruit development (stage 2) or left in the dark, in order to investigate the effect of light quality on flavonoid accumulation in berries at ripeness. After 48 hours treatment under continuous light or darkness, plants were moved to the greenhouse, under natural light conditions. The whole experiment was conducted in temperature controlled conditions of 21 ± 1 °C.

It was observed a tendency of blue light to decrease the level of quercetin 3-*O*-galactose, while that of red wavelengths to increase the levels of myricetin hexoses. On the contrary, the amounts of procyanidin A2 were lower under red light treatment, and procyanidin B1 level was higher under white light treatment compared with all the other light treatments. However, the most prominent effect of monochromatic light treatments was seen on the AC profile. The average accumulation of Dp, Mv and Pt were increased 33%, 46% and 38%, respectively, in berries treated with monochromatic light wavelengths when compared to the berries of the plants grown in white light conditions. No differences were found

on the contrary, for Cy and Pn. Plants left in dark conditions accumulated the same AC amount as plants treated with white light. In the context of single AC compounds, monochromatic light increased Dp, Mv and Pt compounds conjugated with glucose, galactose and arabinose sugars, but had no effect on the acetylated and coumaroylated compounds.

The expression of flavonoid pathway genes *VmCHS*, *VmF3'5'H*, *VmDFR*, *VmANS* and *VmANR*, and the transcription factor *VmMYB2* were also analyzed during the light treatments. Most of the examined genes showed increased expression during the first 12 hours from the beginning of the light treatment, even though variation between samples and time points was high. *VmANS* expression increased up to 3-, 2- and 3.5-fold under monochromatic blue, red and far-red light treatments, respectively, compared to dark treated plants. Under white light, vice versa, the expression was only slightly increased (up to 1.3-fold) compared to dark treated plants.

4.4 Carotenoid accumulation in bilberries under modified light conditions in controlled room temperature (I)

The effect of the different light conditions on the accumulation of carotenoids was studied during two 5-days experiments, the first conducted during the early stage of berry development (stage 2), whereas the second on plant holding berries close to ripeness (stage 5). The expression of the carotenoid pathway genes and the berry carotenoid profile was evaluated in response to different photoperiodic conditions. Continuous white light (long photoperiod) was compared with circadian photoperiod -16 hours day/8 hours night- under white light (short photoperiod). Moreover, supplemental red light (600-800 m) under the shorter photoperiod was tested (I).

Generally, all the light treatments induced an increase in the expression of carotenoid pathway genes, compared to berries placed in darkness (which was used as a control). During early berry development, the long photoperiod, increased markedly the expression of *VmPSY* (10-fold), *VmPDS* (20-fold), *VmCRTISO* (10-fold), and to a small extent also the expression of *VmLCYB* (2-fold) and *VmLCYE* (6-fold) compared to the shorter photoperiod. Under the shorter photoperiod with supplemental red light, the gene *VmPSY* was expressed as under the same photoperiod with only white light, whereas *VmPDS*, *VmCRTISO* and *VmLCYB* were increased by 10-, 5- and 1-fold, respectively.

In almost fully ripe berries (stage 5), the response of carotenoid pathway genes to light conditions showed a different trend. The expression of the genes *VmPSY*, *VmZDS*, *VmCRTISO*, *VmLCYB* and *VmLCYE* was particularly stimulated by the short photoperiod with supplemental red light. On the contrary, under long and shorter photoperiod under white light the gene expression was similar.

The metabolic profile showed to be affected by light, especially during the early stage of bilberry development, when the accumulation of both carotenes and xanthophylls was increased in presence of light. Under long photoperiod, β-carotene, 9z-β-carotene and zeaxanthin were increased compared to shorter photoperiod, whereas antheraxathin and violaxanthin were accumulated more under the shorter photoperiod. In the presence of supplemental red light in the short photoperiod, all the compounds were accumulated in amounts comparable to plants grown in darkness. On the contrary, light treatment did not affect the accumulation of carotenoids in berries close to ripeness.

4.5 Different combinations of light and temperature in field affected anthocyanin accumulation in bilberries and blueberries (III, IV)

In the present study, the AC composition of bilberries and blueberries growing along an altitudinal gradient was studied in relation to changes in environmental light and temperature conditions. Light and temperature vary markedly along an altitudinal gradient, whereas the soil pH ranged between 4.3 and 5.1, which is optimal for the growth of *Vaccinium* species, but did not show any particular trend connected with altitude (IV).

