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Characterization of bioactive compounds and quality attributes in *Vaccinium spp.* during development ripening and storage.

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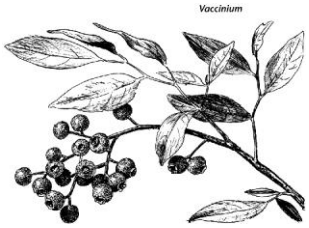
Index



1. Introduction

Blueberry belongs to the division of Spermatophyta. The flower has an ovary, thus placing it in the class Angiospermae, and its seedlings have two cotyledons, putting it in the subclass Dicotyledonae. It is a member of the Ericaceae family of plants comprising mostly woody shrubs that grow naturally on acid soils. This is a large family and is found widely distributed throughout the world.

The genus *Vaccinum* is a widespread genus with over 200 species of evergreen and deciduous woody plants vary in size from dwarf shrubs to trees; it includes many economically important cultivated small fruit species, like blueberries, bilberries and cranberries. Blueberries (*Vaccinium corymbosum*) belong to the subfamily Vacciniaceae, the genus *Vaccinium* and the subgenus *Cyanococcus* (from Greek "*cyano"* (blue) and "*coccus"* (berry). The European blueberry, also called bilberry (*Vaccinium myrtillus*) belongs to the subgenus Euvaccinium. Due to the reduced dimension of plants and fruits, this subgenus is not used for cultivation and the fruits naturally grow as a characteristic field layer species in boreal forests. In Italy the blueberry production includes both highbush blueberry (*Vaccinium corymbosum)* and bilberry (*Vaccinium mirtyllus).*

1.1. Bilberry

Among spontaneous blueberries in Europe, bilberry, also called European blueberry, covers the major commercial interest. East Europe is the area in which bilberry is more widespread. Poland is the most important producer in Europe (10-30000 Mg/year) followed by Finland (15000 Mg/year), Sweden (12000 Mg/year), Slovenia (2000 Mg/year), Italy (440 Mg/year) and Germany (400 Mg/year). Bilberry is a deciduous dwarf shrub (15-60 cm) and a characteristic field layer species in forests. The fruit are dark blue color with colored pulp and can varying in size from 4 to 8 mm and in weight from 0.2 to 0.4 g. Flowering time is from May until June while ripening time is from July until September. Fruits are manually harvested with an average yield of 1 Kg/ hour/man.

Bilberries are very well appreciated for their sensory qualities as well as for the high nutritional value. They are rich in tannins (7-12%), sugars (15-20%), pectin (8-10%), anthocyanins, carotenoids, vitamins (C, B, P) and organic acids. Bilberries can be eaten fresh or used by the food industry for different kind of food preparations such as ice creams, syrups, jams, yogurt and candies.

Nutraceutical properties of bilberry are known since the middle ages, when berries were used for their sensory qualities but also for treatment of diarrhea and colitis, and for their anti-bacterial

properties. The aroma of bilberry is regarded as special and delicious, differing from other blueberries.

Various ecological, physiological and genetic studies have been conducted on bilberry. Research has been performed on its morphology, growth habit, pollination system, and population dynamics (Flower-Ellis 1971, Sjörs 1989, Jacquemart 1994, Tolvanen 1995, Nuortila et al. 2002). Also, its carbon-, nutrition- and water economy has been explored (Havas 1971, Lähdesmäki et al. 1990, Pakonen et al. 1990, Gerdol et al. 2000, Grelet et al. 2001). Many studies concern the response of bilberry to environmental factors like low temperatures (Taulavuori et al. 1997a), pollution (Taulavuori et al. 1997b, Reimann et al. 2001), enhanced UV-B radiation (Taulavuori et al. 1998, Phoenix et al. 2000, 2001) and biotic stresses (Koskimäki et al., 2009). Moreover, several studies have been conducted on the content of phenolic compounds and their organ-specific distribution in *Vaccinium* species (Määttä-Riihinen et al., 2004; Riihinen et al., 2008) as well as on the expression of genes that attend to the accumulation of phenolic compounds during bilberry fruit development (Jaakola et al., 2002). The influence of artificial wounding and solar radiation on biosynthesis of flavonoids and hydroxycinnamic acids was recently studied in bilberry leaves and fruit (Jaakola et al., 2008; Jaakola et al., 2004).

1.2. Blueberry

Highbush blueberry is one of the most commercially significant berry crops. It is mainly cultivated in United States and Canada, but also in Europe, Australia, Chile and New Zealand.

Poland is the biggest producer in Europe (11023 Mg/year), followed by Sweden (2576 Mg/year), Romania (2353 Mg/year), Italy (1509 Mg/year) and Spain (924 Mg/year) (http://faostat.fao.org/ 2009). The most productive regions in Italy are Piemonte, Trentino Alto Adige and Lombardia. Production of this crop is likely to increase in response to increased consumers demand for healthy foods. All the commercial species of blueberry are deciduous woody perennials, with simple leaves arranged alternately on the stem. Leaves dimension and morphology can vary among cultivars and thus can be used as an aid to identification. The plant is a bushy shrub that can reach and exceed 2-3 m in height, plants can be productive for 40-50 years. The root system is shallow and expanded, with fine roots colonized by mycorrhizal fungi in the soil. The blueberry can grow well on sites where most other crops fail. It performs well on loose textured soils (mixtures of sand and peat are optimal). It prefers an organically rich, medium to wet, well-drained soil in full sun to partial shade. Plants appreciate a good organic mulch. Pruning, as needed in late winter,

begins in the third year after planting. Although blueberries are self-fertile, cross-pollination produces the best yields. Flowering time is usually from mid April until the first ten days of May. In Italy blueberries ripen between June and September. The fruit is a round berry, with a weight of 1-3 grams, with a pronounced eye cavity. The characteristics of the eye cavity, and the scar of the stalk are useful in identifying cultivars. The color of fruit varies from blue to blackish blue and is made clearer by a layer of pruine also typical of the cultivar. The pulp is greenish-white, with a sweet-sour taste and contains from 5 to 70 seeds. In the same cultivar, the number of seeds is directly proportional to the size of the fruit. Blueberries are widely appreciated for their sensorial and health-promoting qualities. Many studies has been conducted on the antioxidant properties and phenolic content of blueberry (Howard et al., 2003, Prior et al., 1998), on effects of cold temperature storage (Connor et al., 2002, Krupa and Tomala, 2007) and on cultivation and breeding techniques (see Prodorutti et al., 2007, for a review). Positive effects of blueberry consumption on health and protection from different kind of pathologies have been widely documented (Krikorian et al., 2010; Kim et al., 2010; Adams et al., 2010).

In Italy the production of blueberry starts at the beginning of June until mid/late August. Based on the production period, the cultivars were divided into early- (maturing from early June to early July), medium- (from late June to mid-late July) and late-ripening (late July to early September). Below we report the main characteristics of the cultivars of blueberry (*Vaccinium corymbosum*) that we used in this work:

‘Duke’

It is the most widespread early ripening variety and the most used in new plants. It arises from the intersection of two cultivars, 'Ivanhoe' and 'Earliblue', and its cultivation began in 1985 in New Jersey, USA. The plant is of medium vigor, productive, self-fertile, plants show a good cold tolerance. The berries are well distributed in the canopy, easy to collect, with attractive color, rich in bloom, aromatic, with good sensorial characteristics. It is a variety that ripens very early, in fact, its harvest season is between late June and early July. In the last few years ‘Duke’ has been the most widely cultivated blueberry variety, always ensuring optimum productivity. Moreover, it was chosen in both the U.S. and in Italy and other Countries for its easy cultivation, for its good resistance to cold during the winter months, but especially for excellent quality also in the postharvest.

‘Blue Ray’

'Blue Ray' is a heavy producer cultivar, with high quality, large, powder-blue berries with outstanding dessert flavor. Pink tinged flowers are followed by edible, sweet, round, deep blue berries to 1.5 cm across. It is a midseason blueberry that ripens in early August. The foliage turns a burgundy color in the fall. It is similar to ‘Bluecrop’, but a bit sweeter. This deciduous shrub is dense with an upright, multiple-branched growth habit. The shallow, fibrous roots need constant moisture and good drainage.

‘Brigitta Blue’

Cultivar obtained by free pollination of 'Lateblue' in Michigan and selected in Victoria, Australia. It is in cultivation since 1980. The plant is very vigorous, of medium height, with upright habit, fast growing, self-fertile. It produces berries of good size and high sugar content, easy to collect, suitable for cold storage. It shows a quite high productivity, and cold sensitiveness in winter, so it should usually be grown at altitudes lower than 600 m above sea level.

‘Legacy’

The plant is vigorous, upright and is particularly suited for southern climates of the USA (low-chilling variety, i.e., with low demand for cold). The productivity is high and constant. The fruit is medium size with excellent flavor and sweetness, considered one of the best-tasting varieties, also has a long shelf. The fruit ripens in August, medium-late season. The foliage remains on the bushes during the winter. The plant is resistant to collar rot.

1.3. Bioactive compounds and health-promoting properties

Berry fruits are widely recognized as natural functional products. In fact these fruits represent a rich source of phytochemicals such as polyphenols and ascorbic acid (Tab.1).

Tab. 1 Phenolics, anthocyanins and ascorbic acid content of blueberry and bilberry (modified from Szajdek and Borowska, 2008).

Species	Phenolics (mg/100 g FW)	Anthocyanins (mg/100 g FW)	Ascorbic acid (mg/100 g FW)
Bilberry (*Vaccinium myrtillus*)	181.1–525.0	214.7–299.6	6.1-6.8
Blueberry (*Vaccinium corymbosum*,	261–585	62.6–331.0	12.4–13.1

1.3.1. Polyphenols

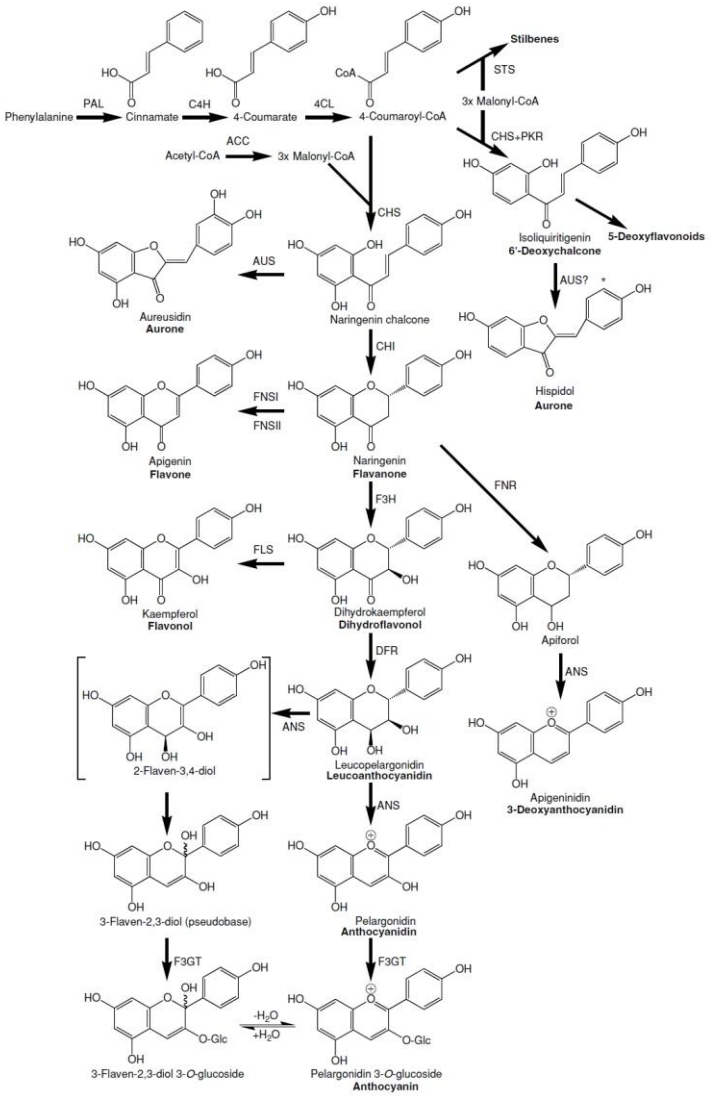


Fig. 1 The phenylpropanoid pathway

Polyphenols are biosynthesized through the phenylpropanoid pathway (Fig.1), which produces a wide range of secondary metabolites that are different in structure and molecular weight, and can be divided in four classes: phenolic acids (such as benzoic and cinnamic acid), tannins, stilbenes and flavonoids (such as anthocyanins, flavonols and flavanols).

The key polyphenols precursors are phenylalanine, obtained via the shikimate and arogenate pathways and malonyl-CoA, derived from citrate produced by the tricarboxylic acid (TCA) cycle. Most of the biosynthetic enzymes in the pathway characterized to date are thought to operate in enzyme complexes located in the cytosol. Flavonoid end products are transported to various sub-cellular or extracellular locations, those flavonoids involved in pigmentation (such as anthocyanins) are generally located into the vacuole.

Polyphenols are very important in the determination of characteristic taste and color of fruits and vegetables. In fact, phenolic acids are responsible for acid taste, tannins give astringency and flavonoids could attribute a bitter taste. Anthocyanins are the main pigments in fruits, vegetables as well as in flowers.

Phenolic compounds are also involved in allelopathic interactions between plants and animals, bacteria and fungi, and have also a role in plant's defense mechanisms.

Berry fruits are characterized by a high content and wide diversity of phenolic compounds (Szajdek and Borowska, 2008), the most common phenolic acids in berry fruits are cinnamic acid and benzoic acid. In presence of polyphenol oxidases phenolic acids lead to browning due to oxidation reactions (Shahidi and Naczk, 2004). Anthocyanins are a subclass of flavonoids derivatives of 2-phenylbenzopyrylium (flavylium cation) (Fig. 2) and valued as pigments in plants. They are important health-promoting phytochemicals and are abundant in berry fruits. In many fruits, these colored compounds accumulate only in the skin, while in bilberry they occur throughout the fruit flesh. In the cells they are located in vacuoles in the form of various sized granules. Anthocyanins consist of an aglycone (anthocyanidin), sugar(s), and, in many cases, acyl group(s) and they differ with regard to the number of hydroxyl groups in a molecule, the degree of methylation of these groups, the type, number and place of attachment of sugar molecules and the type and number of aliphatic or aromatic acids attached to sugars in the molecule (Heim et al., 2002; Kong et al., 2003). The composition of anthocyanins is particularly diversified in the fruit of blueberry and bilberry and more than ten anthocyanins were found in these species (Dugo et al., 2001; Du et al., 2004; Kader et al., 1996; Lohachoompol et al., 2008). While there are six common anthocyanidins, more than 540 anthocyanin pigments have been identified in nature, with most of the structural variation coming from glycosidic substitutions and possible acylation of sugar residues with organic acids. Among the regulatory factors of the anthocyanin pathway which have been characterized so far, MYB and helix-loop-helix (HLH) proteins are predominantly represented (Springob et al., 2003). In a recent paper, the expression pattern and functional analysis of a SQUAMOSA-class MADS box transcription factor, *VmTDR4*, associated with anthocyanin biosynthesis in bilberry was reported. *VmTDR4* was demonstrated to play an important role in the accumulation of anthocyanins during normal ripening in bilberry, probably through direct or indirect control of transcription factors belonging to the R2R3 MYB family (Jaakola et al., 2010).

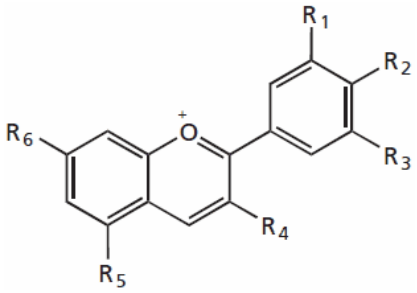


Fig. 2: The flavylium cation

Until recently phenolic compounds were regarded as non-nutritive compounds of fruits and vegetables which often hinder their technological processing. High level of phenolic compounds, in particular tannins, could also have adverse consequences because they inhibit the bioavailability of iron, thiamin and proteins, and block the activity of digestive enzymes in the gastrointestinal tract. Moreover, tannins can interact with proteins leading to astringency (Szajdek and Borowska,

2008). Nowadays the importance of these compounds in human diet is widely recognized. Many researches had been conducted so far to study the health-promoting and antioxidant properties of several classes of phenolic compounds (Leopoldini et al., 2004, Rice-Evans et al., 1997, Borges et al., 2010). As antioxidants, polyphenols can protect cell components from oxidative damage and limit the risk of various degenerative diseases related to oxidative stress (Scalbert et al., 2005).

Numerous studies in animal models and in *vitro* have shown that polyphenols added in diet could limit the development of cancers, cardiovascular diseases, diabetes and osteoporosis (Scalbert et al., 2005). *In vitro* the antioxidant capacity is commonly evaluated as the ability of different phenolic compounds to trap free radicals and reduce other chemicals. Their potency is then compared to that of a reference substance, usually Trolox (a water-soluble derivate of vitamn E). There are several assays which can be used for this purpose (see Sànchez-Moreno, 2002 for a review). However, the value of such measurements to evaluate the health benefits of isolated polyphenols or plant extracts is limited because of the following reasons: quite big differences have been observed using different methods of evaluation; polyphenols are extensively metabolized in the body, thus their structure (and their antioxidant properties) change during digestion and then the bioavailability can change widely from a polyphenol to another.

In conclusion the health benefits of phenolic compounds and, even more, of polyphenols-rich food and beverages cannot merely be evaluated as their antioxidant capacity *in vitro.* A precise examination of the type and amount of different polyphenols in a plant extract or food should be preferred. This does not exclude that the antioxidant properties of these compounds are a key factor that may trigger cell responses at the origin of their biological effects (Scalbert et al., 2005).

The phenolic content in fruits can be affected by several factors like species, variety, agronomical practices, ripeness, climate conditions (Benvenuti et al., 2004; Häkkinen and Törrönen, 2000; Connor et al., 2002; Anttonen and Karjalainen, 2005; Ehala et al., 2005; Castrejón et al., 2008).

1.3.2. Ascorbic acid

Ascorbic acid (AsA) is one of the most important molecules in biological processes. It is found in almost all plants and is involved in many metabolic reactions. Until a few decades ago, the most likely answer to the question of what would be the function of ascorbic acid, was that given by Albert von Szent Gyorgyi, when he won the Nobel Prize in 1937 for having discovered it: "*it is the factor able to cure all of the clinical symptoms known as scurvy, a syndrome that occurs in humans in case of a diet low in fruits and vegetables*". Today the same question might have different answers and few would mention the scurvy, which nowadays is not widespread anymore (Arrigoni

and De Tullio, 2002). The functions attributed to AsA are numerous: it acts as a cofactor for several enzymes, it plays a key role in scavenging free radicals, it acts as a donor/acceptor in electron transport systems located in chloroplasts and plasma membrane and, in some plants , it is the substrate for the synthesis of oxalate and tartrate (Diplock et al. 1998; Foyer et al. 1991; Padh, 1990, Loewus and Loewus, 1987; Foyer, 1993; Bànhegyi et al. 1997; Sauberlich, 1990 ; Noctor and Foyer, 1998; Smirnoff, 1996). Most vertebrates are able to synthesize AsA, unlike many invertebrates. A few mammals, including humans and some primates have lost the ability to biosynthesize it, and then L-AsA is an essential element (vitamin C) for them to be introduced with the diet. This deficiency is due to the lack of the enzyme that catalyzes the last step in the synthesis, L-1,4- gulono lactone oxidase (Gulo). The gene encoding this enzyme is present in the human genome, but is not expressed, because of numerous mutations (Nishikimi and Yagi, 1996; Nishikimi et al. 1992; Nishikimi et al., 1994). Chemically AsA is one of the simplest vitamins, it consists of a structure with six carbon atoms similar to that of hexose sugars, its precursors. More than 45 years ago Isherwood and his colleagues proposed a possible pathway for ascorbic acid biosynthesis in plants (Isherwood et al., 1954). This path was based on the conversion of derivatives of D-galactose, it was similar to what happens in animals and included an inversion in the configuration of the carbon skeleton, from D to L. The data supporting this pathway were concentrated mainly on the last step, where L-galactose-1,4-lactone is oxidized to L-ascorbic acid by the enzyme L-galactose-1,4-lactone dehydrogenase (GLDH). Over the years, very extensive and detailed studies carried by Loewus and his colleagues concluded that the majority (80%) of D-glucose is converted to L-AsA via a path that does not involve the inversion of the carbon skeleton (Saito et al., 1990; Loewus, 1963; Loewus, 1980; Loewus and Loewus, 1987). So it was suggested that the synthesis of ascorbic acid in plants could take place in an opposite manner compared to what usually happens in animals.