Light conditions were measured along the altitudinal gradient, in six locations in the range between 485 m a.s.l. and 1404 m a.s.l. Light intensity increased constantly towards increasing altitudes, and significant changes were recorded between locations at 485 m a.s.l., at 756 m a.s.l. and locations higher than 1034 m a.s.l. The increase was due to a progressive increase of visible light along with altitude, although the blue and red components were not significantly affected with increasing altitude. The UV radiation accounted only for the 1.3-3.8% of the total radiation, and was not significantly affected by altitude. Temperature decreased progressively with increasing altitude.

Bilberry and blueberry showed important differences in the accumulation of ACs along altitudinal gradients. A clear positive trend in the accumulation of ACs was found in bilberries with increasing altitude in both years of study. The trend was due to a significant increase in the tri-hydroxylated anthocyanidins as Dp

($p = 0.0001$ in 2013, $p = 0.0002$ in 2014) and Mv ($p = 0.0002$ in 2013, $p = 0.0026$ in 2014). Also Pt increased in minor level with increasing altitude ($p = 0.001$ in 2013). The accumulation of the mono-hydroxylated Cy and Pn instead was not affected by the altitude. In blueberry, an opposite trend was observed during the two years; in 2013 the accumulation of total AC was positively increasing towards higher altitudes ($p = 0.0006$), while in 2014 accumulation was negatively correlated with altitude ($p = 0.0000$). In blueberry, all classes of anthocyanidins were significantly correlated with the altitude (IV).

In order to study the effect of light quality conditions on AC accumulation in greater depth, we conducted a field experiment with the application of coloured photo-selective nets over two growing seasons (III). As described in Table 1, he nets used were red, which removed part of the blue bands from sunlight spectra, blue, which instead increased the proportion of blue bands reaching the plants and black which reduced the intensity of light by 90% and increased the proportion of blue bands. All the treatments were compared to berries grown under natural sunlight, in open-field conditions. The nets were shown also to modify the temperature around the study plants (Table 1). The temperature under the red net followed the temperatures in the open-field, except during sunlight exposure hours (9 am - 4 pm) when temperature was lowered up to 0.5 °C. Blue and black nets resulted in an overall decreased in air temperature of 0.1-0.5 °C during the night hours. During the day-time temperatures increased up to 1.5 °C during sunlight exposure hours (10 am - 4 pm) and decreased during the following hours under the blue net. On the contrary, under the black net, temperature presented a sharp and linear decrease (as much as to 2.5 °C) during the sunlight exposure hours (9 am – 5 pm) and was increased up to 1.0 °C in the following hours (5 pm – 8 pm) before being decreased during the night hours.

The highest amount of ACs was detected in bilberries grown under the black net both in 2013 and 2014, which was significantly higher ($p<0.01$) than in bilberries grown under the red net or in the open-field. Under the blue net, ACs accumulated at the same level as under the black net in 2013, due to a significant increase in the accumulation of Dps and Mvs over the red net and open-field grown bilberries. An opposite trend was observed in blueberries, where the accumulation of ACs was lowest under the black net, compared to all other treatments in both years. The decrease was accompanied by a significant decrease in Dp ($p<0.01$), Cy and Pn ($p<0.05$). In blueberries ripened under the blue, red and white nets, the content of total ACs was similar, and no differences in anthocyanidin classes were found in both seasons. Significant differences were

recorded in 2013 between blueberries grown in open-field compared to all other treatments ($p<0.05$), as blueberries grown under direct sunlight produced the highest amount of ACs accompanied by highest production of all classes of anthocyanidins. The trend was, however, not repeated in 2014, as blueberries under sunlight accumulated the same amount of ACs as under colored nets (III).

5 Discussion

5.1 Accumulation of flavonoids and carotenoids during berry development and ripening

At ripeness, the secondary metabolite profile of berries and more generally of fruits, is determined by the interplay of multiple factors. A complex program of fruit ripening and maturation regulates major metabolic and structural changes, such as sugar accumulation and softening. Contemporarily, the coordinated expression of the genes of the metabolic pathways leads to accumulation and degradation of secondary metabolites, defining the colour and the aroma of the ripe fruits. In bilberry, the accumulation of flavonoids and carotenoids during berry development represents the same trends as in other berry species, such as blueberry (Castrejon *et al.* 2008, Rowland *et al.* 2012, Zifkin *et al.* 2012), grape (Castellarin *et al.* 2006, Farina *et al.* 2010), strawberry (Zhu *et al.* 2015) and raspberry (Carvalho *et al.* 2013).