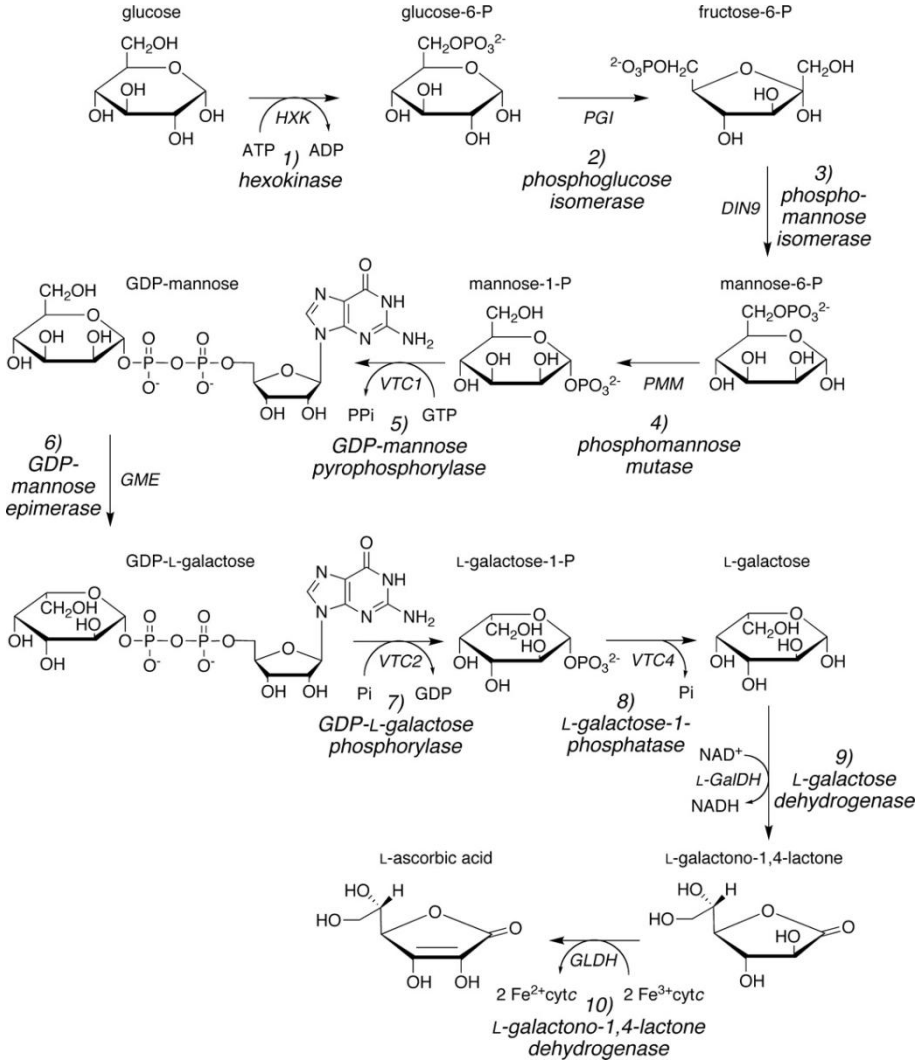


Fig. 3: The main ascorbic acid biosynthetic pathway.

Many of the concerns about the data collected during the last decades, have been clarified with the proposal of a new biosynthetic route, which involves GDP-L-galactose and GDP-D-mannose as precursors (Wheeler et al., 1998) (Fig.3). The first part of this cycle is also involved in the synthesis of precursors of cell wall polysaccharides, while the last steps (from the GDP-L-galactose and up) are purely dedicated to the synthesis of AsA. The enzyme that catalyzes the last step is L-galactose-1 ,4-lactone dehydrogenase (GLDH) as originally suggested by Isherwood, but in this cycle, the synthesis takes place without inversion of the skeleton to six carbon atoms, according to data collected by Loewus. The relevance of this new route also lies in the fact that the synthesis of L-AsA is deeply integrated in the metabolism of carbohydrates, polysaccharides of the wall, and in the glycosylation of proteins (Davey et al., 2000). Nevertheless, some intriguing questions remain unanswered, particularly with regard to the hypothesis that ascorbic acid produced by plants could derive from the conversion of uronic acids. It is well known that plants are able to synthesize

it from D-glucuronic acid derivatives and D-galacturonic (Isherwood et al. 1954; Mapson and Isherwood, 1956; Loewus, 1963; Chattererjee et al. 1960; Isherwood and Mapson, 1962; Loewus et al. 1958; Loewus et al., 1958b). The importance from the physiological point of view of this way should be interpreted with caution, waiting to get more complete data about it. It can be hypothesized that the synthesis of AsA from these precursors has some importance in certain conditions or in specific tissues. For example D-glucuronic acid and D-galacturonic are the major components of non-cellulosic polysaccharides of the cell wall, and their conversion into ascorbic acid may provide a way to recover some of the carbon derived from the disassembly of the wall that may occur during abscission, fruit ripening, softening and cell expansion.

Due to its low electronegativity, AsA can donate electrons to many types of substrates, in fact, it is able to interact with many forms of active oxygen, including $O_2^{\cdot-}$ and H_2O_2 (Halliwell, 1982). The direct product of AsA oxidation is the radical monodehydroascorbate (MDHA) also called ascorbate free radical (AFR). MDHA has normally a short life span, if not rapidly reduced by monodehydroscorbic reductase (MHDAR), it disproportionates into ascorbate and dehydroascorbate (DHA). A combination of *de novo* synthesis and an efficient recycling, thanks to monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) are critical in maintaining AsA levels constant. Monodehydroascorbate can in fact be enzymatically reduced to AsA in a reaction catalyzed by MDHAR, while DHAR catalyze the reduction of DHA to AsA. Reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are produced during normal plant metabolisms and the detoxification from these compounds can be considered as an integral part of the housekeeping duties required for an aerobic existence in eukaryotic cells. Oxidative stress can be defined as an imbalance between the production of ROS and the biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of tissues can cause toxic effects through the production of peroxides and free radicals that can damage all components of the cell, including proteins, lipids, and DNA. Efficient control of ROS levels includes several enzymatic and non-enzymatic systems acting in synchronicity. O_2^- produced in different cellular compartments is rapidly converted to H_2O_2 by the action of superoxide dismutase (SOD). The reduction of superoxide anion is also possible through direct action of AsA. But the reduction of O_2^- just convert one destructive ROS to another (H_2O_2).

Catalases (CAT) are the enzymes able to reduce H_2O_2 to water and molecular oxygen. These enzymes show high maximum catalytic rates but low affinity for substrates and their absence in chloroplasts precludes a role in protection in the Calvin cycle (Noctor and Foyer, 1998).

An alternative way of H_2O_2 detoxification is represented by peroxidases, a big family of enzymes which are ubiquitous in the cell and have a much more higher affinity for H_2O_2 than CAT.

These enzymes requires a reductant molecule since they reduce hydrogen peroxide to water. AsA represents the most important reducing substrate for H_2O_2 detoxification in plants (Noctor and Foyer, 1998). Ascorbate regeneration can occur by reaction with glutathione, which in turn is regenerated by using the reducing power of NADPH. From this observation, Foyer and Halliwell indicated the possible existence of an ascorbate-glutathione cycle (Fig. 4), thus AsA levels in plants could be

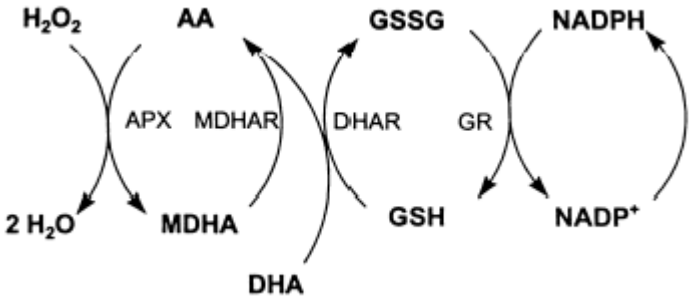


Fig. 4: Halliwell-Asada cycle.

maintained by an efficient balance between biosynthesis and recycling. In the recycling route (cycle of ascorbate-glutathione or Halliwell-Asada cycle), ascorbate peroxidase (APX) catalyzes the oxidation of AsA into monodehydroascorbate (MDHA); as we mentioned, this radical form could be spontaneously converted into AsA and dehydroascorbate (DHA). The enzymatic regeneration of AsA from MDHA and DHA is catalyzed by MDHAR, DHAR, and glutathione reductase (GR). This pathway provides an efficient way of recycling of AsA and detoxification of H_2O_2 (Noctor and Foyer, 1998) with the only consumption of reducing power given by NADH and NADPH.

In a Western-type diet, ascorbic acid is supplied through the consumption of fruits and vegetables, eaten fresh or in the form of juices and processed products. Substantial losses are found in processed fruits. The AsA content in plant-derived foods differs from species to species but is also depending on environmental conditions, cultural practices, however there is no unique model to describe the changes in ascorbic acid content with the progress of maturation (Breene, 1994). Processing and storage techniques may also have an effect on the ascorbate content of produce: preparation of preserved food, dehydration and storage at low temperatures are techniques by which is possible to ensure the supply of plant foods throughout the year. All plant foods are in fact seasonal, and may undergo to rapid changes when stored at room temperature without

adequate treatment. These changes affect the enzymatic reactions that cause variations in color and flavor, as well as have a profound impact on the nutritional value of the product. Losses in AsA content during fruit storage can be limited by stocking the fruit at low temperatures (0-4°C), in controlled atmospheres with low concentrations of O_2 and CO_2 concentrations higher than 10% (Lee and Kader, 2000). Fruits are subjected to a series of changes after harvest, and the ability to maintain the stability of AsA is linked to the enzymatic reactions that lead to its oxidation and reduction. There are products most susceptible to the loss of ascorbate and others, like citrus, characterized by a low pH of the juice which stabilizes AsA, but all the plant products are intended for a progressive decrease of AsA after harvest.

1.4 Plant activators

Plant resistance activators are a class of either natural or synthetic compounds that stimulate active plant defense mechanisms. The induction of pathogen resistance in plants is called "systemic acquired resistance" (SAR). SAR is considered as a long lasting and broad spectrum mechanism of defense from pathogens. The induction of SAR can be achieved by using abiotic agents like salicylic acid, and 2,6-dichloroisonicotinic acid (Ryals et al., 1994) and benzothiadiazole (BTH, Bion®, Fig.5) (Hukkanen et al., 2008). No clear evidences have been so far produced to explain how the resistance is developed by these agents. Most reports describe a correlation between pathogen resistance and the accumulation of pathogen related (PR) proteins or enzymes involved in the scavenging from ROS (Hukkanen et al., 2008). Several studies demonstrated also that these compounds could lead to an increase in plants pathogen resistance and at the same time could stimulate the biosynthesis of secondary metabolites such as flavonoids and other phytochemicals like carotenoids and ascorbic acid (Iriti et al., 2004; Liu et al 2005). The induction of SAR by the use of plant activators is not easily predictable in timing and level of expression and therefore, has not been widely used in many crops to date. Among different classes of plant activators we focused our research on BTH, a functional analogue of the plant endogenous hormone-like compound salicylic acid (SA), that is required for the induction of defense genes leading to SAR establishment. Treatments with exogenous BTH were demonstrated to enhance resveratrol and anthocyanin biosynthesis in grapevine meanwhile improving resistance to *Botrytis* cinerea (Iriti et al., 2004), to induce resistance of peach to *Penicillium expansum* and enhance activity of fruit defense mechanisms (Liu

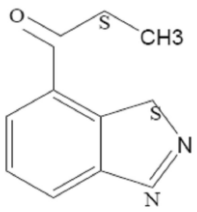


Fig. 5 Benzothiadiazole (BTH).

et al., 2005). Moreover, BTH treatments activate antioxidant enzymes (APX, DHAR, MDHAR; GR) in pea leaves (Clemente-Moreno et al., 2009) and induce enzyme activities related to anthocyanin metabolism in strawberry fruit after harvest (Cao et al., 2010). Although BTH is a synthetic analogue of SA, its use is associated to a very low toxicological risk, it is also rapidly degraded in plant tissues and lacks any antibiotic activity.

Jasmonates (jasmonic acid and methyl jasmonate) (Fig.6) are phytohormones that occur widely in plants. The first description of methyljasmonate (jasmonic acid methyl ester) was as a fragrant constituent of the essential oil of *Jasminium grandiflorum* (Demole et al., 1962), interestingly, it is also a component of female-attracting pheromones in certain moths (Baker et al., 1991). The biosynthesis of jasmonates starts with linolenic acid and proceeds through a number of classes involving lipoxidation, cyclization and β-oxidation (Creelman and Mullet, 1997). They are efficient elicitors or signalling agents and play key roles in plant growth and affect many physiological and biochemical processes in plants (Creelman and Mullet, 1997, Wang et al., 2008). Based on the findings that some defense genes are inducible by jasmonates, it has been speculated that those compounds are involved in pathogen response mechanisms (Peña-Cortez et al. 2005). It has also been demonstrated that treatments with exogenous jasmonates stimulate the accumulation of secondary metabolites in pre- and postharvest. Preharvest treatments with MeJa were effective in enhancing antioxidant activity and flavonoid content in Blackberry (*Rubus* sp.)(Wang et. al., 2008).

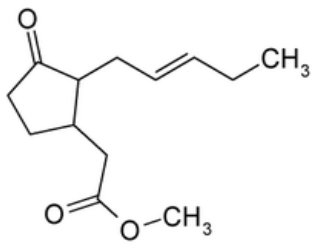


Fig. 6 Methyl jasmonate (MeJa).

1.5 Storage

Blueberry can be easily stored at low temperature (0°-1°C) and high relative humidity (up to 90%) for about a month without significant loss of quality. The main limitations to the preservation at low temperatures is due to fungal development *(Botrytis cinerea, Alternaria spp. Colletotrichum spp.*) and weight loss that causes shrivelling and loss of brightness. Fruit weight loss is mainly due to water loss, which is produced by a difference in vapour pressure between the fruit and the surrounding air. This loss is affected by the area/volume ratio, by mechanical wounding and storage temperature (Wills, McGlasson, Graham, and Joyce, 1998). Small-sized fruits, such as blueberries, show a high area/volume, and they therefore dehydrate more than larger-sized fruits (Chiabrando and Giacalone, 2011; Cabezas, 2004; Navarrete, 2004; Wills et al., 1998). The controlled atmosphere (CA), especially the use of CO_2-enriched (9-10 kPa) and low O_2 (1-4 kPa) gas

mixtures, can reduce or prevent the onset of fungal contaminations. Several researches have demonstrated the useful role of CO_2-rich atmospheres in enhancing blueberry storage life, for long periods, albeit with different responses among varieties (Connor et al., 2002). Controlled atmosphere storage could also affect the levels of nutrients and health-promoting compounds in blueberry. Anthocyanin content and antioxidant capacity resulted higher in blueberries cv 'Bluecrop' after 2-4 weeks of cold storage in CA (Krupa and Tomala, 2005). In the study conducted by Connor and colleagues (2002) ripe fruit from most of the blueberry cultivars tested demonstrated stability in the antioxidant activity, total phenolic content, and anthocyanin content during cold storage, and one cultivar demonstrated an increase during the first 3 weeks of storage. On the other hand high CO_2 concentration (between 10 and 20 kPa) has been demonstrated to have a detrimental effect on AsA content in berry fruits (Agar et al., 1997).

Little is known so far about the effects of a long-term storage of blueberries on quality parameters, and on the levels of health promoting compounds such as ascorbic acid and phenolics.

2. Aim

Blueberry and bilberry possess one of the highest antioxidant capacities in berries due to the high content of anthocyanin pigments; they are also a moderate source of ascorbic acid (AsA). It is accepted that the content of phenolics and ascorbic acid in berries is not only affected by genetic differences and pre-harvest environmental conditions, but also by the degree of maturity at harvest.

In postharvest, different storage conditions may also strongly influence antioxidant capacity of this produce and there are many factors which contribute to improve fruit quality maintenance such as temperature, gas concentration and cultivar.

The aims of the present Ph.D. project were:

- To acquire a better understanding about the changes in bioactive compounds content during fruit development, ripening and storage of blueberry by measuring the accumulation of compounds (phenolics and ascorbic acid), enzymatic activities and expression profiling of genes related to ascorbic acid biosynthesis and recycling during development and ripening;
- To acquire a better knowledge on the effects and mechanisms of action of plant activators that could be effective in stimulating the accumulation of health-promoting compounds. Integration of transcripts and metabolites data analyses in order to find possible correlations between gene upregulation and metabolites accumulation following to treatments with plant defence activators such as methyljasmonate and benzothidiazole;
- To define optimal storage conditions to extend the shelf life of fresh produce while ensuring both commercial and nutraceutical quality. Application of controlled atmosphere technologies in long term cold storage of different cultivars of blueberry. Analyses of quality attributes and health promoting compounds;
- To identify commercial cultivars of blueberry with the highest attitude to storage;
- To detect possible biochemical indexes useful to point out the onset of product degradation during storage.

3. Ascorbic Acid Metabolism During Bilberry (*Vaccinium myrtillus* L.) Fruit Development

3.1. Introduction

Bilberry (*Vaccinium myrtillus* L.), also known as European blueberry, is a woody shrub widespread in northern Europe. Its berries are valued for their nutraceutical properties and are among the fruits with the highest antioxidant activity due to their high phenolic compounds content, particularly anthocyanins (Prior, Cao, Martin, Sofic, and McEwen, 1998). The berries are also a moderate source of another antioxidant compound, ascorbic acid (AsA). Since humans are not able to biosynthesize this compound, plant-derived AsA, along with its oxidized form (dehydroascorbic acid), represents an essential vitamin in diet (vitamin C). AsA can act as an antioxidant in a wide number of enzymatic and non-enzymatic reactions in humans (Ishikawa, Dowdle, and Smirnoff, 2006), and it is also active in the non-enzymatic regeneration of other antioxidant molecules such as α-tocopherol (vitamin E). In addition, as a strong antioxidant, AsA has a beneficial role in reducing the risk derived from cardiovascular, age-related and chronic human disorders and acts as a co-substrate in reactions catalyzed by several dioxygenases (De Tullio and Arrigoni, 2004). It has also been reported that AsA may have a role in the prevention of certain forms of cancer (De Tullio and Arrigoni, 2004).

AsA has several essential functions in plant physiology. Most importantly, AsA functions as a cofactor for enzymes involved in cell expansion and cell division and is a co-substrate for the biosynthesis of important plant hormones such as ethylene and gibberellic acid (Davey et al., 2000). It is also an effective radical scavenger that is able to interact with the reactive oxygen species (ROS) that are produced during cell wall lignification, cell division, photosynthesis and stress responses (Davey et al., 2000; Halliwell, 1996). Moreover, in some plant species, AsA appears to be the substrate for oxalate and tartrate biosynthesis (Davey et al., 2000).

In plants, the main biosynthetic route of AsA proceeds from mannose-6-phosphate to AsA via GDP-mannose and GDP-galactose (Fig. 7) (Ishikawa et al., 2006). However, many studies suggest the co-existence of several alternative pathways for AsA in plants (Davey et al., 2000; Ishikawa et al., 2006). The AsA levels in plants are maintained by an efficient balance between biosynthesis and recycling. In the recycling route, ascorbate peroxidase (APX) catalyzes the oxidation of AsA into monodehydroascorbate (MDHA), a radical form that is spontaneously converted into AsA and dehydroascorbate (DHA) (Fig. 7). The enzymatic regeneration of AsA from MDHA and DHA is

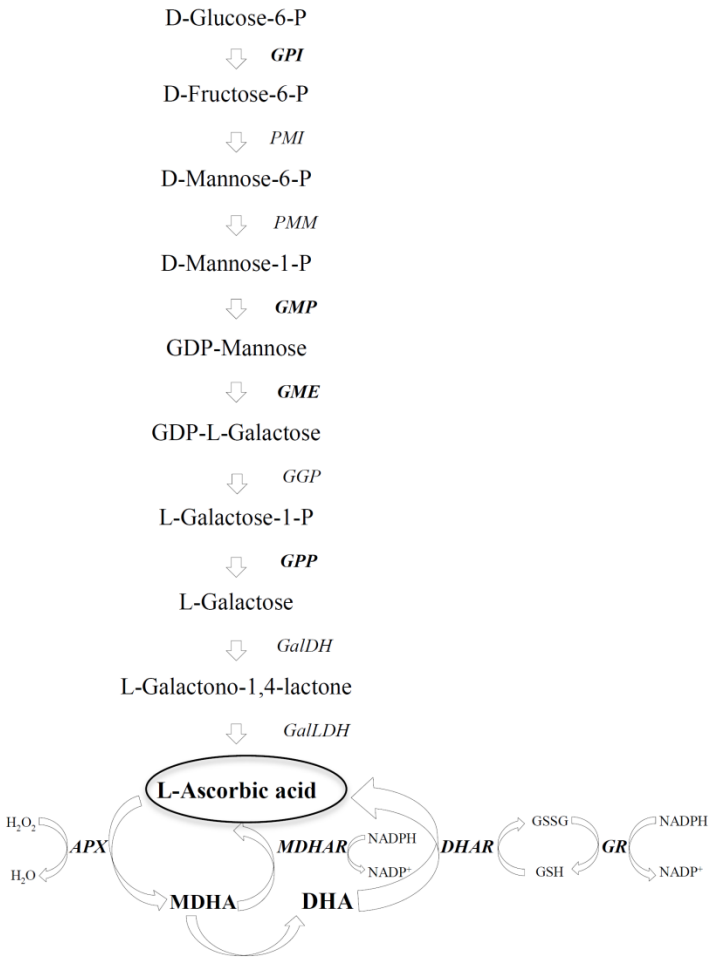


Fig. 7: The main AsA biosynthetic and recycling pathway

catalyzed by monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). The recycling pathway, also known as ascorbate-glutathione cycle, provides an efficient way of recycling of AsA and detoxification of H_2O_2 (Noctor and Foyer, 1998). Several studies have been conducted on the content of phenolic compounds and their organ-specific distribution in *Vaccinium* species (Määttä-Riihinen et al.,2004; Riihinenet al., 2008) as well as on the expression of genes that attend to the accumulation of phenolic compounds during bilberry fruit development (Jaakola et al., 2002). However, little is known about the molecular mechanisms that regulate the biosynthesis and recycling of AsA in these small fruits during development and ripening. The aim of the present work was to study the expression of the genes that encode enzymes from the main AsA biosynthetic and recycling routes in relation to the AsA content in bilberry fruit at four different developmental stages, that is, from unripe green to over-ripe berries. The AsA accumulation and the expression of the AsA-related genes in different fruit tissues (pulp and skin) of over-ripe berries were analysed as well. Moreover, the activities of the antioxidant enzymes APX, MDHAR, DHAR and GR catalyzing the oxidation and recycling of AsA were measured.

3.2. Materials and methods

3.2.1. Plant material

Bilberry fruits growing naturally in the forest in Oulu, Finland (65°01’ N, 25° 28’ E), were harvested, from June to August 2010, at four different ripening stages; 1: unripe green, 2: unripe purple, 3: ripe, 4: over-ripe (Fig. 8). In order to determine the AsA content and measure the expression of

the AsA-related genes in different fruit tissues, pulp and skin from over-ripe berries (stage 4) were separated after harvesting. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until used for the analyses.

3.2.2. RNA isolation

Total RNA was isolated from intact berries as well as from skin and pulp samples using the CTAB method described by Jaakola et al. (2001). The quality of the RNA was verified by measuring the absorbance spectrum with NanoDrop N-1000 spectrophotometer (NanoDrop technologies, Wilmington, DE, USA). The RNA was reverse-transcribed to obtain cDNA using the SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). The cDNA was purified from the genomic DNA according to Jaakola et al.(2004).

3.2.3. Quantitative RT-PCR (qRT-PCR)

A bilberry fruit specific EST database created at the University of Oulu was used for identifying the sequences of the genes related to AsA biosynthesis and to design gene-specific primers for qRT-PCR analyses. The EST database was created using the 454 GS-FLX platform (Roche Applied Sciences, Indianapolis, IN, USA). Contigs were assembled using MIRA (Chevreux et al., 2004), and highly similar mRNA sequences were identified using BLAST (NCBI). The gene-specific primers designed for glucose-6-phosphate isomerase (*GPI*), GDP-D-mannose pyrophosphorylase (*GMP*), GDP-D-mannose-3´, 5´-epimerase (*GME*), L-Galactose-1-phosphate phosphatase (*GPP*), ascorbate peroxidase (*APX*), monodehydroascorbate reductase (*MDHAR*), and dehydroascorbate reductase (*DHAR*) are shown in Table 2. In order to test the efficiency of the primers and normalization of the results in each experiment, a standard curve was created from different dilutions of cDNA from ripe bilberry fruit samples. Specificities of the amplified PCR products were verified using melting curve analysis. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a reference gene for the relative quantification of PCR products (Jaakola et al., 2002, Tab. 2). The results were calculated with LightCycler®480 (Roche) software, using the calibrator-normalized PCR efficiency-corrected method (technical Note No. LC 13/2001, Roche Applied Science).

Tab. 2: Gene-specific primer sequences used for the qRT-PCR analysis.

Gene	*Primers 5'>3'*	*bp*	*Tm (°C)*
GPI	Forward: ATGCATCTCGGAAGAAAGGA	20	59.77
	Reverse: GACAGTCGTGCCTGCACTTA	20	60.06
GMP	Forward: GCCCCTTGTTGATTTTGGTA	20	59.80
	Reverse: CGGTCTCTTGAGAGCATGTG	20	59.57
GME	Forward: GTGGGAACACAAGCTCCAGT	20	60.16
	Reverse: CCCATCTCCATAAATCCCAAT	21	59.86
GPP	Forward: TCTCAGTCGGAGCTTGTGAA	20	59.70
	Reverse: ATGCCACAGAGATCCAATGC	20	61.05
APX	Forward: GCATTCCGATCACTCTCTGC	20	60.93
	Reverse: ACGGAGCATAATTGGAGCAC	20	60.10
MDHAR	Forward: TTTCTGGAGAGTGGGACACC	20	60.09
	Reverse: CACGCGTGAAGTACGACAAC	20	60.37
DHAR	Forward: TTGGATCTGAACCCAGAAGG	20	60.04
	Reverse: TCACTGGGATCCTTGCTCTT	20	59.80
GAPDH	Forward: CAAACTGTCTTGCCCCACTT	20	63.98
	Reverse: CAGGCAACACCTTACCAACA	20	63.53

3.2.4. Determination of AsA content

AsA extraction was performed according to Sinelli et al. (2008). Briefly, 3 g of berries were homogenized in a mortar containing 4 mL of cold, 6% (w/v) metaphosphoric acid and centrifuged at 10,000 × *g* at 4 °C. The pellet was washed with 3 mL of 6% cold metaphosphoric acid and centrifuged at 10,000 × *g* at 4 °C. The supernatants were combined and cold 6% metaphosphoric acid was added to a final volume of 10 mL. The extracts were filtered through a 0.45 µm syringe filter before injection to the high-performance liquid chromatography (HPLC). The extraction was carried out in triplicate.

The HPLC- diode array detector (DAD) analysis for the determination of AsA content was performed, with minor modifications, according to Sinelli et al. (2008). An Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) fitted with Agilent 1100 autosampler, an Agilent 1100 binary pump, and an Agilent 1100 DAD was used in this study. The HPLC separation was performed with Inertsil ODS-3 4.6 × 250 mm column (GL Sciences, Tokyo, Japan) with 5 µm particle size. The temperature of the column oven was set at 20 °C, and the injection volume was 10 µL. Data were acquired and processed using Hewlett Packard ChemStation software (Agilent Technologies).

3.2.5. Analysis of water content

To measure the water content of the samples, the intact berries as well as the skin and pulp samples were freeze-dried (Flexi-Dry, FTS Systems, Stone Ridge, NY, USA) and weighted before and after the procedure.

3.2.6. Analysis of enzyme activities

Quadruplicates samples of berries (5 g) were homogenized in a mortar at 4 °C with 10 mL of 100 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 25% glycerol (w/v), 0.25% Triton X-100 (w/v) and 1 g of polyvinyl-polypyrrolidone (PVPP). Just before the extraction, 2 mM β-mercaptoethanol, 1 mM PMSF (dissolved in DMSO), and 1 mM sodium ascorbate were added to the buffer. The homogenate was filtered through two layers of cheesecloth and centrifuged at 20,000 x *g* at 4 °C for 30 min to separate insoluble material. The supernatant was then stored at −80 °C until used for analyses of enzyme activities.

Protein content was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard. The enzyme activities were measured with a Cary 50 Bio spectrophotometer (UV-Visible) (Varian Australia Ptv Ltd., Victoria, Australia).

3.2.7. APX (EC 1.11.1.11) activity assay

APX activity was assayed by measuring the decrease in the ascorbate concentration at 290 nm (extinction coefficient: 2.8 mM^{-1} cm^{-1}) according to Nakano and Asada (1981) with slight modifications. The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.0) supplemented with 0.1 mM EDTA and 0.5 mM sodium ascorbate. The reaction was triggered by adding 0.1 mM H_2O_2.

3.2.8. MDHAR (EC 1.6.5.4) activity assay

The activity of MDHAR was measured by observing the decrease in absorbance at 340 nm due to oxidation of NADH (extinction coefficient: 6.22 mM^{-1} cm^{-1}) according to Arrigoni et al. (1981) with slight modifications. The MDHA was generated by the ascorbate/ascorbate oxidase (A.O.) complex. The assay mixture consisted of 50 mM Tris-HCl buffer (pH 7.6) supplemented with 2.5 mM sodium ascorbate and 0.1 mM NADH. The reaction was triggered by adding 0.14 U of A.O.

3.2.9. DHAR (EC 1.8.5.1) activity assay

DHAR activity was measured by observing the increase in absorbance at 265 nm due to the formation of ascorbate (extinction coefficient: 14 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) supplemented with 0.1 mM EDTA and 0.2 mM DHA. The reaction was triggered by the addition of 2.5 mM glutathione (GSH) according to the method of Nakano and Asada (1981).

3.2.10. GR (EC 1.6.4.2) activity assay

The GR activity was calculated by measuring the decrease in absorbance at 340 nm due to oxidation of NADPH (extinction coefficient: 6.22 mM^{-1} cm^{-1}) as described by Donahue et al. (1997). The reaction buffer consisted of 100 mM Tris-HCl (pH 7.8), 2 mM EDTA and 0.5 mM glutathione disulfide (GSSG).

3.2.11. Statistical analysis

Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). Significant differences were calculated by Duncan's mean test. Differences at $p \leq 0.05$ were considered as significant.

3.3. Results

The protein content in bilberry fruits varied markedly during the ripening period, and the highest concentrations were found at the ripening stages 3 and 4 (Fig. 8). Also, the fresh weight/dry weight ratio of fruits increased during the ripening process, reaching the highest value at the ripening stage 4 (Fig. 8).

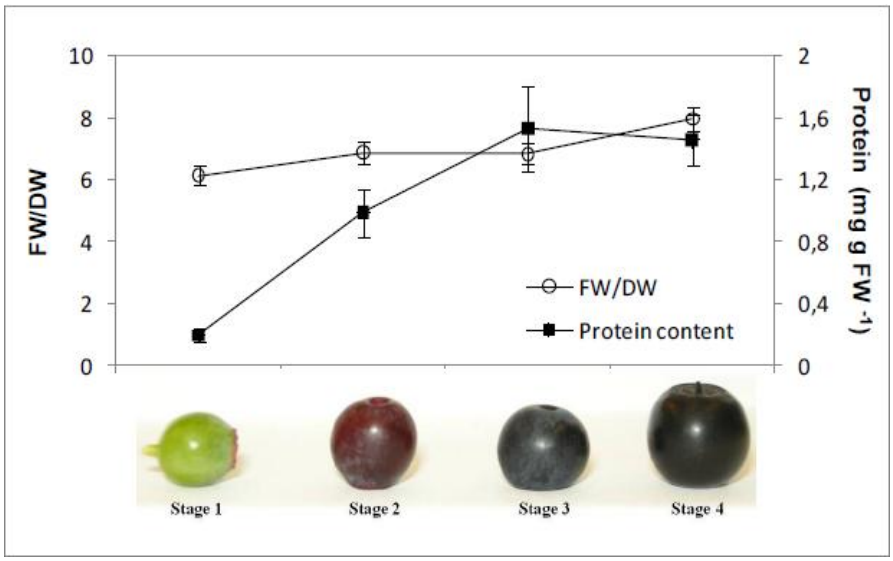


Fig. 8: Protein content and fresh weight/dry weight ratio in different ripening stages of bilberry fruit (Stage 1: unripe green, stage 2: unripe purple, stage 3: ripe, stage 4: over-ripe). Values represent means ± SD (*n = 3*).

The AsA concentrations were measured from all four stages of berry ripening (Fig. 8). The unripe green fruits showed the highest AsA content in both relation to fresh weight and dry weight (Tab. 3). A significant decrease was recorded from the first to the second stage, but during the later

stages of the ripening process, the AsA level remained relatively stable. In the stages 3 and 4, when the fruits are normally consumed, the AsA content was over 6 mg 100 g^{-1} FW (~50 mg 100 g^{-1} DW). In over-ripe berries (stage 4), AsA was mostly accumulating in the berry skin, in which the concentration was almost twice as high as that in pulp.

Tab. 3: AsA content (mg 100 g-1 FW and mg 100 g-1 DW) in bilberry fruit at different ripening stages and in different tissues. Values represent means ± SD (*n = 3*). Different letters indicate significant differences (p ≤ 0.05) according to the Duncan's mean test.

Ripening stage/Tissue	AsA (mg 100 g^{-1})	
	FW	DW
Stage 1 unripe green	8.25 ± 0.43a	50.67 ± 2.64a
Stage 2 unripe purple	6.52 ± 0.47b	44.90 ± 3.26b
Stage 3 ripe	6.81 ± 0.36b	46.61 ± 2.45ab
Stage 4 over-ripe	6.14 ± 0.22b	49.03 ± 1.78ab
Berry skin (Stage 4 over-ripe)	3.81 ± 0.47a	33.32 ± 4.11a
Berry pulp (Stage 4 over-ripe)	2.22 ± 0.03b	16.92 ± 0.25b

The expression of the genes encoding some of the enzymes involved in the main AsA biosynthetic and recycling routes was studied during the bilberry fruit ripening process (Fig. 9). Transcripts of *GPI* were approximately three times higher at the second stage of ripening when compared to the other developmental stages (Fig. 9A). In contrast, *GMP* appeared to be highly expressed in the first stage of ripening, but the expression decreased in stages 2 and 3 increasing again at the over-ripe stage (Fig. 9B). Both *GME* and *GPP* showed the highest expression values in unripe berries (stages 1 and 2) which was followed by a decrease in expression levels during the ripening process (Fig. 9C and D). The expression of *APX*, the gene encoding the first enzyme in the AsA recycling pathway, reached the highest levels in stages 1 and 3 (Fig. 9E). Part of the AsA oxidized by APX can be recycled by the enzymatic action of MDHAR and DHAR. *MDHAR* showed the highest transcript abundance in stage 2 (Fig. 9F). In contrast, *DHAR* was highly expressed in stages 1 and 3, thus showing an expression pattern very similar to *APX* (Fig. 9G). The expression data of all the analyzed genes from both the main biosynthesis and the recycling pathway showed higher transcripts abundance in berry skin in comparison to pulp (Fig. 9H). For example, more than three-fold higher expression level for *GPI*, five-fold for *GME*, three-fold for *GPP* and six-fold for *MDHAR* were detected in skin compared to pulp.

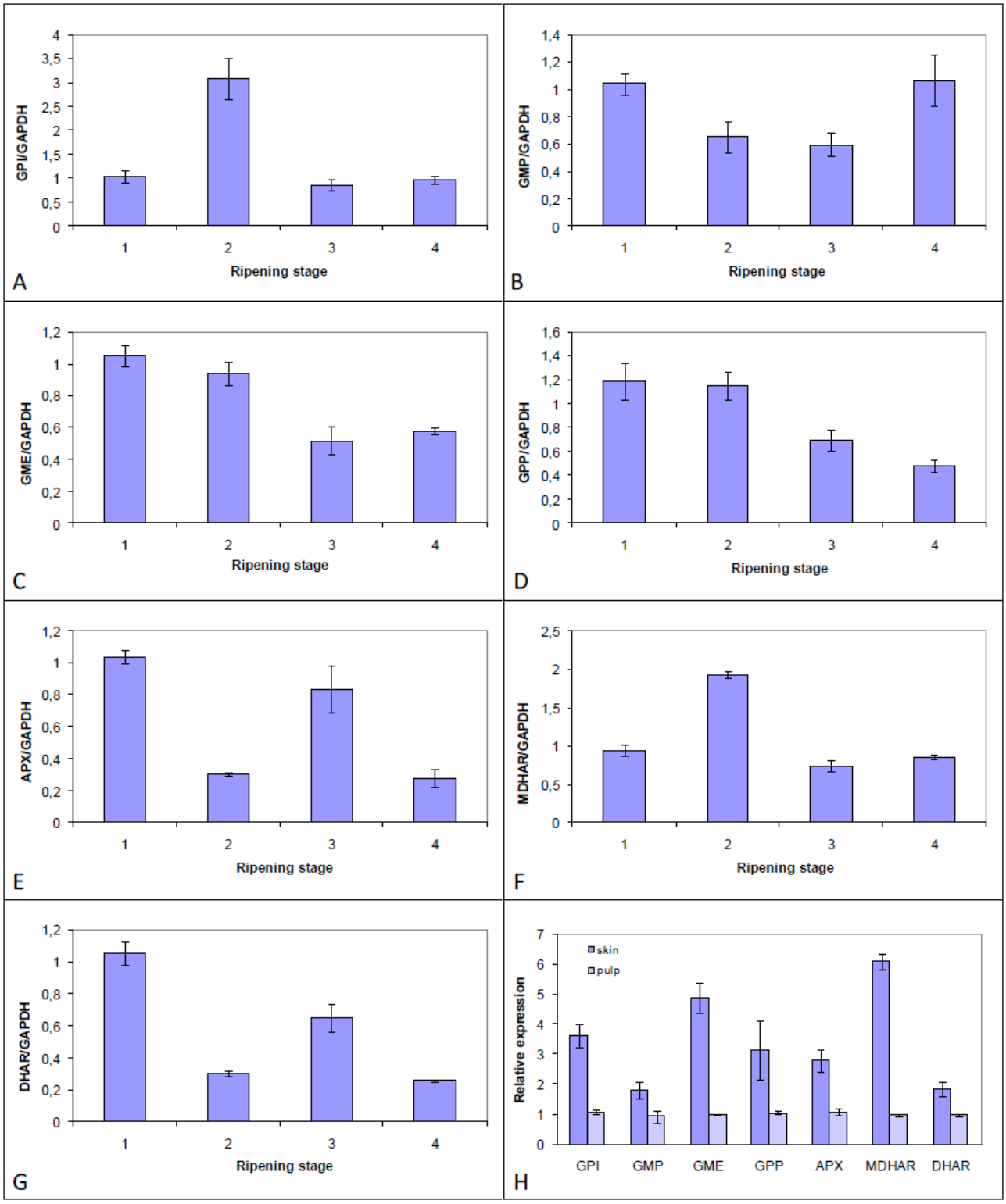


Fig. 9: Expression of the AsA biosynthetic genes A) GPI, B) GMP, C) GME, D) GPP, and recycling genes E) APX, F) MDHAR and G) DHAR during bilberry fruit ripening and in H) over-ripe bilberry fruits in two different tissues: skin and pulp. Values represent means ± SD (*n = 3*).

The activities of the antioxidant enzymes from AsA recycling pathway during the bilberry fruit ripening process are presented in Fig. 10. For all the enzymes in the recycling route, except for GR, the highest activity values were registered in the green unripe berries. Both APX and DHAR

activities decreased significantly from the first to the second stage but remained stable during the later stages of ripening (Fig. 10A and C). The decrease of MDHAR activity from initial stage 1 to 2 was followed by an increment in the activity in stages 3 and 4 (Fig. 10B). GR activity, in contrast, was characterized by a gradual increase from stage 1 to stage 3 followed by a decrease at stage 4 to values equal to those observed in stage 2 (Fig. 10D).

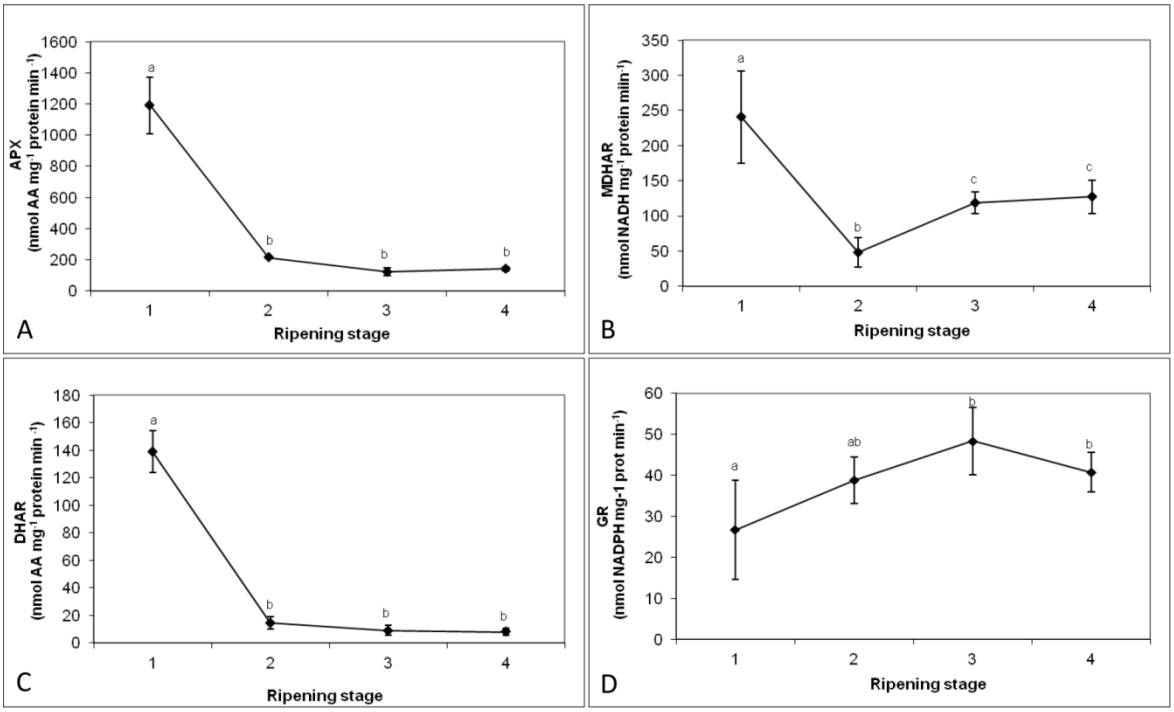


Fig. 10: Activities of A) APX, B) MDHAR, C) DHAR and D) GR during bilberry fruit ripening. Values represent means ± SD (*n = 4*). Different letters indicate significant differences (p ≤ 0.05) according to the Duncan's mean test.

3.4. Discussion

AsA content is an important trait in crops because it has a central role in the plant physiology and it is an essential vitamin in human diet. In order to develop health promoting crops with high nutritional value and quality traits, a better understanding of the mechanisms leading to the accumulation of AsA is crucial. The AsA level in plants is mainly determined by genotype but is also influenced by different environmental factors such as light (Badejo, Fujikawa, and Esaka, 2009; Li et al., 2009), date of harvest (Felicetti and Mattheis, 2010), and both biotic (Davey, Auwerkerken, and Keulemans, 2007) and abiotic stresses (Gautier et al., 2010).

In the present study, the highest AsA content in bilberry fruits was measured in unripe green fruits. A significant decrease in the AsA level was recorded from the first to the second stage, but the AsA content remained relatively stable during the later stages of ripening. This result clearly differs from the data obtained from grapes and tomato, in which the AsA levels increased progressively during fruit development (Cruz-Rus Botella, Valpuesta, and Gomez-Jimenez, 2010;

Ioannidi et al., 2009). Our previous studies on blueberry cultivars (*V. corymbosum*) showed a strong increase in the AsA levels during the berry ripening process (data not published). AsA accumulation in bilberry fruits seems to be more similar to apple fruit (Li, Ma, Zhang, and Pu, 2008) and acerola (Badejo et al., 2009; Eltelib, Badejo, Fujikawa, and Esaka, 2011), in which the AsA levels in young fruits are higher compared to mature fruits.