At the early stages of ripening, bilberries contain high yields of hydroxycinnamic acids, proanthocyanidins, quercetin-3-*O*-glucuronides, carotenoids and xanthophylls (Table 2, I). At the onset of ripening, the level of quercetin derivatives (in particular quercetin-3-*O*-glucuronide) decreases significantly between the early and late ripening stages, whereas the level of more hydroxylated myricetin-based flavonols increase during ripening, as found by Jaakola et al. (Jaakola *et al.* 2002). Consistently with the metabolic profile, also the expression of the flavonoid pathway genes *VmCHS, VmDFR* and *VmANS* increase (Jaakola *et al.* 2010). The flavonoid 3′5′ hydroxylase (*F3’5’H*) gene catalyses two hydroxylations in the 3’- and 5’-positions of flavonoids leading to the myricetin and delphinidin branch. Although no specific studies have been performed on the time-course of *VmF3’5’H* gene expression during bilberry fruit development, studies conducted on blueberry and grape berries showed that the gene is weakly expressed during the earliest ripening stages and is abundant only during the late ripening stages, closely paralleling the appearance of ACs (Zifkin *et al.* 2012). In blueberry, as the fruit ripens and the exocarp colour changes from mostly green to partially pink, blue-purple Dp-type ACs begin to accumulate. The appearance of the tri-hydroxylated anthocyanidin (Dp) and derivatives (Mv and Pt) is coordinated with, and likely driven by, the abundance of *VcF3'5'H* transcripts at stage S5 (Zifkin *et al.* 2012). Based on conservation of the flavonoid pathway in diverse

species, the *VmF3'5'H* gene is expected to increase in the late ripening stages of bilberry explaining the shift from quercetin- to myricetin-derivatives at the ripe stage (Table 2). During ripening, the expression of the *VmANR* gene decreases consistently along with the reduced amount of proanthocyanidins in ripe bilberries (Jaakola *et al.* 2010).

Differently from the flavonoid pathway, the expression of carotenoid biosynthetic genes is not consistent with the carotenoid metabolic profile. As fruit development proceeds, the expression of the carotenoid biosynthetic genes increases whereas carotenoids decrease. However, the expression of the *VmCCD1* gene increased in ripening stages as the 9-*cis*-epoxycarotenoid dioxygenase *VmNCED1* and neoxanthin synthase *VmNSY* (Karppinen *et al.* 2013) and similarly to *VvCCD1* in grape berries (Lashbrooke *et al.* 2013). CCD catalyses the cleavage of a variety of carotenoids at the 5',6' and 9',10' double bond position, which results in the production of a variety of flavour and aroma compounds (Lashbrooke *et al.* 2013). In grape berries it was found that *VvCCD1* has ε-carotene and β-carotene as substrates, suggesting that the increased activity of *VmCCD1*, together with the increased activity of *VmNCED1* and *VmNSY*, is involved in the decrease of carotenoid content during bilberry ripening (Lashbrooke *et al.* 2013). Nevertheless, the analysis of volatile compounds accumulation would confirm the involvement of *VmCCD1* in the production of aroma compounds during bilberry ripening.

Several *in vitro* and *in vivo* studies have confirmed that consumption of antioxidant molecules through diet show anti-cancer properties and offer multiple benefits for human health (Li *et al.* 2015). Considering the present study, results emphasize bilberries as excellent sources of antioxidants readily absorbed by the human body (Bowen *et al.* 2015). Indeed, lutein and β-carotene, which are highly absorbable by the human intestinal tract (Bowen *et al.* 2015) accounted for 65% of the total carotenoids of bilberry. Also, despite the low absorbability of ACs (Li *et al.* 2015), the 50-60% of bilberry ACs is composed of Dps, one of the most absorbable AC forms (McGhie & Walton 2007).