The expression of the AsA-related genes of the main biosynthetic route was studied during bilberry fruit ripening and also compared to AsA levels. As many of the encoded enzymes of the pathway are also involved in other metabolic routes, and since the levels of AsA are also highly affected by the recycling route, total consistency with the AsA level is not expected. For example, *GPI,* which is the most distinct enzyme in the main biosynthetic route leading to AsA, is also strongly implicated in the sugar metabolism. The transcripts of *GMP* in bilberry fruit were found to be related with AsA levels, particularly in the early stages of development (stages 1-3). This is consistent with earlier studies where *GMP* was found to show a strong correlation with the AsA levels during acerola fruit development and it was suggested to play a major role in AsA biosynthetic pathway in acerola fruit (Badejo, Jeong, Goto-Yamamoto, and Esaka, 2007; Badejo et al., 2009). However, in tomato, a negative correlation between *GMP* expression and AsA accumulation was detected (Ioannidi et al, 2009), which suggests different regulatory mechanism of *GMP* between plant species.

The expression of both *GME* and *GPP* showed a pattern similar to the AsA levels, with the highest transcript levels at the early stage of ripening followed by a slight decrease at the last stages. Similar expression pattern for *GME* has been observed in grape berries (Cruz-Rus et al., 2010) and in tomato (Ioannidi et al., 2009). The *GME* is supposed to be the key enzyme in the major AsA biosynthetic pathway but it also has a role in the biosynthesis of non-cellulosic cell-wall polysaccharides (Gilbert et al., 2009). Thus, it represents a significant linkage between the two metabolic pathways. *GPP* is suggested to play a regulatory role in AsA biosynthetic pathway during tomato fruit ripening (Ioannidi et al., 2009) and it was also demonstrated to be required for maximal AsA accumulation in *Arabidopsis* (Conklin et al., 2006).

The partial inconsistency between the expression of the genes in the main AsA biosynthetic route and the AsA accumulation during bilberry fruit ripening can also be consequence of post-transcriptional or post-translational modifications taking place in gene regulation. In a recent study by Imai, Ban, Terakami, Yamamoto and Moriguchi (2009) on peach fruit, suggestions were made that the turnover rate of the main AsA biosynthetic enzymes results in no apparent

relationship with gene expression levels. This aspect may play some role in AsA biosynthetic routes that possibly exist in bilberry.

In addition to the main AsA biosynthetic route, the recycling pathway also affects the levels of AsA. In order to obtain more detailed information about the AsA metabolism in bilberry fruits, the gene expression and activities of enzymes in recycling pathway was also investigated. The expression patterns of the two important genes in recycling pathway, APX and DHAR, appeared to be consistent although the enzymes have opposite effects on the AsA level. At the first stage of ripening, the activity patterns of the two enzymes were similar, showing high expression level. The MDHAR also showed the highest activity at first stage of ripening. Thus, the great activity level of both DHAR and MDHAR at the first developmental stage seems to guarantee the high AsA level despite of the strong activity of APX. GR, the last enzyme in the AsA recycling pathway, showed a different activity pattern compared to the other enzymes of the recycling pathway. Furthermore, GR has other important roles in plants, such as involvement in the cell's scavenging system for reactive oxygen compounds in plants by reducing GSSG to GSH (Rao and Reddy 2008).

The gene expression and enzyme activity patterns of the recycling enzymes were not strictly correlated to each other. The expression patterns and enzyme activities of both APX and DHAR correlated with each other in stages 1, 2 and 4 but not in stage 3. Furthermore, the expression pattern of *MDHAR* appears to behave in the opposite way when compared to its activity and the AsA level. In addition, recent studies of relationship between *MDHAR1* transcript levels and AsA levels in tomato fruits also showed a negative correlation (Ioannidi et al., 2009). In contrast to these results, a high correlation between enzyme activity and mRNA abundance of *MDHAR* and *DHAR* in response to ripening and stresses was observed in acerola fruits (Eltelib et al., 2011). On the other hand, a negative correlation was also shown between *MDHAR* activity and the AsA content in acerola over-ripe fruits (Eltelib et al., 2011). Eltelib et al. (2011) explained the mechanism of regulation in acerola to be due to a possible stimulation of low amount of AsA on the transcription and the enzyme activity of MDHAR. However, in our study, the MDHAR activity is better correlated to the AsA levels in all the stages and, therefore, a different mechanism of regulation should be hypothesized in bilberry.

In order to obtain some information about the distribution of this important nutrient at the time when the berries are commonly consumed, the content of AsA and the AsA related gene expression were measured in two different fruit tissues, skin and pulp, at the end of ripening. The higher AsA levels and the transcript abundance of all the studied AsA biosynthesis genes were

observed in skin compared with pulp. This was expected since the fruit skin represents the primary fence against biotic and abiotic stresses and thus requires a high accumulation of antioxidants and bioactive defence compounds, as it has already been hypothesized in earlier studies conducted with apple (Felicetti and Mattheis, 2010; Li et al., 2008).

In conclusion, the present work is the first to report the study on the AsA metabolism in bilberry. The biosynthesis of AsA is at its highest at the beginning of the berry development and stays at a fairly stable level over the later ripening stages. In ripe bilberries, the content of AsA is almost twofold in the skin when compared to the berry pulp in good correlation with the higher expression of all examined AsA pathway genes in the skin. Also especially at the early stages of development, the expression of the key enzyme genes in the main AsA biosynthetic route was consistent with the detected AsA levels.

This thesis chapter is based on the following original manuscript, which is now under review.

Cocetta, G., Karppinen, K., Suokas, M., Hohtola, A., Häggman, H., Spinardi, A., Mignani,I., Jaakola, L. (2011). **Ascorbic Acid Metabolism during Bilberry (*Vaccinium myrtillus* L.) Fruit Development.** 2011. (*Journal of Plant Physiology* JPLPH-D-11-00559)

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4. Effect of BTH on Ascorbic Acid Content and Recycling and on Phenolics Content During Blueberry (*Vaccinium corymbosum* L.) Fruit Development

4.1. Introduction

The consumption of fruits plays an important role as a health-promoting factor and is mainly associated with the antioxidant activity of several compounds which are largely present in vegetal produce. Blueberries (*Vaccinium corymbosum* L.) are considered one of the fruits with the highest antioxidant potentials, owing to the high phenolic content and to the moderate presence of ascorbic acid. Ascorbic acid (AsA, vitamin C) is an essential constituent of the human diet, since humans are unable to synthesize it. AsA is one of the most important free radical scavengers in plants, animals and humans. In mammals, AsA is considered to play a significant role in protecting against various oxidative stress-related diseases such as cancers, cardiovascular diseases, aging, as well as stimulating the immune system. Furthermore, as the most effective and least toxic antioxidant, AsA interacts in plants (enzymatically and non-enzymatically) with damaging oxygen radicals and their derivatives, so-called reactive oxygen species (ROS), originated as byproducts of normal cellular metabolism in chloroplasts, mitochondria and peroxisomes. The toxicity of ROS is linked to their ability to initiate radical cascade reactions which can cause lipid peroxidation, protein and DNA damage and lead finally to cell death. Oxidative stress is a condition originated by the intracellular accumulation of toxic levels of ROS through saturation of the antioxidant enzymatic and non-enzymatic defence mechanisms. Ascorbate is oxidized by enzymatic or non-enzymatic reactions. Ascorbate peroxidase (APX) mediates the scavenging of hydrogen peroxide to water with the simultaneous oxidation of AsA with a high specificity. Ascorbate oxidation always leads to the intermediate monodehydroascorbate (MDHA) radical which normally has a short life span and, if not rapidly reduced by MDHA reductase (MDHAR), spontaneously dismutates into ascorbate and the labile dehydroascorbate (DHA). DHA is reduced to ascorbate by the action of DHA reductase (DHAR), using glutathione as the reducing substrate. DHA can undergo irreversible hydrolysis to form 2,3-diketo-L-gulonic acid with consequent loss of biological activity (Bode et al., 1990) or is directly catabolized to a number of breakdown products including oxalate and tartrate (Loewus, 1988).

Due to the interaction with ROS, AsA is involved also in the modulation of processes such as cell division, lignification and in incompatible plant-pathogen interactions (Conklin, 2001). During incompatible plant-pathogen interactions, the recognition of an invading pathogen stimulates an 'oxidative burst' and a coordinated defense response, mediated by ROS.

Blueberries are a rich source of phenolic compounds, a broad class of secondary metabolites synthesized in the phenylpropanoid pathway and acting as antioxidants. Structurally, these substances are characterized by at least one aromatic ring linked to one or more hydroxyl groups. Recent studies have demonstrated that the presence of phenols in food is particularly important for their oxidative stability and anti-microbial protection. Phenolic compounds can act as antibiotics, as a fence against UV radiation, as insect repellent and signal molecules in plant-microorganism interactions. They are also constituents of complex polymeric structures like lignin and suberin and they have a prominent role in determining color and flavor of fruit and vegetables. Phenolic pigments are involved in pollination and seed dispersal by attracting insects and herbivores. Anthocyanins are phenolic compounds of particular interest within the group of flavonoids. They are the main pigments in many fruits and vegetables, their color varies from red to blue depending on the pH of the medium in which they are located and on the formation of salts with metals present in those tissues. Color is only one type of signal used to attract insects; phenolic substances such as vanillin, can act as odors that attract pollinators to the flowers. In general, the role of secondary metabolites in plant defense mechanisms is related to their particular chemical and physical characteristics that make them poisonous or repellent. For example, during fruit ripening several changes in the phenolic compounds pool take place determining a decrease in astringency (deterrent for animals who want to eat of the fruit not yet fully developed) and increase in the visual appeal with most color deriving from anthocyanins. The presence of phenolic compounds in epidermal cells of plant tissues is effective in preventing mutagenesis and phenomena like the formation of pyrimidine dimers, DNA damage induced by exposure to UV-B and UV-C radiation and is able to prevent photo-destruction of coenzymes NAD or NADP. Another possible advantage for cellular constituents, due to the presence of phenolic substances, is derived from their antioxidant properties and its ability to chelate metals. These properties result in a reduction of the probability of photo-oxidation of some compounds in conditions of high light intensities. Researches carried out on leaves exposed to high light intensities suggested that flavonoids may act as antioxidants in response to such stresses (Tattini et al., 2004). Studies conducted on tomato and watermelon (Rivero et al., 2000) showed that

thermal stress causes an accumulation of phenolic compounds such as flavonoids and phenylpropanoids. Phenylalanine ammonia lyase (PAL), the key enzyme of the biosynthesis of phenolic compounds is influenced by many environmental factors, such as light (through its effects on phytochrome), temperature, concentration of nutrients (Hahlbrock et al., 1989). Also stress caused by nitrogen deficiency may influence the content of flavonoids (Løvdal et al., 2009). A further property of phenols is to act as antifungal agents, different classes of phenolic substances have antimicrobial activity that can effectively combat fungal infections, bacterial or viral infections. The sequence of events that constitute the immune response to infection, may include in succession: death and necrosis of the host cell, accumulation of toxic phenols, changes in host cell walls by phenolic substituents (esterification reactions) or creation of physical barriers and, finally, production of specific substances such as phytoalexins.

This inducible defence mechanism that provides a long-lasting, systemic resistance against broad spectrum of pathogens is called Systemic acquired resistance (SAR). The treatment of plants with various biotic and abiotic agents (e.g. plant pathogens, nonpathogens, plant extracts, cell wall fragments and synthetic chemicals) can trigger SAR and lead to the induction of resistance to subsequent pathogen attack. In the vast majority of cases, SAR depends on the early increase of the endogenously synthesized hormonelike compound salicylic acid, which induces a specific set of defense genes. These genes include those coding for pathogenesis-related-proteins (PR) and key enzymes of secondary metabolic pathways, thus improving phytoalexin synthesis and, in turn, plant resistance. However, some studies suggest that induced resistance by pathogens or synthetic SAR inducers affect plant metabolism broadly, exerting effects on primary as well as on secondary metabolism. Among the synthetic activators (inducers) of plant disease resistance, the benzothiadiazole (BTH) acibenzolar-S-methyl, is a well-studied functional analogue of salicylic acid (Schurter et al.,1987; Friedrich et al.,1996) that has been shown to induce SAR in monocots and in many horticultural crops and fruit trees.

BTH has been shown to systemically protect wheat against powdery mildew infection (Görlach et al.1996), tobacco against TMV (Friedrich et al. 1996), *Arabidopsis* against *Pseudomonas syringae* (Lawton et al.1996) and tomato against Cucumber mosaic virus (Anfoka, 2000). The effect of BTH on antioxidative metabolism has also been described. For example, it increased POX, SOD and ascorbic acid content in peach fruit (Liu et al. 2005). Treatment of soybean cells resulted in increased glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and glutathione-S-transferase (GST) activities, as well as higher ascorbate and glutathione content

(Knörzer et al. 1999). However, BTH was found to inactivate catalase and ascorbate peroxidase, the two major H_2O_2 scavenger enzymes; accordingly, an increase in H_2O_2 occurred in treated tissues (Wendehenne et al. 1998), suggesting that changes in H_2O_2 levels or in cellular redox state may be involved in BTH/SA-mediated activation of certain defence responses (Wendehenne et al. 1998). BTH treatments were also demonstrated to be effective on phenolic metabolism, in fact BTH induced enzyme activities related to anthocyanin metabolism in strawberry fruit after harvest (Cao et al., 2010), enhanced resveratrol and anthocyanin biosynthesis in grapevine, meanwhile improving resistance to *Botrytis cinerea* (Iriti et al., 2004) and increases proanthocyanidins in grape (Iriti et al., 2005).

The aim of this study was to investigate the effects of spray treatments with BTH on health-promoting properties of two different blueberry cultivars at different stages of ripening. We measured the content of ascorbic acid and the specific activities of the enzymes involved in its recycling route. We also studied the effect of treatment on phenolic compounds by measuring the levels of total phenolics, total flavonoids and total anthocyanins. Changes in some quality parameters such as total titratable acidity and soluble sugars content were also measured.

4.2. Matherial and Methods

4.2.1. Plant material

Blueberry (*Vaccinium corymbosum*, L.) were grown in Berbenno (SO), in northern Lombardy at 650 meters on sea level. Berry samples were collected from plants from two different cultivars, 'Brigitta' and 'Duke' 24 hours after spray treatments with 0.118 mM BTH (trade name: Bion®, Syngenta). Berries from untreated plants were also collected as a control. After harvest, fruit were sorted in four different ripening stages (Fig. 11).

Fig. 11: Ripening stages: Stage 1 unripe green, Stage 2 unripe purple, Stage 3 ripe, Stage 4 full ripe.

Berries of every different ripening stage were then divided, weighed, placed in plastic bags and stored at

-80 °C until laboratory analysis.

4.2.2. Total titratable acidity (TTA) determination

The total titratable acidity (TTA) was determined on 5 g of berries from stages 3 and 4 following homogenization of the berries with an equal weight of water for 5 min. The homogenate was titrated to pH 8.3 with 0.1 N NaOH with a Crison automatic titrator (Crison Instruments A. G., Baar, Switzerland), and TTA was calculated and expressed as milliequivalents per 100 grams of fresh weight.

4.2.3. Analysis of water content

To measure the water content of the samples, the intact berries were freeze-dried and weighted before and after the procedure.

4.2.4. Total soluble solids (TSS) determination

Total soluble solids, expressed as °Brix, were determined by a hand refractometer (Atago mod., N1, Tokyo, Japan) on juice obtained from squeezing 5 g of berries.

4.2.5. Determination of AsA content

For ascorbate analysis, 7.5 g of blueberries were homogenized in a mortar with 10 mL of cold 6% (w/v) metaphosphoric acid and centrifuged at 10,000 × *g* at 4 ◦C. The supernatant was transferred into a 25-mL volumetric flask at 4 ◦C. The pellet obtained by centrifugation was washed with 8 mL of cold metaphosphoric acid solution and centrifuged. The supernatants were combined and cold 6% metaphosphoric acid was added to a final volume of 25 mL. After filtration through 0.2-µmnylon filter, a 10 µ L sample aliquot was injected onto an Inertsil ODS-3 (5 µm; 4.6mm×250mm) GL Science column at 20 ◦C attached to a Series 200 LC pump (PerkinElmer, Norwalk, CT, USA). The column was eluted with 0.02 M-orthophosphoric acid at a flowrate of 0.7 mL/min and ascorbic acid was monitored at 254 nm with a UV-975 intelligent UV–vis detector (Jasco model 7800, Tokyo, Japan). Peaks were converted to concentrations by using the dilution of stock ascorbic acid to construct a standard curve. Chromatographic data were stored and processed with a Perkin Elmer TotalChrom 6.3 data processor (PerkinElmer, Norwalk, CT, USA) (Sinelli et al., 2008).

4.2.6. Analysis of enzyme activities

Quadruplicates samples of berries (5 g) from stages 3 and 4 were homogenized in a mortar at 4 °C with 10 mL of 100 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 25% glycerol

(w/v), 0.25% Triton X-100 (w/v) and 1 g of polyvinylpolypyrrolidone (PVPP). Just before the extraction, 2 mM β-mercaptoethanol, 1 mM PMSF (dissolved in DMSO), and 1 mM sodium ascorbate were added to the buffer. The homogenate was filtered through two layers of cheesecloth and centrifuged at 20,000 *g* at 4 °C for 30 min to separate insoluble material. The supernatant was then stored at –80 °C until used for analyses of enzyme activities.

Protein content was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard. The enzyme activities were measured with a Cary 50 Bio spectrophotometer (UV-Visible) (Varian Australia Ptv Ltd., Victoria, Australia).

4.2.7. APX (EC 1.11.1.11) activity assay

APX activity was assayed by measuring the decrease in the ascorbate concentration at 290 nm (extinction coefficient: 2.8 mM^{-1} cm^{-1}) according to Nakano and Asada (1981) with slight modifications. The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.0) supplemented with 0.1 mM EDTA and 0.5 mM sodium ascorbate. The reaction was triggered by adding 0.1 mM H_2O_2.

4.2.8. MDHAR (EC 1.6.5.4) activity assay

The activity of MDHAR was measured by observing the decrease in absorbance at 340 nm due to oxidation of NADH (extinction coefficient: 6.22 mM^{-1} cm^{-1}) according to Arrigoni, Dipierro, and Borracino (1981) with slight modifications. The MDHA was generated by the ascorbate/ascorbate oxidase (A.O.) complex. The assay mixture consisted of 50 mM Tris-HCl buffer (pH 7.6) supplemented with 2.5 mM sodium ascorbate and 0.1 mM NADH. The reaction was triggered by adding 0.14 U of A.O.

4.2.9. DHAR (EC 1.8.5.1) activity assay

DHAR activity was measured by observing the increase in absorbance at 265 nm due to the formation of ascorbate (extinction coefficient: 14 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) supplemented with 0.1 mM EDTA and 0.2 mM DHA. The reaction was triggered by the addition of 2.5 mM glutathione (GSH) according to the method of Nakano and Asada (1981).

4.2.10. GR (EC 1.6.4.2) activity assay

The GR activity was calculated by measuring the decrease in absorbance at 340 nm due to oxidation of NADPH (extinction coefficient: 6.22 mM^{-1} cm^{-1}) as described by Donahue et al. (1997). The reaction buffer consisted of 100 mM Tris-HCl (pH 7.8), 2 mM EDTA and 0.5 mM glutathione disulfide (GSSG). The reaction was initiated by adding 0,05 mM NADPH.

4.2.11. Determination of total phenolics, total flavonoids and total anthocyanins

For spectrophotometric analysis, 5 g of homogenized berries were extracted with 25 or 50 mL of acidified methanol (1% HCl) by mixing for one hour, then centrifuged at 10,000*g* for 10min at 15°C. Total phenolic content was determined according to the Folin-Ciocalteau method (Waterhouse, 2005). One milliliter of Folin-Ciocalteau reagent, 5mL of distilled water and 2mL of 20% Na_2CO_3 were added to 0,1mL of extract in a 20 mL volumetric flask and immediately diluted to the final volume with distilled water. The optical density, after 90 minutes, was measured at 700 nm on a UV–vis spectrophotometer (Jasco model 7800, Tokyo, Japan). Results were expressed as milligrams of gallic acid per 100 grams of fresh weight. Total flavonoids were evaluated spectrophotometrically at 280 nm from berries from stages 3 and 4. A catechin standard curve was set and results were reported as milligrams of catechin per 100 grams of fresh weight (Iriti et al., 2005). The total anthocyanins (ACY) were estimated by the pH differential method (Cheng and Breen, 1991). Absorbance was measured on a UV-vis spectrophotometer (Jasco model 7800, Tokyo, Japan) at 520 nm and at 700 nm in buffers at pH 1.0 and 4.5, using the following equations:

$$A=[(A520 - A700)\ pH1.0 - (A520 - A700)\ pH4.5]$$

$$ACY\ (mg/l) = (A \times MW \times DF \times 1000)/(\varepsilon \times 1)$$

with a molar extinction coefficient (ε) of cyanidin-3-glucoside of 29600. Results were expressed as milligrams of cyanidin-3-glucoside equivalent per 100 grams of fresh weight.

4.1.12 Statistical analysis

Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). Significant differences were calculated by Duncan's mean test. Differences at $p \leq 0.05$ were considered as significant.

4.3. Results and Discussion

4.3.1. Total titratable acidity (TTA)

We observed that the titratable acidity decreased most markedly in 'Duke' moving from third to fourth ripening stage (Fig. 12). 'Brigitta' showed the highest values and maintained them even at the most advanced stage of maturation, this feature could be linked to the good attitude of 'Brigitta' to storage. From our analyses it was confirmed that the total titratable acidity, an important index of maturation along with total soluble solids, decreases during maturation, as also reported in other studies on *Vaccinium corymbosum* L. (Castrejon et al., 2008; Remberg et al., 2006).

'Duke' treated with BTH tends to have more acidity, especially in third stage. 'Brigitta' treated in the third stage also showed more acidity, while the fourth had lower values compared the control.

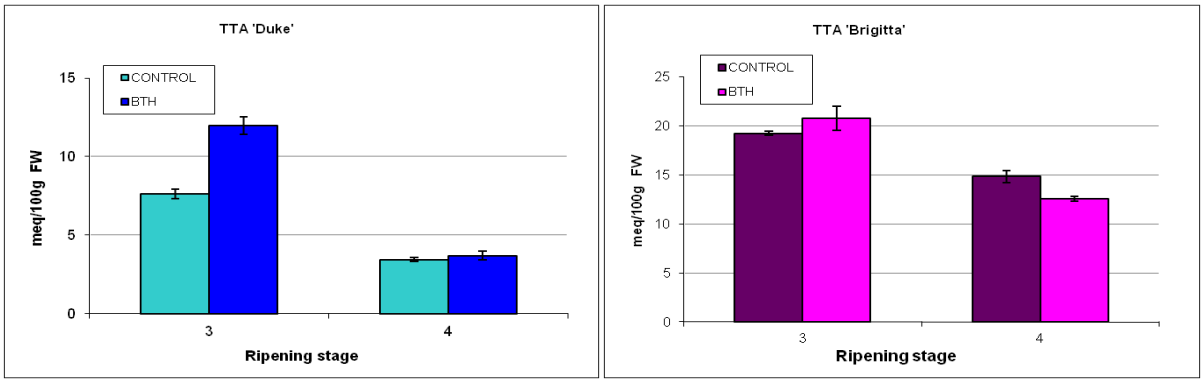


Fig. 12: Total titratable acidity (TTA). Values represent means ± SE (*n = 4*).

4.3.2. Analysis of water content

Generally the percentage of dry matter (Tab. 4) increased along ripening consistently with data previously reported for blueberry (Kalt and Mc Donalds, 1996). In 'Duke' the percentage of dry matter in berries treated with BTH showed lower values compared with controls in all ripening stages, but in stage 3 this difference was not statistically relevant. In control samples from 'Brigitta' the levels of dry matter measured along ripening were not statistically different, while in treated samples, at the end of the the ripening process, an increment in dry matter was detected. In this cultivar the treatment determined lower percentage of dry weight in stages 1 and 2 but not in samples from the stages 3 and 4.

4.3.3. Total soluble solids (TSS)

In both the cultivars we observed an increase of the soluble solids content throughout berry ripening (Tab.4) and this is consistent with data previously published (Shutak et al. 1956; Castrejon et al., 2008). There were no significant differences between cultivars for untreated samples, on the contrary berries of 'Duke' treated at stage 3 and 4 with BTH had lower soluble solids content respect to the same berries of 'Brigitta'. In the fourth ripening stage, 'Brigitta' and 'Duke' showed an average content of soluble solids of about 10° Brix, this value was also reported by Prior and colleagues (1998) for blueberries. Treatment with BTH had significant effects on both cultivars, in all stages of maturity except stage 2 and 4 for 'Duke'. It should be specified that the two cultivars, however, showed an opposite behavior: in 'Brigitta', in fact, the berries treated with BTH showed a higher sugar content in both the third and fourth stage, while in 'Duke' non-treated berries resulted to be more rich in soluble solids at stage 3.

4.3.4. AsA content

In both of the cultivars there was a sharp increment in AsA levels along ripening with the lowest levels in samples from stages 1 and two. This data are quite different compared with what we observed during development and ripening of bilberry (*Vaccinium myrtillus*) in which the levels of AsA were not changing dramatically and showed the highest levels at the first ripening stage. The ascorbic acid content in 'Duke' and 'Brigitta' (Tab. 4) was about 2.5 mg/100 g FW in berries from the third ripening stage, while fully mature 'Duke' showed higher levels compared with 'Brigitta' (4.2 mg/100 g FW and 3 mg/100 g FW respectively). Our results are slightly different from what reported by other authors. For example Prior and colleagues (1998) found in ripe berries from the cultivar 'Duke' higher contents of ascorbic acid (about 7.3 mg/100 g FW), this is probably due to many factors which can affect the accumulation of this compound, such as different climatic conditions, cultural practices and different stage of ripening at harvest. The values measured for the two cultivars differ statistically at the fourth ripening stage. Variability in the concentration of ascorbic acid between cultivars was also reported in a study on two blueberry cultivars, 'Darrow' and 'Bluecrop', which investigated the effect of the genetic diversity and of date of harvest on ascorbate and phenolic compounds (Lata et al., 2005) and also by Prior and colleagues in their studies on *Vaccinium spp.* (1998).

For each cultivar, samples from the last ripening stage showed a significant difference compared to other stages, considering both treated and non treated berries, the only case in which there was no difference between the fourth and third stage was found in 'Brigitta' after treatment.

Treatment with BTH had no significant effect in 'Brigitta' along all the ripening process. For 'Duke', treatment decreased significantly AsA content in the first ripening stage, on the contrary in the fourth stage the level of AsA was higher in treated berries, nevertheless without showing a statistically detectable difference.

Tab. 4: Effect of BTH treatment on dry matter (%), soluble solids content (°Brix) and ascorbic acid content in 'Brigitta' and 'Duke' at different ripening stages. (Values represent means ± SE (*n = 4*). Different letters indicate significant differences (p ≤ 0.05) according to the Duncan's mean test.

Cv/Ripening stage	Dry matter (%)		Soluble solids (°Brix)		AsA (mg 100 g^{-1} FW)	
	Control	BTH	Control	BTH	Control	BTH
Duke						
Stage 1 unripe green	11.13±0.03a	10.52±0.03a	6.53±0.07a	5.80±0.11a	0.53 ± 0.08a	0.15 ± 0.02a
Stage 2 unripe purple	11.90±0.12b	11.41±0.01b	7.07±0.29b	6.47±0.27a	1.24 ± 0.27ab	1.07 ± 0.24a
Stage 3 ripe	12.09±0.01b	11.28±0.42b	8.03±0.39c	7.95±0.42b	2.29 ± 0.27b	2.52 ± 0.25b
Stage 4 full ripe	13.97±0.34c	12.78±0.13c	9.53±0.52d	9.67±0.40c	4.25 ± 0.37c	5.04 ± 0.36c
Brigitta						
Stage 1 unripe green	12.08±0.21a	10.30±0.03a	5,80±0.11a	6.33±0.18a	0.31 ± 0.22a	0.09 ± 0.04a
Stage 2 unripe purple	13.49±0.65a	11.09±0.01a	6.73±0.13b	6.87±0.18a	0.75 ± 0.18a	0.58 ± 0.25a
Stage 3 ripe	12.87±0.53a	13.17±0.49b	8.53±0.28c	9.33±0.21b	2.40 ± 0.22b	2.39 ± 0.32b
Stage 4 full ripe	13.85±0.62a	14.46±0.57c	10.20±0.09d	10.60±0.15c	3.00 ± 0.20c	3.01 ± 0.35b

4.3.5. Enzyme activities

Significant differences in the activity of enzymes involved in the AsA recycling through the ascorbate-glutathione cycle were detected among blueberry cultivars and among ripening stages as well (Fig.13). BTH resulted to be effective in stimulating the enzymatic activities but different cultivars react differently to treatment at different ripening stages.

APX is the first enzyme in the cycle, it shows high affinity for AsA as a substrate and is very important in the detoxification from H_2O_2. BTH is thought to inhibit the activity of APX (Wendehenne et al., 1998), leading to a burst in H_2O_2 production. In the third ripening stage we measured higher activities with respect to the last stage, in both the cultivars, 'Brigitta' showed slightly higher activities compared with 'Duke'. At this stage BTH had no effect on the APX activity. Along the ripening process the APX activity decreased in both, 'Brigitta' and 'Duke', but in fully ripe samples treated with BTH the values were significantly higher. Those data are not supporting the hypothesis of an inhibitory effect on the activity of APX by BTH. We have not measured the levels

of H_2O_2 but since we collected samples 24 hours after treatment and treatments were repeated starting from the beginning of fruit pigmentation throughout the harvest season, is possible to speculate that after an initial suppression of the APX activity, the high levels of H_2O_2 lead to an increment in the enzymatic activity as we observed in full ripe samples.De Pinto et al.(2006) pointed out the relevance of different timing and amounts of H_2O_2 as critical points for APX behaviour. The constant production of low amounts of this reactive species, determines a transient rise in APX. Such a rise is aimed at restoring redox impairment due to H_2O_2 overproduction. An activation (intensification, strengthening) of the antioxidant metabolism has been reported widely in the literature as a first line of defence against moderate oxidative stress (Shigeoka et al., 2002; Morita et al, 1999). It is likely that the regulation of APX expression and activity is part of a mechanism for controlling the balance between beneficial and detrimental roles of H_2O_2 in plant cells.

MDHAR is the enzyme that allows the regeneration of AsA from MDHA. A rapid regeneration of AsA is necessary to maintain its antioxidant potential (Wang et al., 2011). MDHAR was reported to be influenced by many stresses and treatments. For example exposure of mature green tomatoes to low oxygen, regardless of concentration, resulted in a decrease of MDHAR transcript levels after 1 h (Ioannidi et al., 2010). In a recent study Stevens and colleagues (2008) showed that MDHAR activity levels correlate with reduced AsA levels in tomato fruit under chilling stress. From our results MDHAR activity appeared to be positively correlated with AsA accumulation, in the last two ripening stages. In both the cultivar levels of activity increased from stage 3 to 4, but this pattern appeared much more evident in ‘Duke’ compared with ‘Brigitta’. Treatment with BTH was effective in stimulating the MDHAR activity in both of the cultivars only on the third stage of ripening, while no differences were observed in samples from stage 4.

DHAR is the enzyme committed to catalyze the reduction of dehydroascorbate to AsA by using the reducing power deriving from the oxidation of glutathione. Thus DHAR represents a valuable component involved in the protection of cells from oxidative stress. Increased DHAR activity was reported in response to various ROS-inducing stresses, including hydrogen peroxide, ozone, salt, drought and low temperature treatments (Eltayeb et al., 2006). Our study revealed that the DHAR activity was different among cultivars. In fact ‘Duke’ showed the highest levels of activity in both the ripening stages, but appeared not to be sensitive to BTH. On the other hand ‘Brigitta’ had lower levels in third and fourth ripening stage, but was positively affected by BTH treatment showing significant increments in activity values.

GR catalyzes the regeneration of oxidized glutathione derived from the activity of DHAR, thus it is important in maintaining the efficiency of the recycling pathway of ascorbate. A past study revealed a positive correlation between GR activity and AsA levels (Wang et al., 2011). BTH was reported to increase the activity of GR in strawberry fruit during storage (Cao et al. 2010). In our experiment we did not observed great changes in GR activity and the values we recorded were quite similar between cultivars and at different ripening stages. The only significant change was observed in 'Duke' at the last stage of ripening in which the levels increased in treated berries compared with the control.

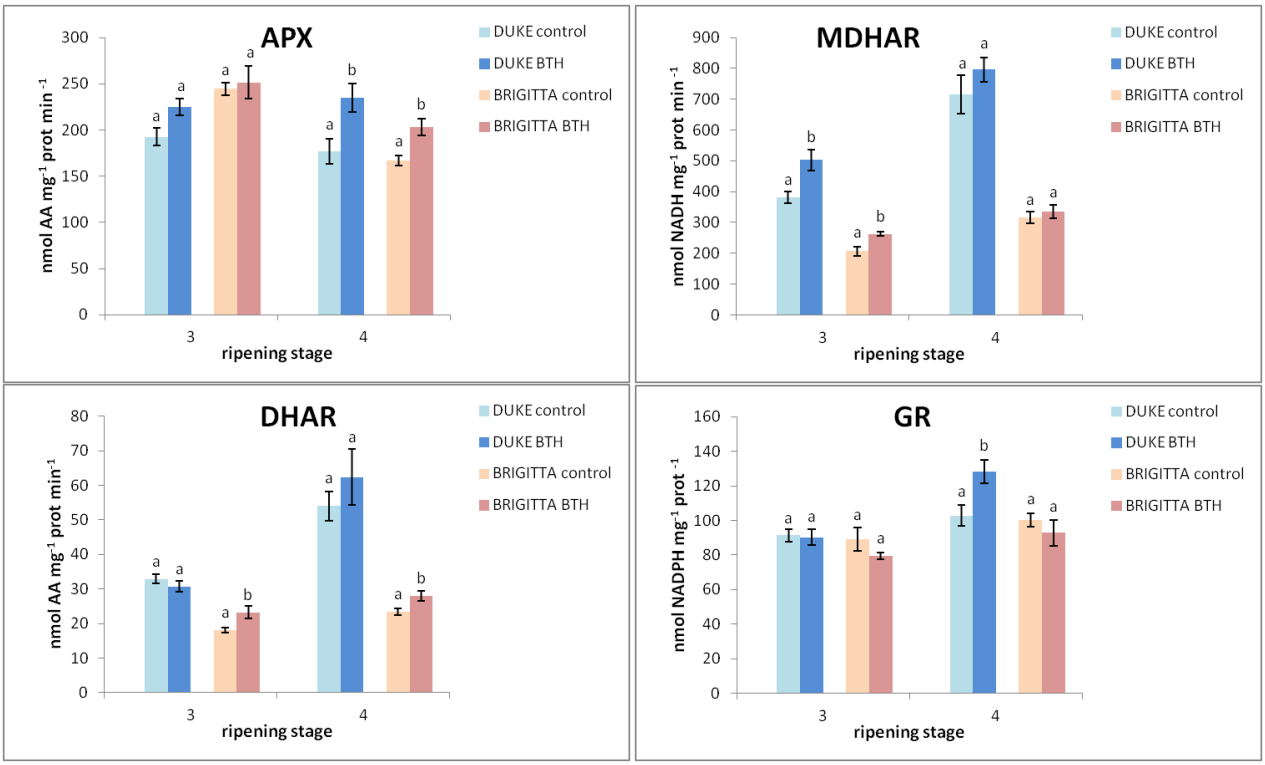


Fig. 13: Effect of BTH on activities of APX, MDHAR, DHAR and GR in 'Duke' and 'Brigitta' at two ripening stages. Values represent means ± SE (*n = 4*). Different letters indicate significant differences (p ≤ 0.05) according to the Duncan's mean test.

4.3.6. Total phenolics, total flavonoids and total anthocyanins

The stress response triggered by BTH can result in an activation of the secondary metabolic pathway that leads to accumulation of phenolic compounds. In fact, treatments with BTH were reported to be effective in enhancing PAL activity and increasing the levels of total phenols, flavonoids and anthocyanins. In this work we measured the levels of phenolic compounds during the ripening process and we evaluated the effectiveness of BTH in stimulating those parameters.

The lower levels of total polyphenols (Fig. 14) were found in 'Brigitta', particularly in the third ripening stage (150.5 mg/100 g FW). Along the process of maturation 'Duke' maintained higher levels of polyphenols, but on the other hand the levels decreased sharply at the second and third

ripening stage (about 270 mg/100 g FW in control plants). The amount of total phenols that we measured is comparable with those reported by Prior and his colleagues (1998) for 'Duke' in their study (306 mg / 100 g FW). Strawberry has been reported to have an higher content of total polyphenols at the early stages of maturation (Halbwirth et al., 2006). In 'Duke' and 'Brigitta', during ripening, after a consistent decrease, total polyphenols increased to reach in stage 4 the same levels as in stage 1.

Moreover, the differences we found with other published studies dealing with other blueberry cultivars prove that polyphenol content depends on genetic background, as previously suggested (Prior et al. 1998; Taruscio et al., 2004).

The BTH treatment was effective in 'Brigitta' in stimulating phenolic production and this allowed to accumulate significantly higher amounts of total phenols in the first, third and fourth stages. On the other hand, no effect was detected in 'Duke' after BTH treatment.

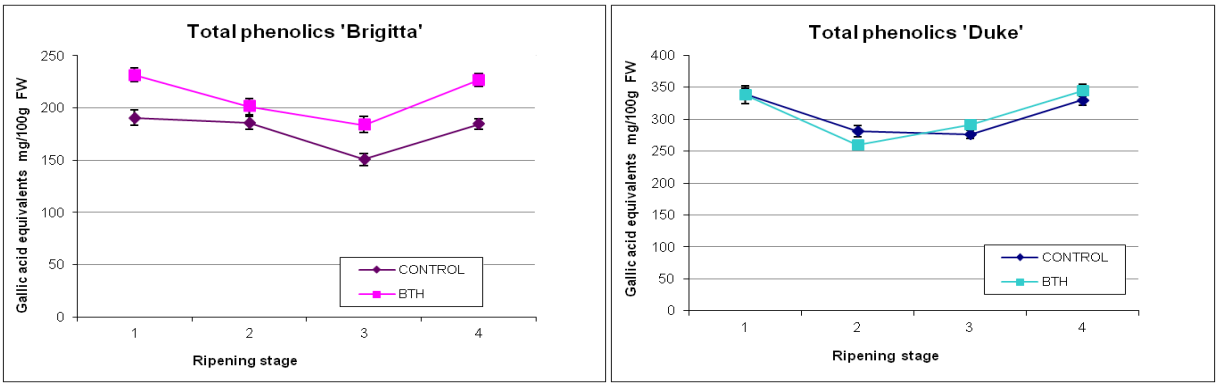


Fig. 14: Effect of BTH treatment on total phenolics content in two cultivars 'Duke' and 'Brigitta'. Values represent means ± SE (*n = 4*).

Flavonoids represent a valuable part of the pool of phenolic compounds in blueberry. In this study the amount of total flavonoids and the effect of BTH were investigated in the two blueberry cultivars at the end of the ripening progress (stages 3 and 4), when berries are commonly consumed (Fig. 15). The levels we found are comparable with those reported for blueberry in a recent study (Marinova et al., 2005). The content of flavonoids increased at the end of ripening and at the last stage the higher levels were registred in both of the cultivars. 'Duke' showed higher concentrations compared with 'Brigitta', confirming this cultivar as the better source of phenols and the most rich in flavonoids (319.4 mg/100 g FW at stage 4).

Treatment with BTH had significant effects only in the third stage of 'Brigitta', in which the berries have a higher flavonoids content compared to control, although the trend is toward

an increment in flavonoids also in 'Duke' at the third ripening stage of ripening and in 'Brigitta' at the fourth.

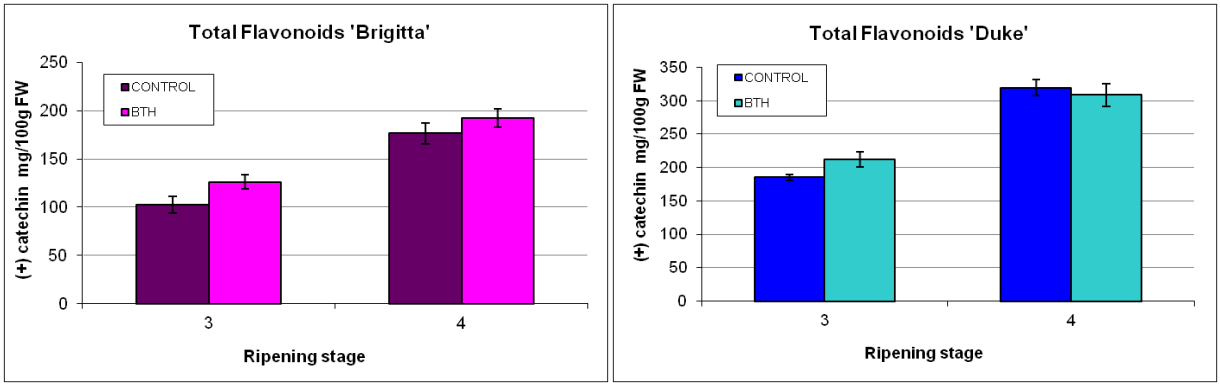


Fig. 15: Effect of BTH treatment on total flavonoids content in two cultivars 'Duke' and 'Brigitta'. Values represent means ± SE (*n = 4*).

Among phenolic compounds, the flavonoid subclass of anthocyanins is one of the most interesting and representative in blueberry.

The content of total anthocyanin increased switching from the second to the fourth ripening stage in both of the cultivars tested (Fig. 16), as reported by Macheix and colleagues (1990), which showed that the more colorful berries are also the most rich in anthocyanins. Also the study of Prior (1998), showed that anthocyanins levels increase during maturation in several *Vaccinium* species (*Vaccinium corymbosum* L. (Highbush), *Vaccinium ashei Reade* (Rabbiteye), *Vaccinium angustifolium* (Lowbush) and *Vaccinium myrtillus* L. (Bilberry)).

Within each cultivar, the three stages of maturation considered differ statistically. The content of anthocyanins was reported to have a wide range of variation, Mazza and colleagues (1993), reported for *Vaccinium corymbosum* L. values ranging from 25 to 495 mg/100 g FW, Prior and his colleagues showed contents of anthocyanins, from 93 to 235 mg/100 g FW).

In our study, at all the considered stages of maturity, cultivars differ significantly in anthocyanin content, with greater differences at the last ripening stage in which 'Duke' (164 mg/100 g FW) was confirmed to be a better source of this class of compounds compared to 'Brigitta' (95 mg/100 g FW).

The increase in anthocyanin content, associated with a decrease of other phenolic substances during maturation was also reported in other studies (Kalt et al., 2003) and

might suggests that changes in the pool of total phenolics with a greater accumulation of anthocyanins could typically occur during ripening, (Castrejon et al., 2008).