5.2 The role of light in accumulation of flavonoid and carotenoid compounds in berries

Light regulates many physiological processes during berry ripening, and both photoperiod and light spectrum affect the accumulation of secondary metabolite compounds in bilberry. The present results showed that carotenoid biosynthetic

genes are not only regulated at developmental level, but they are also light inducible, as they were highly expressed in berries exposed to light compared to the ones grown in darkness (I). Controlled light experiments showed that a long photoperiod (24 hours of continuous light) markedly increased the expression of the carotenoid pathway genes *VmPSY*, *VmPDS*, *VmCRTISO*, *VmLCYB* and *VmLCYE* in green unripe berries, compared to shorter photoperiod with 16 hours of day and 8 hours of night, leading to a higher accumulation of carotenoids at the early stages of berry ripening (I). Earlier experiments conducted in phytotrones showed that a long photoperiod stimulates the accumulation of ACs in bilberry clones from Nordic countries, increasing the accumulation of ACs compared to a shorter photoperiod (Uleberg *et al.* 2012).

Light quality also affected flavonoid and carotenoid biosynthesis in bilberries. The total AC content in ripe berries was significantly increased in berries under monochromatic lights of the blue, red and far-red spectra, in comparison to fruits under white light or in darkness. The increase regarded the classes of the tri-hydroxylated anthocyanidins Dp-, Pt- and Mv-glycosides, but not the mono-hydroxylated forms Cy and Pn which were accumulated at the same level of fruits under white light or in darkness. The expression of the *VmCHS, VmF3'5'H*, *VmDFR* and *VmANR* genes was less influenced by the light treatments, which was consistent with the detected levels of flavonols and proanthocyanidins, which were not markedly affected by the different light treatments (II). The expression of *VmANS*, which is the key gene in the biosynthesis of ACs, clearly increased under monochromatic light treatments, whereas under white light and in the dark treatment it was not influenced in response to light. Blue, red and far-red light all up-regulated the expression of *VmANS* already within the first 6 h after the beginning of the light treatment and also throughout the 2-day treatment. According to Jaakola *et al.* (2010), *VmANS* is expressed at low level in bilberry fruits at the early stages of fruit development compared with the ripening berries. However, the early stages of berry development appeared to be reactive to the monochromatic light treatments leading to the accumulation of ACs by increasing the expression of *VmANS* (II). As well, in study I, the exposition of bilberry plants to white light with additional light in the red-far red wavelenghts, showed to increase the expression of the biosynthetic genes *VmPDS*, *VmCRTISO*, *VmLCYB* in unripe berries, and *VmPSY*, *VmPDS*, *VmCRTISO*, *VmLCYB* and *VmLCYE* in ripe berries, although the carotenoid content was not increased.

The application of monochromatic wavelengths using LED lights has also been used in other studies concerning the effect of selected wavelengths on metabolite accumulation in fruits, which supports the present results (I, II). Kondo *et al.* (2014) showed that in grape berries additional red light affected the expression of *VvNED1* and levels of ABA, which is produced via the carotenoid pathway. Red light has been shown to induce carotenoid biosynthesis also in tomato (Liu *et al.* 2009) and citrus fruits (Ma *et al.* 2012). Blue light has been found to significantly increase the biosynthesis of ACs also in strawberries (Kadomura-Ishikawa *et al.* 2013) and grape berries (Kondo *et al.* 2014), while in cranberries, red and far-red light increased the AC accumulation over white light (Zhou & Singh 2002).

Generally, it appears that the early stages of bilberry development are highly reactive to light, which increases the expression of genes involved in the accumulation of flavonoids and carotenoids and later the metabolite accumulation in the berries. Moreover, short exposition to specific portions of the light spectrum during the early berry development has high effects on the final secondary metabolite profile of bilberries.

5.3 Interaction of light and temperature

Despite the strong role of developmental regulation in the accumulation of flavonoids during berry ripening, numerous studies in the past few years have shown that light intensity, photoperiod, light quality and temperature may induce quantitative and/or qualitative changes in the AC profile of apples, grape berries, strawberries and bilberries (Jaakola 2013). In bilberry, indeed, long photoperiod (24 hours) increased the levels of all measured ACs, except Cy derivatives (Uleberg *et al.* 2012). In study II, it was shown that also specific portions of the light spectrum increased markedly the accumulation of Dp-glycosides and their derivatives (Mvs and Pts) in bilberries, but not Cys and Pns. Therefore, the light quality environment has a great impact on both quantitative and qualitative changes in the AC profile of bilberries.