We did not mention any data on the amount of anthocyanins in unripe green berries (stage 1) as those pigments were not detectable. In fact unripe berries were reported to completely lack of those pigments (Kalt et al., 2003).

BTH had a positive effect on anthocyanins levels, at the third ripening stage in 'Brigitta' and 'Duke' as well as on the fourth stage in 'Brigitta'. These data are supporting the hypothesis that BTH has the ability to stimulate the production of phenolic antioxidants and pigments in some berry fruits as previously reported (Irit et al., 2004).

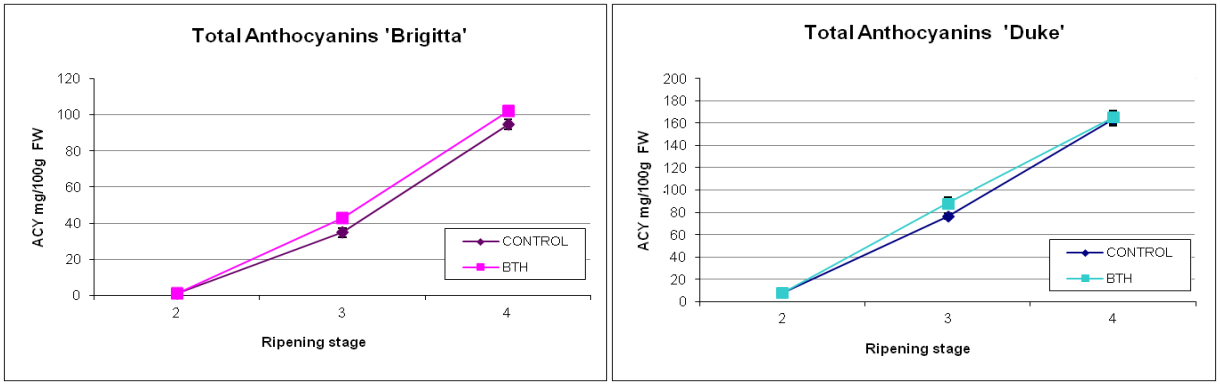


Fig. 16: Effect of BTH treatment on total anthocyanins content in two cultivars 'Duke' and 'Brigitta'. Values represent means ± SE (*n = 4*).

4.4. Conclusions

In this study we evaluated the effect of BTH treatment on two blueberry cultivars during ripening. Analyses were directed to the study of accumulation of health-promoting compounds (such as polyphenols, anthocyanins, flavonoids and ascorbic acid) and important parameters considered as indices of maturity (such as soluble solids and titratable acidity). Moreover we studied the effect of treatment on the enzymatic system committed in the detoxification from H_2O_2 by measuring the specific activities of APX, MDHAR, DHAR and GR.

At the end of the ripening process, the soluble solids content increased in all cultivars while the titratable acidity decreased. 'Brigitta' had the highest values of acidity and keep them until the last stage of maturation, this feature might explain the greater storage attitude of this cultivar. In 'Brigitta', BTH had a stimulating effect on both the parameters mentioned above (with the only exception of a slight decrement in titratable acidity in the fourth ripening stage). In 'Duke', no

significant effects were detected after treatment, the only exception was an increase in titratable acidity in samples from the third stage.

Ascorbic acid content can be affected by many factors such as genetic diversity, climate conditions, stage of ripening, biotic and abiotic stresses. In this study we observed for all cultivars an increase in AsA levels along the maturation, this increment was more marked in 'Duke', which also showed the highest values in full ripen berries. The treatment with BTH caused an increment in the ascorbic acid content only in 'Duke' at the end of ripening. From this results we can deduce that BTH did not directly affect the AsA levels. However the treatment appeared to be effective in stimulating the activities of the enzymes involved in AsA recycling pathway and in the detoxification from H_2O_2 indicating a possible role of this enzymatic system in plant-pathogen responses triggered by BTH and in restoring AsA levels after an oxidative stress. Our data showed differences in the responses for different cultivars and different stages of maturation and in some cases an increment in the activity of certain enzymes has helped to maintain the cycle effective, despite the drop in activity observed in other enzymes. The total polyphenols levels were interested by a decline during ripening in both of the cultivars we studied, showing an upturn at the end of ripening. The effect of treatment with BTH only affected the polyphenol content in 'Brigitta'.

The levels of flavonoids in ‘Duke’ were almost double then those measured in ‘Brigitta’, but on the other hand ‘Brigitta’ resulted to be positively influenced by treatment with BTH.

The total anthocyanins increased during maturation, in all cases analyzed. At the end of ripening, 'Duke' showed the highest content of anthocyanins compared with 'Brigitta'. BTH was effective in stimulating the synthesis of anthocyanins at the third ripening stage for both cultivars and fourth stage of 'Brigitta', so, the response of blueberry to BTH treatment could be considered cultivar-dependent.

The different trends of total phenolics, total flavonoids and anthocyanins observed along ripening shows that the phenolic pool varied consistently during the process.

Blueberry is confirmed to be an high-valuable nutraceutical product, however it shows significant differences in antioxidants content depending on genetic diversity and in response to different cultivation practices.

In this study BTH stimulated the accumulation of anthocyanins and of polyphenols, in the last stages of ripening (especially in 'Brigitta'). It was also active in stimulating the enzymatic mechanisms of response to oxidative stress.

The increase in content of antioxidants in fruits represent an interesting challenge which can lead to beneficial effects on human health, as well as on qualitative aspects of the fruits, such as color or storage performance. From our results, in blueberry, an activation of secondary metabolism, with a consequent increase in polyphenolic compounds, did not affect the primary metabolites accumulation such as soluble solids and organic acids, which were not negatively affected following the treatment. It is important to point out how different cultivars behave differently during ripening and, even more, in response to treatment. In fact, in many cases 'Duke' and 'Brigitta' showed different or even opposite behavior after treatment with BTH, suggesting that the responses are modulated differently according to different genotypes.

Collectively our results could be useful in breeding programs aiming to develope cultivars with optimal traits in terms of health-promoting components and quality attributes.

5. Effects of Methyljasmonate on Phenolic Metabolism in Blueberry (*Vaccinium corymbosum*, L.)

5.1. Introduction

Blueberries (*Vaccinium corymbosum,* L.) are very appreciated for their health promoting traits. They have been demonstrated to be one of the richest sources of health-promoting compounds and antioxidants among fruits (Prior et al., 1998). In fact they show a high content of phenolic compounds and a moderate content of ascorbic acid (vitamin C) and carotenoids (Szajde, and Borowska, 2008). Phenolics, which include flavonoids and their subclass, anthocyanins, are secondary metabolites that frequently serve as pigments in plants, but are also involved in many biological interactions. Their biosynthesis occurs through the phenylpropanoid pathway (Fig. 17).

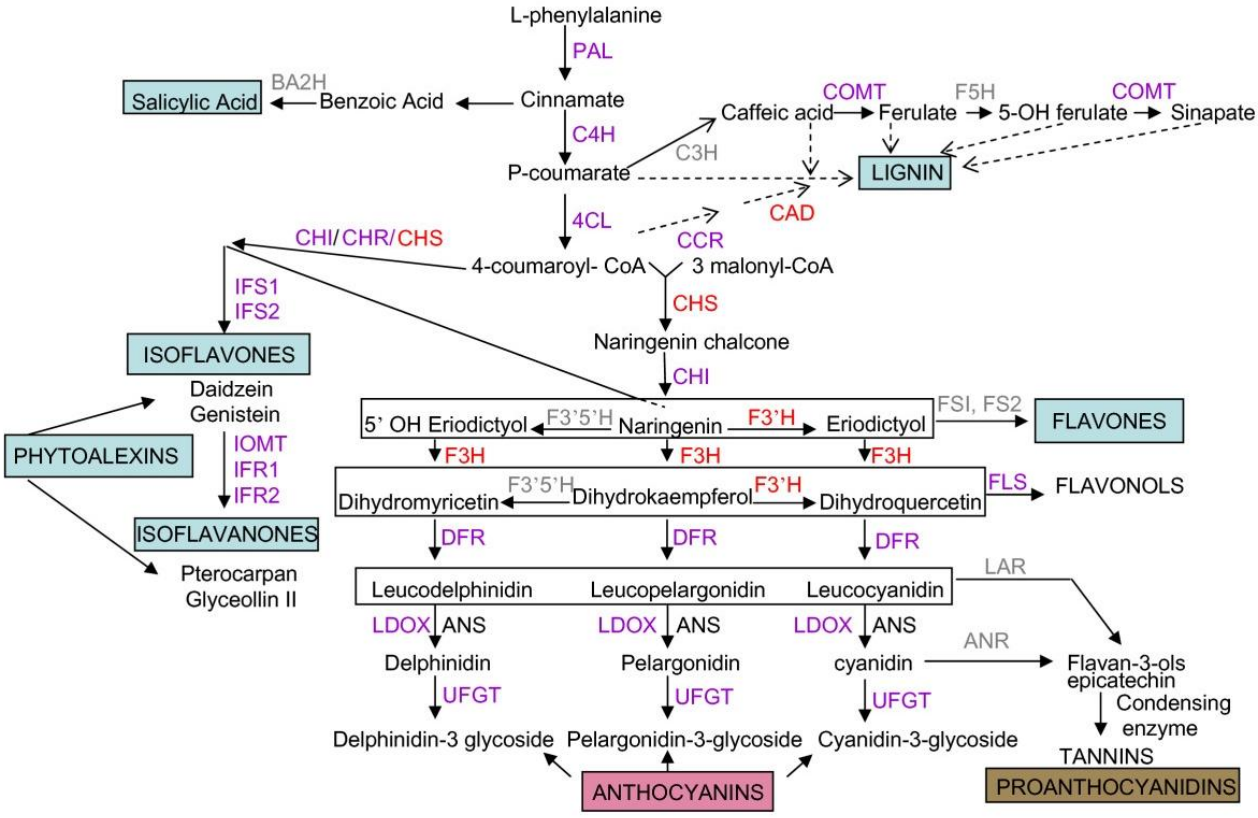


Fig.17. The phenylpropanoid metabolic pathway

Recently more attention has been paid to the health-promoting and antioxidant compounds contained in fruits because epidemiological studies revealed that high polyphenol-rich fruit intake appears to be positively correlated with reduced effects of many human pathologies (Scalbert et

al., 2005). A significant positive correlation was reported between antioxidant capacity and anthocyanins and total phenolics in ripe blueberries (Prior et al., 1998).

Methyljasmonate (MeJa) is a phytohormone which plays a key role in plant growth and in many physiological and biochemical processes. A new potential concept for enhance antioxidant capacity of fruit products is based on the use of chemical compounds which activate the plant's own defence systems. The use of plant activating compounds such as MeJa stimulated the studies of the biochemical and genetic basis of plant response following their use in pre- and postharvest treatments. It has been shown that MeJa treatments stimulate secondary metabolites production in many plant species. Recent studies demonstrated that preharvest treatments with two different concentrations (0.01 and 0.1 mM) of MeJa enhance antioxidant activity and flavonoids content in blackberries (Wang et al., 2008). Moreover raspberries treated during storage with MeJa showed the highest ascorbate content, antioxidant activity and enzymatic activity of the Halliwell Asada-cycle (Wang et al., 2006); application of MeJa also significantly enhanced the level of resveratrol in strawberry fruit (Wang et al., 2007) and raspberry fruits treated with MeJa maintained higher levels of antioxidant capacity, total anthocyanins compared to untreated fruits during storage (Ghasemnezhad and Javaherdashti, 2008). Other studies demonstrated that MeJa was effective in stimulating different responses in higher plants. Several lines of evidence suggest that this compound play an important role as signal molecules in plant defence mechanisms (Tripathy and Dubey, 2004). Jasmonates have been shown to activate genes encoding antifungal proteins and several other genes involved in phytoalexin biosynthesis (Tripathy and Dubey, 2004). Exogenous applications of jasmonates caused the accumulation of paclitaxel and related taxanes, alchaloids, rosmarinic acid and anthocyanins, in several plant species (Creelman and Mullet, 1997).

In this study we hypothesized that spray applications of a MeJa solution on blueberry plants during ripening could stimulate the biosynthesis of health-promoting phenolic compounds at harvest. This could happen through the activation of key genes of the phenylpropanoid metabolic pathway. Our purpose was to determinate the changes in expression patterns of the key genes in the phenolic biosynthetic pathway in blueberries treated with MeJa, at different time points. Moreover, we measured the levels of different classes of phenolic compounds (such as phenolic acids, flavonols, flavan-3-ols, gallic acid esters and stilbenes) in order to evaluate the effectiveness of the treatment.

5.2. Materials and methods

5.2.1. Plant material

Blueberry plants (*Vaccinium corymbosum,* L.) were grown in an experimental field under natural conditions. One cultivar ('Blue Ray') was studied on the first year (summer '2009') and two cultivars ('Blue Ray' and 'Duke') were treated and analyzed in the second's year experiment (summer 2010).

5.2.2. Chemical treatments

Six plants for each cultivar were treated with MeJa 0.1 mM plus 0.05% Tween-20, the solution was applied as a foliage-berry spray to runoff when berries were starting to develop color (varìason). The control plants were sprayed with a 0.05% Tween-20 solution. Berries were picked up randomly 3 h, 6 h, 9 h and 24 h after treatment, immediately frozen in liquid N_2, stored at -80°C and used for molecular analyses. Four days and one week after the treatment, berries from each plant were harvested, and sorted in four ripening stages (Stage 1: unripe green, Stage 2: unripe purple, Stage 3: ripe, Stage 4: full ripe), then the last two stages, corresponding to commercial maturity (Stages 3 and 4), were selected and stored at -80°C for HPLC analyses.

The field experiments and treatment were carried out at the same location over two growing seasons.

5.2.3. Total RNA isolation and gene expression analyses

Total RNA was extracted from berries (about 2-3 g) according to Wan and Wilkins (1994) with slight modifications. Berries were grinded in a mortar under liquid N_2 and the powder transferred to 5 volumes of a 80°C pre-heated extraction buffer (0.2 M sodium borate decahydrate/ 30 mM [ethylenebis(oxyethylenenitrilo)] tetraacetic acid/ 1 % (w/v) sodium lauryl sulphate/ 1 % (w/v) deoxycholic acid/ 10 mM dithiothreitol (DTT)/ 1 % (w/v) Igepal/ 2% (w/v) polyvinylpyrrolidone-40). The extracts were vortexed for 30 s to thoroughly mix, then 0.015% (w/v) proteinase K (Sigma, Italy) was added before the tubes were gently inverted and placed horizontally in a shaking incubator at 37°C for 1.5 h. Then 0.08 volumes of 2 M KCl were added and the extracts were incubated on ice for 30 min, then centrifuged at 12,000 x g for 30 min and the aqueous phases transferred into tubes containing one volume of 4 M LiCl (Sigma, Italy), and precipitated overnight. The next morning, the precipitate was pelleted by centrifugation at 12,000 x g for 30 min at 4°C, resuspended in 600 µl of DEPC-treated water and passed directly into 1 volume of chloroform

(Sigma, Italy). The extracts were then vortexed, the resulting turbid solution was centrifuged at 13,500 x g for 10 min at 4°C, the supernatant containing the RNA removed, precipitated in isopropanol and 3M sodium acetate on ice for 30 min, washed in 80 % (v/v) ethanol and resuspended in 80 µL sterile water. RNA was quantified by measuring the absorbance spectrum with NanoDrop N-1000 spectrophotometer (NanoDrop technologies). The quality of the isolated RNA was verified by electrophoresis on a denaturing agarose gel.

5.2.4. Gene expression analyses

Gene expression were determined by qRT-PCR (ABI7300, Applied Biosystem, Italy) using specific primers. Primers were designed for phenylanlanine ammonia lyase (*VmPAL*, AY123770.1), chalcone synthase (*VmCHS*, AY123765.1), flavanone 3β-hyddroxylase (*VmF3H*, AY123766.1), dihydroflavonol 4-reductase (*VmDFR*, AY123767.1), anthocyanidin synthase (*VmANS*, AY123768.1), anthocyanidin reductase (*VmANR*, FJ666338.1), chalcone reductase (*VcCCR*, FJ197338.1). (Tab. 5).

Tab. 5: Primers pair used for qRT-PCR. All primers were designed using primer select software (Lasergene) or Primer3 on line software (http://fokker.wi.mit.edu/primer3/input.htm).

Primer	*Sequence (5'->3')*	*bp*	*Tm (°C)*
VmPAL for	TTACAACAATGGGTTGCCCT	20	64
VmPAL rev	CCTGGTTGTGTGTCAGCACT	20	64
VmCHS for	AGGACCCAAGGCCATCA	17	64.4
VmCHS rev	ATCATGAGTCGCTTCACGG	19	64
VmF3H for	TGGGATTGGAAGAAGACAGG	20	64
VmF3H rev	ATGGGTTCTTGGGCCTAATC	20	63.8
VmDFR for	GAAGTGATCAAGCCGACGAT	20	64.2
VmDFR rev	ATCCAAGTCGCTCCAGTTGT	20	63.7
VmANS for	GCCCTCAACCGGAGCTTGCC	20	74.2
VmANS rev	CGACCGTGTCGCCAATGTGC	20	74.4
VmANR for	GCTGGTGTTTCTCCCACAAT	20	63.9
VmANR rev	GATTCTTTTTCCGCCACAAA	20	63.7
VcCCR for	CACTCTCAACGCCAGCATAA	20	64
VcCCR rev	AATTTCAACCACATCTCCGC	20	63.8
VmGAPDH for	ACTGTCTTGCCCCACTTGCCA	21	71.2
VmGAPDH rev	ACCAACAGCCTTGGCAGCACC	21	72.7

Five µg of total RNA were reverse transcribed using Superscript III (Invitrogen, Italy) and a mix of random primers and oligo dT. In order to avoid genomic DNA amplification total RNA was treated with DNase I (Sigma, Italy).

The SYBR green chemistry was used for gene expression analyses. Dissociation curves have been performed to check the absence of primer dimers and other amplification by-products.

The amplification program was set to: 1 cycle at 50°C for 2 min then at 95°C for 2 min; 40 cycles at 95°C for 30sec; 55 °C for 1 min and 72°C for 30 sec (signal acquisition stage); 72°C, 10 min and dissociation curve. Glyceraldehyde-3-phosphate dehydrogenase (*VmGADPH*, AY123769.1) was used as internal control (Jaakola et al., 2002, Tab. 5).

5.2.5 Analysis of Phenolic Acids, Flavonols, Flavan-3-ols, Gallic Acid Esters and Stilbenes

An extraction method was used for the HPLC analysis of phenolic compounds in berry samples (Kammerer et al., 2004). Frozen berries (10 g) were ground using a mortar and pestle and macerated (24 h) in 50 mL of acidified methanol (0.1 % HCl). Each sample was evaporated to dryness using a rotary evaporator and dissolved in 20 mL of acidified water (pH 3). Mixture was then extracted three times with diethyl ether and three times with ethyl acetate. Sodium sulphate was added to the extracts in order to completely dehydrate. Each sample was again evaporated to dryness in a rotary evaporator and dissolved in 4 mL of a 50% methanol solution, filtered through a 0.45 µm syringe filter before injection into HPLC.

HPLC separation of the phenolic compounds was achieved on a Nova-Pack C18 column (4 µm X 30 cm X 3,9 mm) protected with a guard column (Waters). The mobile phase was A: water/acetic acid (98:2), B: 0.5% acetic acid dissolved in a water/acetonitrile solution (50:50), the applied gradient was:

Time (min)	%A	%B
0	10	90
10	15	85
13	15	58
20	24	76
40	30	70
60	55	45
75	100	0
83	100	0
85	10	90
90	10	90

Analysis of chromatograms was performed using a specific software (CramQuest). The identification of phenolic compounds in the chromatograms was based on the retention times and

on the comparison of the shapes of their UV/Vis spectra with those of the representative standards and of earlier data previously published.

5.3. Results and Discussion

5.3.1. Transcript accumulation of phenylpropanoids-related genes

We measured the transcripts abundance of the main genes in the phenylpropanoid pathway for two different blueberry cultivars, 'Blue Ray' and 'Duke' after a spray treatment with a MeJa solution. In order to determine the most suitable time-point for gene expression analysis, the patterns of expression were tested in a time-course experiment, samples of treated and control berries were collected and analyzed 3, 6, 9 and 24 h after treatment. Data referred to 'Blue Ray' were collected over two different growing season (2009 and 2010), while data referred to 'Duke' were collected during one growing season (2010).

PAL is one of the best characterized enzymes of plant secondary metabolism. It converts L-phenylalanin into *trans*-cinnamate (*E*-cinnamate) by the *trans*-elimination of ammonia and the pro-3*S* proton. PAL can also show a similar activity like tyrosine ammonia lyase (TAL) against tyrosine and TAL enzyme can also show PAL activity (Davies and Schwinn, 2006).

During the first year of our experiments 'Blue Ray' samples showed a 2,5-fold increment of *PAL* transcripts 9 hours after treatment (Fig. 18). During the second year's experiments the effect of the treatment on *PAL* transcripts appeared to be confirmed, with a strong (10-fold) increment of expression on treated samples after 9 hours, moreover, on the second year's samples a lower induction was registered after 3 hours and a slight repression of the gene was observed after 24 hours. The effect of induction on *PAL* expression was recorded also in 'Duke' with a 2-fold increment of transcripts on treated samples 9 hours after the treatment.

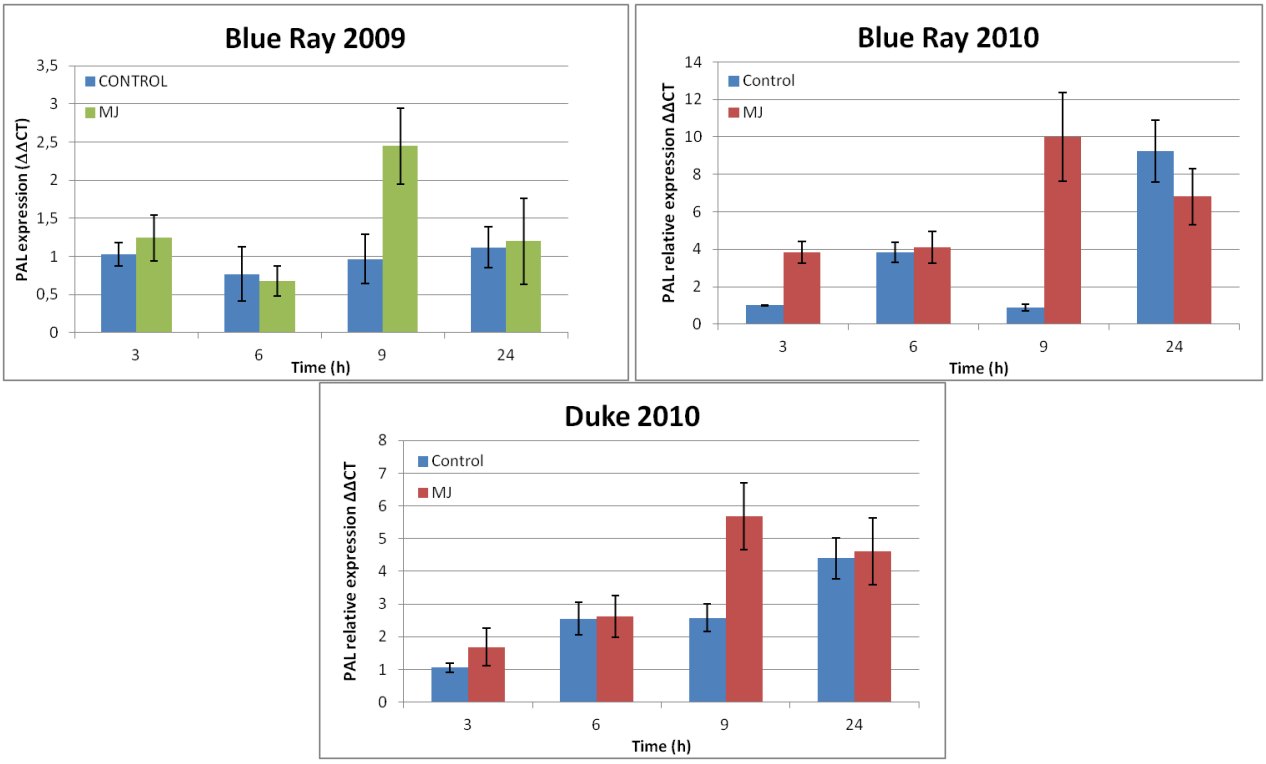


Fig. 18: PAL gene expression. Analyses were performed using qRT-PCR at different time course (0-24 h) in control (0.05% Tween-20) or 0.1 mM MeJa treatment. Data are means with standard deviations of ΔΔCt between genes and GAPDH as internal control. Reactions were repeated four times using two biological samples.

CHS carries out a series of sequential decarboxylation and condensation reactions, using 4-coumaroyl-CoA (in most species) and three molecules of malonyl-CoA, to produce a polyketide intermediate that then undergoes cyclization and aromatization reactions that form the A-ring and the resultant chalcone structure (naringerin chalcone). The key role of CHS in flavonoids biosynthesis has made it a focus of research for many years, and it is now very well characterized (Davies and Schwinn, 2006).

The treatment with MeJa appeared to be effective in stimulating the expression of *CHS,* in both the cultivars studied (Fig. 19). In 'Blue Ray' the most marked effect was registered after 9 hours with an 8-fold increase of transcripts on the first year's experiment and with a stronger effect on the second year, with a 28-fold increment of transcript levels at the same time point. During the second year's experiment a repression of the *CHS* transcripts was registered for 'Blue Ray' 6 and 24 hours after treatment. 'Duke' showed a significant increment of transcripts (5-fold) after 9 hours, even if it was less marked compared with the one observed in 'Blue Ray'.

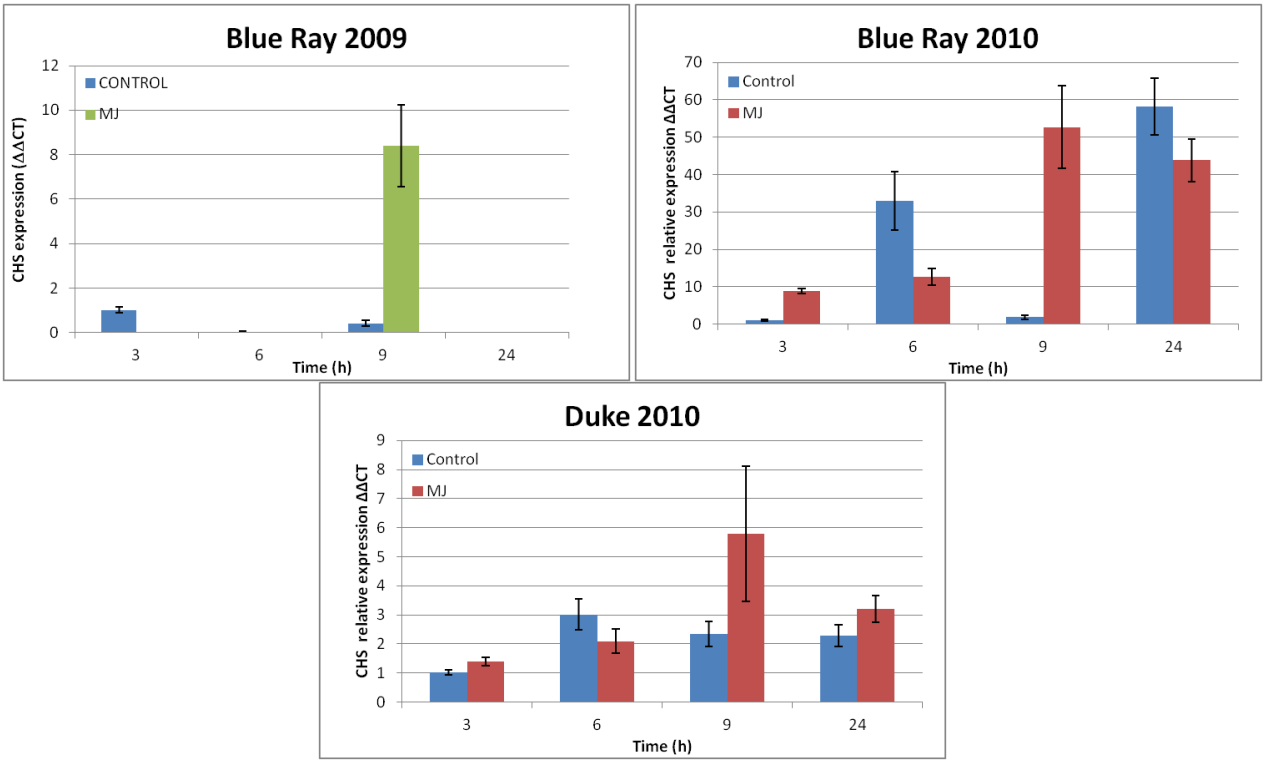


Fig. 19: CHS gene expression. Analyses were performed using qRT-PCR at different time course (0-24 h) in control (0.05% Tween-20) or 0.1 mM MeJa treatment. Data are means with standard deviations of ΔΔCt between genes and GAPDH as internal control. Reactions were repeated four times using two biological samples.

(2*S*)-Flavanones are converted stereospecifically to the respective (2*R*, 3*R*)-dihydroflavonols (DHFs) by F3H, thus this enzyme is required for the biosynthesis of flavonols, catechins and proanthocyanidins. *F3H* transcripts were reported to be coordinated with flavonols formation in Maize anthers (Debo et al., 1995); in bilberry leaves exposure to sunlight was reported to stimulate *F3H* expression and increase flavonols accumulation (Jaakola et al., 2004). Our study revealed a controversial response of *F3H* to MeJa treatment. Relative expression data are shown in Figure 20. In 'Blue Ray' during the first year's experiment no significant effect was reported, while in the second year's experiment we observed a decrease in transcripts levels following to the treatment in both of the cultivars. This repression was stronger in 'Blue Ray' and was observed 9 hours after the treatment, in 'Duke' the down regulation occurred 6 hours after treatment and was less marked.

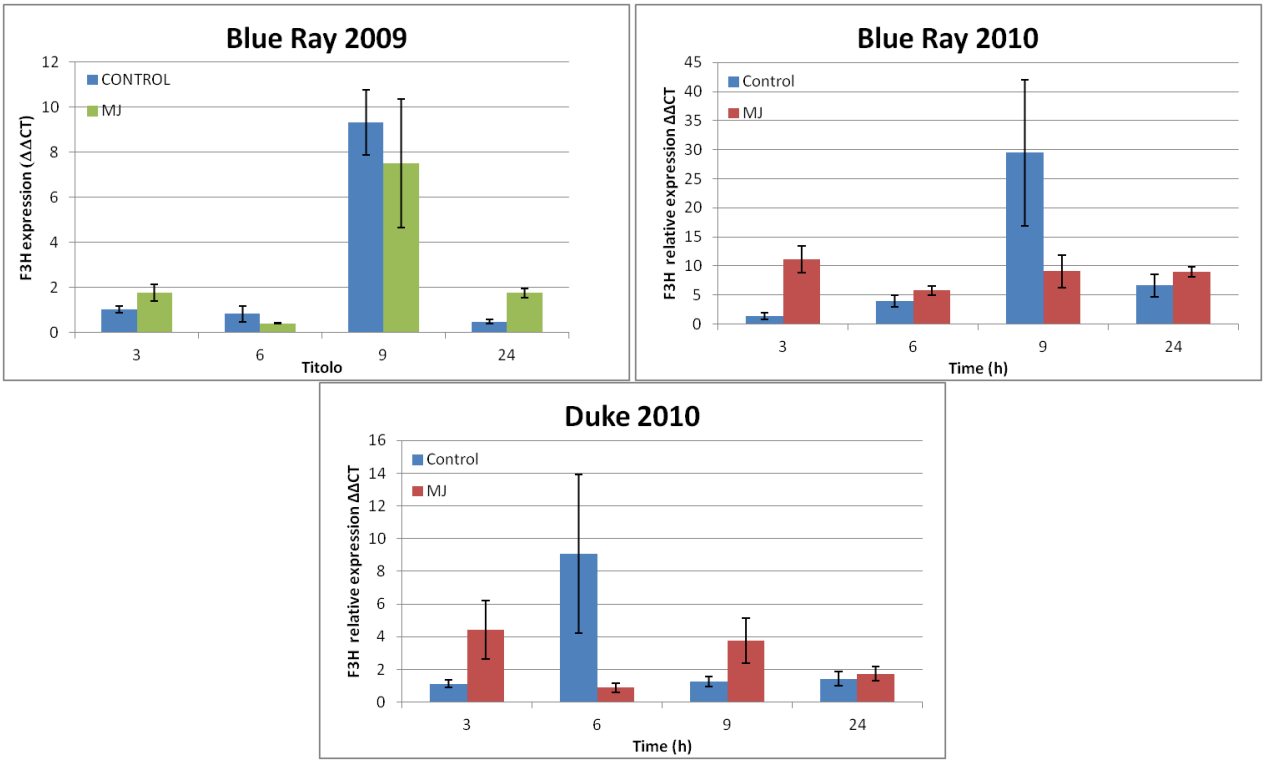


Fig. 20: F3H gene expression. Analyses were performed using qRT-PCR at different time course (0-24 h) in control (0.05% Tween-20) or 0.1 mM MaJa treatment. Data are means with standard deviations of ΔΔCt between genes and GAPDH as internal control. Reactions were repeated four times using two biological samples.

DFR is the enzyme which catalyzes the stereospecific conversion of (2R,3R)-trans-DHF's to the respective (2R,3S,4S)-flavan-2,3-trans-3,4-cis-diols (leucoanthocyanidins) and so it represents the first step toward the biosynthesis of anthocyanins. In a study conducted on bilberry leaves *DFR* gene expression was reported to be minimally influenced by solar radiation in comparison with *PAL CHS* and *F3H* (Jaakola et al., 2004). In a study conducted on *Arabidopsis,* jasmonate in presence of sucrose resulted to be effective in stimulating the expression of *DFR* and other downstream genes in the anthocyanins biosynthetic route (Loreti et al., 2008). In our experiments MeJa was effective in stimulating the expression of *DFR* in both cultivars (Fig. 21). In 'Blue Ray' samples from the first year's experiment a slight (2,3-fold) increment in transcripts was recorded 24 hours after the treatment, while in the second year's experiment the effect was observed in both cultivars 9 hours after MeJa foliage spray application.

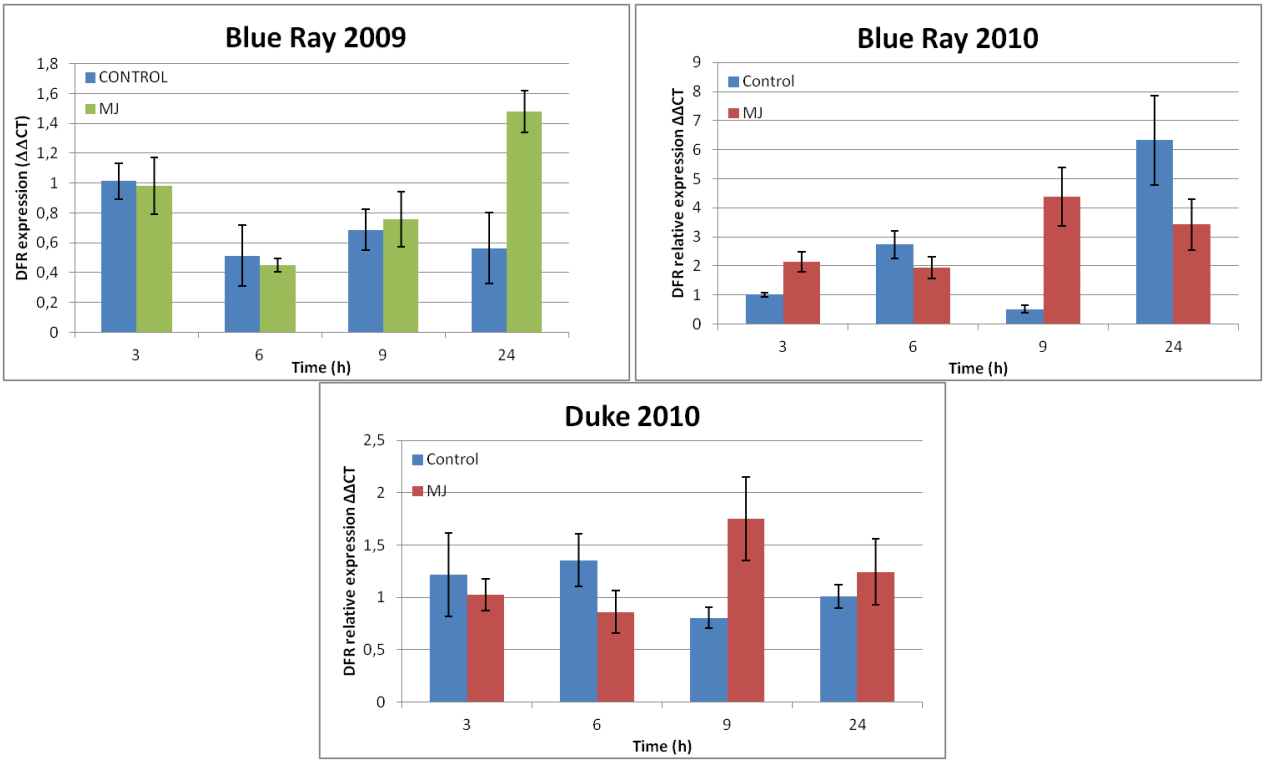


Fig. 21: DFR gene expression. Analyses were performed using qRT-PCR at different time course (0-24 h) in control (0.05% Tween-20) or 0.1 mM MeJa treatment. Data are means with standard deviations of ΔΔCt between genes and GAPDH as internal control. Reactions were repeated four times using two biological samples.

The role of ANS in the biosynthetic pathway is to catalyze the reduction of leucoanthocyanidins to the corresponding anthocyanidins. Artificial infection of *Vaccinium vitis-idaea* L. with *Exobasidium* species carried to high levels of expression of *ANS* (Pehkonen et al., 2008), indicating a possible involvement of anthocyanins in plant responses to stress or a role in defence against pathogens. The effect of solar radiation on the expression of *ANS* in bilberry leaves was recorded as a slight increment of transcript, but this was lower than the effect observed for *PAL*, *CHS* and *F3H* (Jaakola et al., 2004). We registered an 80-fold increment on *ANS* transcripts that was triggered by MeJa in berries from 'Blue Ray' 9 hours after treatment during the second year's experiment (Fig. 22). Data from 'Blue Ray' in the first year and from 'Duke' also indicate the effectiveness of MeJa treatment: high gene expression was recorded at the same time point (9 hours) but the increment was lower.

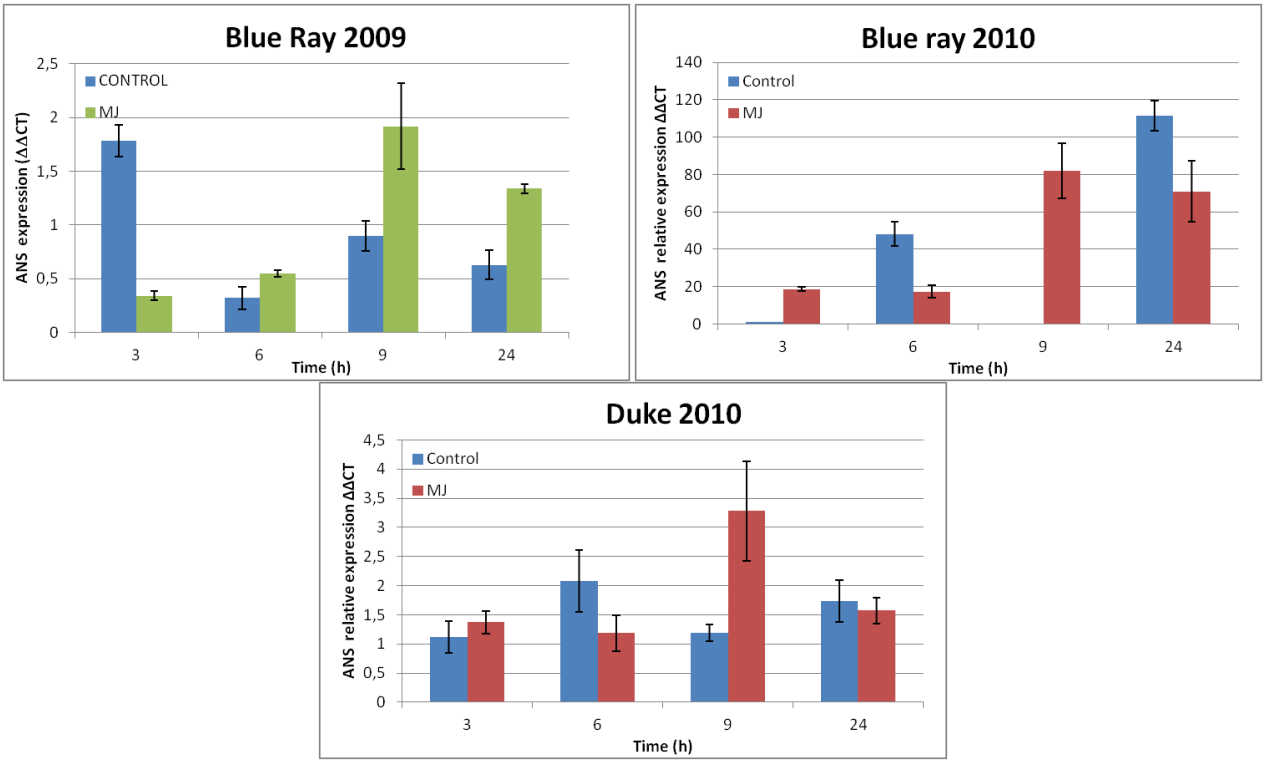


Fig. 22: ANS gene expression. Analyses were performed using qRT-PCR at different time course (0-24 h) in control (0.05% Tween-20) or 0.1 mM MeJa treatment. Data are means with standard deviations of ΔΔCt between genes and GAPDH as internal control. Reactions were repeated four times using two biological samples.

The conversion of anthocyanidins to the corresponding 2,3-cis-flavan-3-ols is catalyzed by ANR. It contributes, together with leucoanthocyanidin reductase (LAR), to biosynthesis of catechins and proanthocyanidins. Recent studies suggest an involvement of abscissic acid (ABA) in controlling the proanthocyanidin biosynthesis coregulating *ANR* and *LAR* in grape skin (Lacampagne et al., 2010). In the study ABA was shown to affect tannin content and to be involved in tannins biosynthesis by decreasing LAR and ANR activity and repressing the expression of related genes a few days after application. From our results it looks that MeJa had a different effect on *ANR*. The expression of *ANR* (Fig. 23) was augmented following to MeJa treatment in 'Blue Ray' as well as in 'Duke', and the effect was much more evident in 'Duke' samples. In both cases the most relevant changes in expression levels were registered 9 hours after spray application.