The role of light quality, combined with light intensity and temperature, in AC accumulation was further studied using photo-selective nets in field conditions (III). As the early stages of berry development showed to be the most reactive to different light quality conditions (II), nets were applied over bilberry plants from the early stages of berry development (stage 2) until berries were ripe (stage 6). The highest AC contents were reached under the black and blue nets,

which presented a similar temperature trend, i.e. lowered temperatures during the night, and a marked difference between maximum and minimum temperatures during daily hours. In addition, despite that the light intensity was not the same, both nets transmitted higher proportions in the blue bands of the light spectra. In bilberries under these light-temperature combinations, the anthocyanidin composition of Dps, Pts and Mvs was significantly increased, whereas Cys and Pns were not affected. Recent studies on berry species, such as grape (Kondo *et al.* 2014) and strawberry (Kadomura-Ishikawa *et al.* 2013) support the importance of the blue irradiance spectra in the AC biosynthesis. The present results indicate that predominance of blue wavebands and low temperature positively regulated the AC composition of bilberries. This aspect can be further supported by results of the same study (III), as under the red net where temperatures were similar to open-field conditions throughout the day and where the blue bands were removed from the light spectra, the content and profile of ACs was most similar to bilberries grown in open-field conditions.

The combination of environmental factors was also suggested to explain the recent findings on changes in bilberry AC profile along a latitudinal gradient (Lätti *et al.* 2008, Åkerstrom *et al.* 2010). The concentrations of the tri-hydroxylated Dp-, Pt- and Mv-glycosides were markedly increased moving from the latitude of 52°N towards the latitude 69°N, whereas Cy and Pn were less affected. The final results indicated that the total AC amount increases with increasing latitude. Bilberry plants moved from the locations along the latitudinal gradient to a common test field at 65°N, showed that this phenomenon is under genetic control as an increasing trend in AC content was still detected towards the more northern clones (Åkerstrom *et al*. 2010). However, climatic factors are involved, as differences in temperature, day length and spectral composition of sunlight are naturally and closely correlated with latitude (McKown *et al*. 2014).

The same trend was found along an altitudinal gradient established in the Alps of Italy in study IV. Six natural bilberry populations between 1166 m a.s.l. and 1829 m a.s.l. showed a clear positive trend in the accumulation of ACs with increasing elevation, over the two year study. The higher AC content was due to a significant increase in the tri-hydroxylated anthocyanidins Dp- and Mv-glycosides, and to a minor level also Pt. The accumulation of the mono-hydroxylated Cy and Pn instead, was not affected by altitude (IV). The AC profile was variable within bilberry individuals growing on the same site, especially in populations growing over 1500 m a.s.l. Thus, the results emphasize the importance of genetic background in the AC accumulation. However, at

increasing elevations, a progressive increase in the intensity of the solar radiation with decreasing temperature, suggests that environmental factors have a greater effect on the accumulation of tri-hydroxylated anthocyanidins in bilberry.

Moreover, when plants from the same populations were moved to lower altitudes (485 m a.s.l.), in open-field conditions, berries accumulated lower amounts of ACs (III), confirming the environmental role in the determination of the final AC profile of berries. The results of the present work suggest that temperature has a major effect on accumulation of ACs, as the difference in the average daily temperature in the original locations (above 1166 m a.s.l.) was at least 5 °C lower than in the test field (485 m a.s.l.) and the AC accumulation was almost doubled in the original site. In support of this, the bilberries ripened in the test field under a black net, which decreased the outside temperature by about 2.5 °C during middle hours of the day and by about 0.5 °C during the night, accumulated amounts of ACs similar to the original locations due to an increase in the synthesis of Dp-based ACs (III-IV). This is in line with the results reported in controlled temperature conditions by Uleberg *et al.* (2012), showing that in bilberries, Dp derivatives are produced in higher amounts at lower (12 °C) compared to higher temperatures (18 °C). Bilberry individuals growing at the highest altitudes (between 1600 m a.s.l. and 1829 m a.s.l.) accumulated the highest AC content among all samples, with the highest content of Dp derivatives, which could be the result of a mechanism of genetic adaptation to the low temperatures recorded in the same locations.

Low temperatures and predominance of blue wavebands seem to favor the expression of the *F3'5'H* gene, which is responsible for the hydroxylation at the 3' and 5' positions of the B-ring of the precursor dihydrokaempferol into Dp and the derivatives Mv and Pt. The enzyme also shifts the biosynthesis from the Cy and Pn branch towards the branch of the pathway producing Dp, Mv and Pt as final core structures of ACs (Castellarin *et al.* 2006).

In studies III and IV, the trials were extended to cultivated highbush blueberries (cv. Brigitta Blue) in order to compare the effect of light-temperature combinations on different *Vaccinium* species. Results showed a different response of bilberry and blueberry to the same environmental conditions. Although bilberries accumulated the highest content of ACs under the black net, the same conditions were instead not favorable for blueberries, whose maturation was delayed for at least two weeks, and sugar and AC accumulation were significantly decreased compared to berries grown in sunlight conditions. On the contrary, the use of colored nets induced an increase in berry size and weight. The increase in

the berry volume also increased the berry water content and therefore the AC content in blueberries on weight bases resulted to be lower under the colored nets compared to berries grown under sunlight, although statistical analysis correlated positively the AC accumulation with the size of blueberries (III). The altitudinal studies showed that in blueberry the accumulation of AC was also highly determined by temperature (IV).

6 Conclusions and future perspectives

In the present study, different AC profiles were found among *Vaccinium* species (bilberry vs blueberry) and also among individuals of the same bilberry population. The results gave further evidence on the role of environmental factors in activation of the tri-hydroxylated branch of the flavonoid pathway. Temperature was shown to play a major role in the specific accumulation of Dp-derived ACs in natural bilberry populations. However, the spectral composition of light also plays a role, as the increased proportion of blue wavelengths in the light spectrum, induced an increased content of Dp-based ACs at ripeness. The effect of light quality on the accumulation of flavonols, proanthocyanidins and carotenoids was less pronounced, although red wavelengths of the light spectra affected the accumulation of these metabolites. The accumulation of other phenolic compounds found in bilberry, such as hydroxycinnamic acids, flavanones and stilbenes instead was not affected by light quality.

The study shows that the same bilberry populations and also blueberries accumulate different amounts of ACs when grown in different locations, under different environmental conditions. Temperature is confirmed to play a major role in the accumulation of ACs in *Vaccinium* species, whereas the response to light conditions is clearly species-dependent. These results, therefore, give exciting prospects for further gene x environment interaction studies on the regulation of the metabolic pathways of *Vaccinium* species. The gene expression profile of the structural genes and the transcription factors from secondary metabolic pathways in response to the different environmental conditions considered in the present study, would explain to which extent light and temperature affect the accumulation of secondary metabolites at gene level. In bilberry, particular emphasis should be pointed to the expression of the key gene *VmF3'5'H* to confirm the activation of the tri-hydroxylated branch of the flavonoid pathway.

If trends in global warming continue into the future, the distribution of bilberry populations may change both in Nordic countries and Alpine environments. The emerging attempts of semi-cultivation practices contribute to domestication of bilberry and enable preservation of natural populations (or diversity). The present study indicates that for production of high-quality berries with regards to AC production, bilberry plants located at high latitudes or altitude should be preferred for propagation. However, environmental conditions must be taken into consideration during field establishment in relation to bilberry genotypes. For this purpose, the metabolite profile of individuals within each

bilberry population may allow to select good genotypes which could be used for cultivation and breeding purposes. Coloured photo-selective nets could be used as a complementary agricultural practice in semi-cultivated field trials for bilberry and for *Vaccinium* species in general. The advantage of netting is that light and temperature conditions can be regulated over the crop, giving also the possibility to widen the locations for cultivation. In this case, extending the gene x environment studies to other *Vaccinium* species and to particular cultivars of agronomic interest, would offer the possibility to select the optimal light-temperature conditions in order to achieve optimal berry production and enhance the quality and the potential health benefit of the berries under cultivation.

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Original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

I Karppinen K, Zoratti L, Sarala M, Carvalho E, Pukki J, Mentula H, Martens S, Häggman H & Jaakola L (2015) Carotenoid metabolism during bilberry fruit development is regulated by biosynthesis and degradation. (manuscript)

II Zoratti L, Sarala M, Carvalho E, Karppinen K, Martens S, Giongo L, Häggman H & Jaakola L (2014) Monochromatic light increases anthocyanin content during fruit development in bilberry. BMC Plant Biology 14:377.

III Zoratti L, Jaakola L, Häggman H & Giongo L (2015) Modification of sunlight radiation through colored photo-selective nets affects anthocyanin profile in *Vaccinium* spp. berries. PLOS ONE DOI: 10.1371/journal.pone.0135935

IV Zoratti L, Jaakola L, Häggman H & Giongo L (2015) Anthocyanin profile in berries of wild and cultivated *Vaccinium* spp. along altitudinal gradients in the Alps. (manuscript)

638. Kortelainen, Tuomas (2014) On iteration-based security flaws in modern hash functions

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