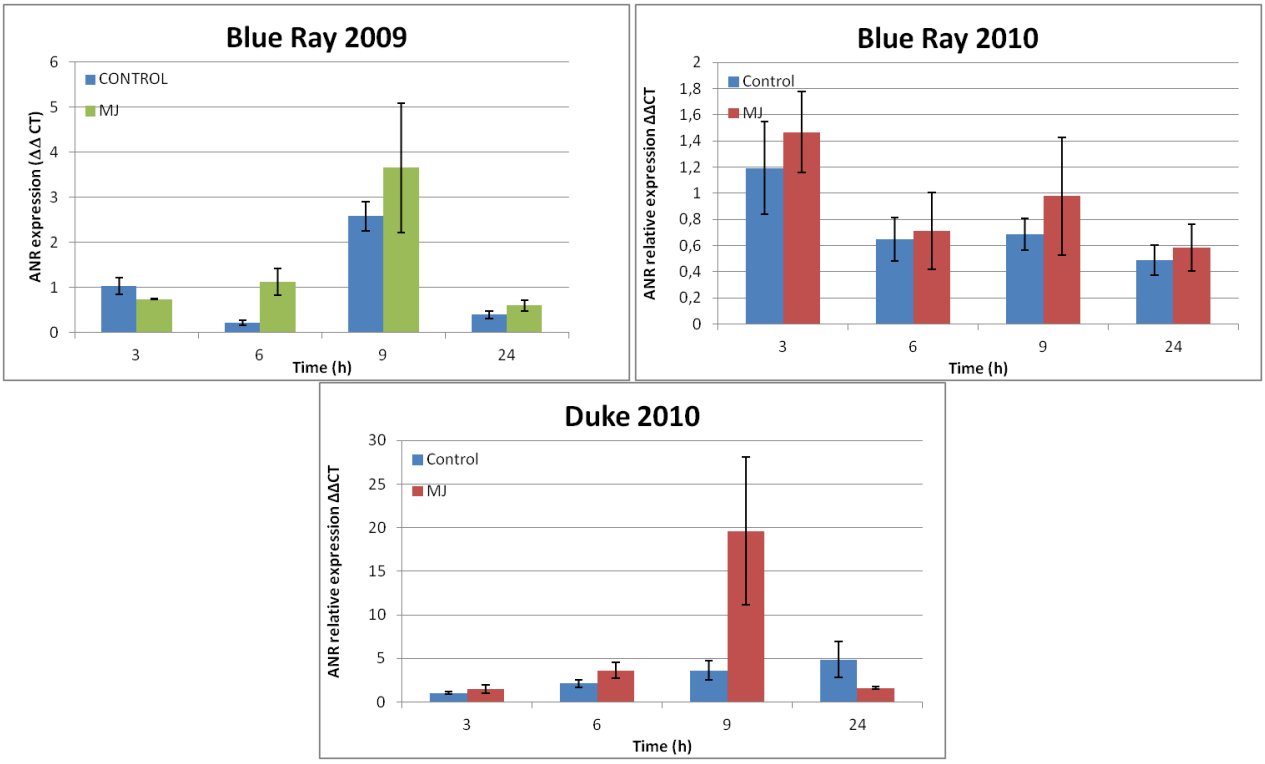


Fig. 23: ANR gene expression. Analyses were performed using qRT-PCR at different time course (0-24 h) in control (0.05% Tween-20) or 0.1 mM MeJa treatment. Data are means with standard deviations of ΔΔCt between genes and GAPDH as internal control. Reactions were repeated four times using two biological samples.

In this study we also evaluated the hypothesis that MeJa might stimulate the branch of the phenylpropanoid pathway which leads to the biosynthesis lignin. Lignin biosynthesis has received growing attention in the cell wall field because lignin is a limiting factor in a number of agro-industrial processes. Cinnamoyl-CoA reductase (CCR) is the entry point for the lignin-specific branch of the phenylpropanoid pathway and is considered to be a key enzyme controlling the quantity and quality of lignins (Tamasloukht et al., 2011). From the preliminary evaluation that we conducted on this study it is possible to observe that MeJa stimulated this branch of the pathway (Fig. 24). In fact we registered a great increment in *CCR* transcripts especially in the experiment conducted in 2010 on 'Blue Ray' when treated berries showed a 400-fold increment of transcripts 3 hours after the treatment. In the same experiment 'Duke' did not show any relevant change in *CCR* expression. In the experiment conducted in 2009 the expression of this gene in 'Blue Ray' was affected by treatment but with a less marked response and at different time points, showing the higher accumulation of transcripts 9 hours after MeJa application.

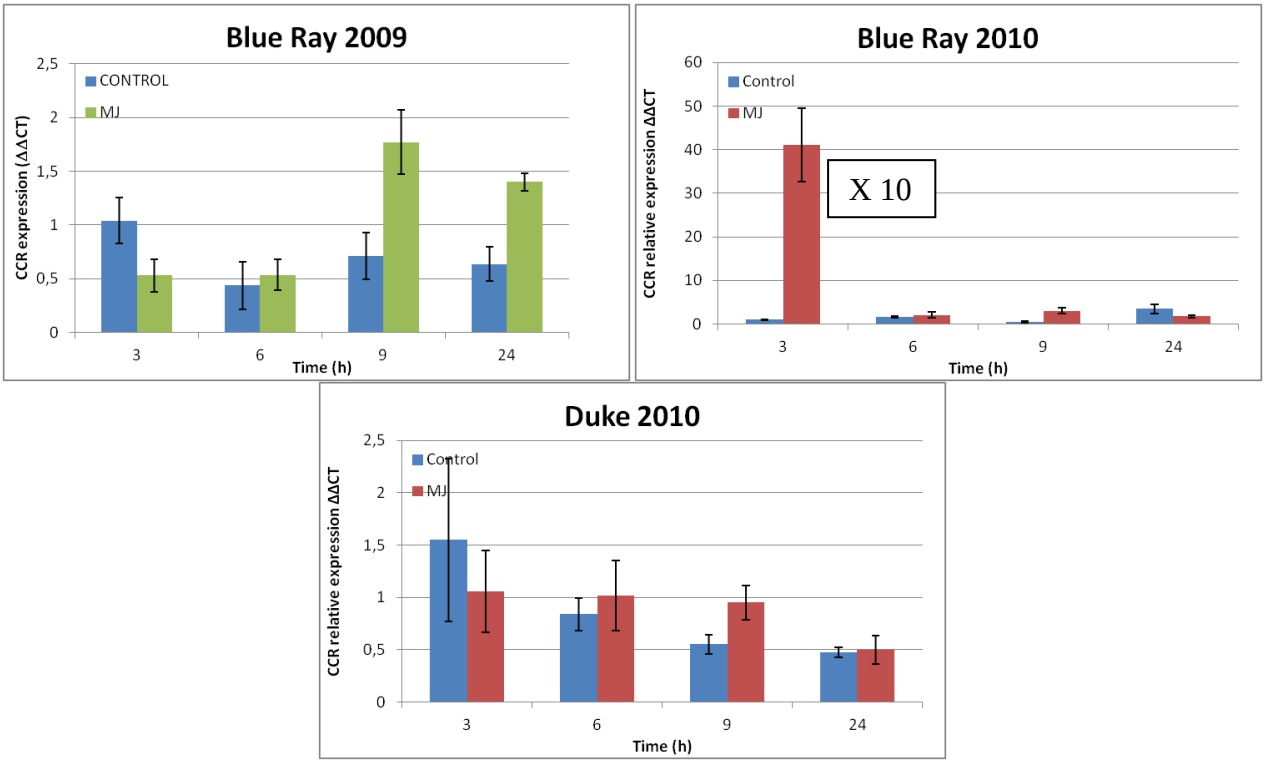


Fig. 24: CCR gene expression. Analyses were performed using qRT-PCR at different time course (0-24 h) in control (0.05% Tween-20) or 0.1 mM MeJa treatment. Data are means with standard deviations of ΔΔCt between genes and GAPDH as internal control. Reactions were repeated four times using two biological samples.

5.3.2. Analysis of Phenolic Acids, Flavonols, Flavan-3-ols, Gallic Acid Esters and Stilbenes

In this study we measured the effects of MeJa on the phenolic profile in two blueberry cultivars by measuring changes in levels of different classes of phenolic compounds 3 days (Tab. 6) and one week (Tab. 7) after the treatment. The data were collected during one growing season (2010). The method we used for extraction and HPLC separation allowed us to identify 20 compounds belonging to the classes of phenolic acids, flavan-3-ols, gallic acid esters, flavonols and stilbenes. The main part of those compounds was extracted and recovered in the ethyl acetate fraction.

Tab. 6: Contents of phenolic compounds in blueberries (cv. 'Duke' and 'Blue Ray') 3 days after treatment with 0.1 mM MeJa. Mean values from duplicate assays are expressed as catechin equivalents (µg/g of fresh weight).

		DUKE				BLUE RAY			
	Ripening stage	**3**		**4**		**3**		**4**	
		Control	**MeJa**	**Control**	**MeJa**	**Control**	**MeJa**	**Control**	**MeJa**
Phenolic Acids	gallic acid	23	25	36	58	11	10	19	207
	vanillic acid	82	78	47	52	39	39	25	31
	syringic acid	25	15	18	21	33	41	17	30
	caffeic acid	34	51	44	52	75	65	62	65
	caftaric acid	26	20	21	21	10	8	5	6
	trans p-cumaric acid	28	11	24	38	47	47	9	16
	ferulic acid	51	17	49	56	23	34	29	15
	TOTAL	***269***	***217***	***238***	***297***	***237***	***244***	***165***	***369***
Flavonols	quercetin 3-o-glucoside	568	571	303	389	330	334	301	283
	kaempferol 3-o glucoside	64	71	43	44	72	72	66	37
	myricetin3-o glucoside	66	61	118	175	82	135	203	221
	TOTAL	***698***	***704***	***463***	***609***	***483***	***541***	***570***	***541***
Flavan-3-Ols (Catechins) (monomers and polymers)	catechin	29	20	17	18	18	9	6	6
	epicatechin	772	979	439	507	568	679	600	474
	gallocatechin	5	6	6	7	5	7	10	5
	catechin 3-o-gallate	70	44	53	40	119	71	92	60
	epicatechin 3-o-gallate	17	5	19	21	21	17	24	13
	proanthocyanidin B1	33	25	54	76	27	30	32	49
	proanthocyanidin B2	3764	3162	1899	1974	5770	6443	5366	4927
	TOTAL	***4691***	***4241***	***2487***	***2645***	***6528***	***7256***	***6131***	***5535***
Stilbenes	*trans*-resveratrol	63	59	53	62	78	89	81	52
	TOTAL	**63**	**59**	**53**	**62**	**78**	**89**	**81**	**52**
Gallic Acid Esters	ethyl gallate	35	24	35	36	33	33	14	17
	methyl gallate	5	6	5	7	6	8	5	5
	TOTAL	***40***	***30***	***40***	***43***	***39***	***41***	***19***	***22***

Tab. 7: Contents of phenolic compounds in blueberries (cv. 'Duke' and 'Blue Ray') one week after treatment with 0.1 mM MeJa. Mean values from duplicate assays are expressed as catechin equivalents (µg/g of fresh weight).

		DUKE				BLUE RAY			
	Ripening stage	**3**		**4**		**3**		**4**	
		Control	**MeJa**	**Control**	**MeJa**	**Control**	**MeJa**	**Control**	**MeJa**
Phenolic Acids	gallic acid	14	12	31	32	9	15	23	20
	vanillic acid	84	86	80	61	49	44	40	33
	syringic acid	13	20	19	12	6	51	30	35
	caffeic acid	33	32	67	46	91	91	24	33
	caftaric acid	18	23	25	20	9	12	1	7
	trans p-cumaric acid	10	23	19	11	68	62	28	32
	ferulic acid	64	53	62	45	83	54	29	22
	TOTAL	***236***	***250***	***303***	***227***	***315***	***331***	***175***	***180***
Flavonols	quercetin 3-o-glucoside	646	602	514	406	444	354	248	274
	kaempferol 3-o glucoside	60	73	41	34	83	141	64	48
	myricetin3-o glucoside	84	70	173	165	179	160	218	260
	TOTAL	***790***	***745***	***728***	***605***	***706***	***655***	***530***	***583***
Flavan-3-Ols (Catechins) (monomers and polymers)	catechin	9	19	16	9	13	16	11	6
	epicatechin	499	705	445	353	933	1065	589	714
	gallocatechin	36	7	17	6	6	7	9	5
	catechin 3-o-gallate	67	64	51	13	109	76	59	54
	epicatechin 3-o-gallate	9	21	30	23	31	26	13	10
	proanthocyanidin B1	21	22	41	49	41	48	46	65
	proanthocyanidin B2	4232	3472	3505	2405	7612	6575	4688	5162
	TOTAL	***4873***	***4310***	***4106***	***2858***	***8746***	***7812***	***5415***	***6016***
Stilbenes	*trans*-resveratrol	64	63	62	52	104	97	79	59
	TOTAL	**64**	**63**	**62**	**52**	**104**	**97**	**79**	**59**
Gallic Acid Esters	ethyl gallate	29	34	20	15	28	26	10	10
	methyl gallate	4	5	4	6	8	10	8	6
	TOTAL	***32***	***39***	***24***	***21***	***37***	***36***	***18***	***17***

Seven phenolic acids were identified in this work: gallic, vanillic and syringic, belonging to the subclass of hydroxybenzoic acid derivates (HBA); caffeic, caftaric, trans-*p*-cumaric and ferulic from the subclass of hydroxycinnamic acids derivates (HCA). Seventeen phenolic acids were identified in berry fruits in a previous study (Zadernowski et al., 2005) while only *p*-cumaric, ferulic and caffeic were identified in a study conducted on 18 different berry species (Määttä-Riihinen et al., 2004). The type of phenolic acids as well as the levels, that we measured, are comparable with those reported in previous studies. Treatment with MeJa affected the amount of phenolic acids in a different way depending on the cultivar, on the ripening stage and on the time elapsed from treatment. Three days after treatment the total amount of phenolic acids increased in all the conditions with the only exception of stage 3 of 'Duke', in which values decreased by 19% compared with control. In 'Blue Ray' a much relevant positive effect was observed in berries from

stage 4 where levels were more than double compared with control, this was mainly due to a 10-fold increment in levels of gallic acid. One week after the treatment no relevant differences between treated and control samples were registered, the only exception is represented by stage 4 of 'Duke' in which levels decreased by around 25%. PAL is the enzyme which mediates the formation of cinnamic acid from phenylalanine, this represents a pivotal branch point of primary and secondary metabolism and is the first and most important regulatory step in the formation of many phenolic acids (Mandal et al., 2010). An increase in levels of phenolic acids could then be correlated to the higher transcripts accumulation of *PAL* that we recorded in this experiment. This hypothesis is supported by other similar results found in literature, for example methyl jasmonate dramatically enhanced phenolic acids accumulation in *Salvia miltiorrhiza*. Meantime, several phenolic acid biosynthetic gene transcripts (such as *PAL*) were coordinately induced (Xiao et al., 2009).

In our study the class of flavonols was mainly represented by quercetin-3-o-glucoside, followed by kaempferol-3-o-glucoside and myricetin-3-o-glucoside. Quercetin resulted to be the most prevalent flavonol in blueberry in previous studies (Määttä-Riihinen et al., 2004) while in the same study kaempferol was not detected. The total amount of flavonols increased 3 days after MeJa treatment only in 'Duke' at the latest ripening stage. Among the compounds in this class myricetin-3-o-glucoside appeared to be the more sensitive to treatment and showed the highest increases. One week after the treatment only slight changes in flavonols levels were detected. *CHS* and *F3H* were reported to be among the genes necessary to the biosynthesis of flavonols (Pollastri and Tattini, 2011). From our findings the correlation between the expression of those genes and flavonols accumulation is not yet clear, *CHS* resulted strongly affected by the treatment with an increase in transcripts, while *F3H* appeared to be repressed by MeJa 9 and 6 hours after treatment in 'Blue Ray' and 'Duke' respectively, but flavonols levels after three days and one week resulted generally quite stable or showed slight changes as mentioned above.

Flavan-3-ols (catechins) resulted the most abundant class among the phenolic compounds that we identified. Epicatechin and proanthocyanidin B2 were the most prevalent compounds among monomers and polimers respectively. High content of epicatechin is a typical feature in berries from *Vaccinum* species, as reported by Määttä-Riihinen and colleagues (2004). Also in a Dutch study epicatechin dominated among flavan-3-ols in plant-derived foodstuffs (Arts et al., 2000). In general the treatment with MeJa determined only little changes in catechins accumulation. The most significant change induced by treatment was observed in 'Duke' at the last ripening stage

one week after treatment, with a decrement (30%) in levels. Three days after treatment proanthocyanidin B2 resulted quite stable without showing great changes due to MeJa, on the other hand epicatechin showed a positive response to treatment with slight increments. Only in 'Blue Ray' at stage 4 we registered a decrement in epicatechin accumulation following the treatment. One week after treatment proanthocyanidin B2 showed a reduction in 'Duke' at both ripening stages while epicatechin showed a general positive effect due to the treatment with the highest increment in 'Duke' at the third ripening stage. Catechins derive from leucocyanidin and anthocyanidins which accumulate through the reaction catalyzed by the enzymes DFR and ANS and are converted to flavan-3-ols by the reactions catalized by ANR and LAR. Positive correlation between catechin contents and gene expression suggested also a possible role of enzymes like PAL and C4H in catechins biosynthesis and a crosstalk between phenylpropanoid and flavonoid pathways (Singh et al., 2009). In this study an increment in transcripts of *ANR* and *DFR* was stimulated by MeJa, this could in part correlate with the changes in accumulation of catechins that we observed.

In our samples the class of stilbenes was only represented by *trans*-resveratrol. This compound has been widely recognized as a high-valuable health-promoting component in berry fruits. The levels we found in our samples ranged between 60 and 100 µg/g FW and resulted higher compared with whose reported in other studies (Može et al., 2011; Wang et al., 2010) which were between 2 and 4 µg/g FW. Numerous factors, such as varietals and regional differences, the degree maturity at harvest, as well as the analytical procedure used for extraction and quantification of phenolics might contribute to these differences as referred by Zadernowski and colleagues in their study (2005). In our study the treatment with MeJa affected very weakly the content of *trans*-resveratrol. Another study evaluated the effectiveness of allyl isothiocyanate as a potential elicitor able to enhance phenolic content in blueberry, but this did not affect the content of *trans*-resveratrol in 'Duke' blueberry (Wang et al., 2010).

Esters of gallic acid represent minor compounds and, in our study, resulted to be present at low concentrations in blueberry. We found two compounds belonging to this class, ethyl gallate and methyl gallate, the former has been reported to be involved in the amelioration of oxidative damage in *in vitro* models (Kaur et al., 2011), the latter was identified as the active constituent of *Pistacia integerrima* for mediating its anti-inflammatory activity (Mehla et al., 2010). We did not observe any relevant effects of MeJa treatments on the accumulation of those compounds.

5.4 Conclusions

In this work we evaluated the effects of treatments with methyl jasmonate (0.1 mM) on the phenolic profile as well as on the gene expression of the key genes in the phenylpropanoyd pathway in two cultivars of blueberry (*Vaccinium corymbosum*).

Quantitative RT-PCR analyses revealed that MeJa strongly enhanced the expression of *PAL*, *CHS* and *ANS* in both the cultivars with a transient increment in transcripts levels 9 hours after the treatment. The treatment appeared to be effective also in stimulating the expression of *DFR* and *ANR* at the same time point.

Interestingly we found a strong effect of MeJa on the expression of *CCR,* the first enzyme in the branch of the pathway which leads to the accumulation of lignins. This data could reveal a competitive relation between different branches of the pathway and, in the future, could be useful in manipulating the pathway toward the accumulation of desired products.

Metabolic profiling analyses performed with HPLC technique allowed us to identify 20 phenolic compounds belonging to the classes of phenolic acids, flavonols, flavan-3-ols (monomers and polymers), gallic acid esters and stilbenes. Among every class we individuated the most prevalent compounds in both cultivars of blueberry.

MeJa stimulated the accumulation of phenolic acids, and the highest levels were measured three days after the treatment. Flavonols levels were also higher in 'Duke' three days after treatment. Effects of MeJa were detected on the levels of flavan-3-ols, however different cultivars at different ripening stages were differently stimulated by the treatment. From our results blueberry resulted to be a valuable source of *trans*-resveratrol, but no effect of the treatment was detected on the accumulation of this compound belonging to the the class of stilbens. Data obtained from transcripts and metabolite analyses resulted to be well-paralleled and allowed us to make comparisons and observe good correlations in the so called gene-to-metabolite networks for secondary metabolism in blueberry (Rischer et al., 2006). However more detailed analyses, for example on the regulatory factors of this pathway remains to be performed as an interesting challenge for future analyses.

6. Long-term Cold Storage of Highbush Blueberry (*Vaccinium corymbosum*, L.)

6.1. Introduction

Blueberry (*Vaccinium corymbosum*, L.) is a fruit crop rich in antioxidants and vitamins (such as phenolic compounds and ascorbic acid). The quality of this fruit and the content of health-promoting compounds are influenced by many factors, such as environmental conditions, genetic diversity and degree of maturity at harvest (Connor et al. 2002, Ehlenfeld and Prior, 2002). Blueberry was often reported as an high perishable produce, thus its commercial value could be strongly affected by storage conditions. In postharvest, quality and product losses are mainly due to dehydration, weight loss, shrivel and fungal spoilage. Shelf life extension can be achieved by the use of cold storage (0-1°C) and controlled atmosphere (CA) with low oxygen (1-4- kPa O_2) and high carbon dioxide (9-12 kPa CO_2) concentrations. The effects of storage were reported to be different between different cultivars (Connor et al., 2002). The content in anthocyanins increased during the first 2-4 weeks of storage, and the levels were higher in fruit stored under controlled atmosphere with low O_2 and high CO_2 levels (Krupa and Tomala, 2007). Higher titratable acidity values were reported in blueberries stored under CA, compared with controls stored in natural atmosphere (Chiabrado and Giacalone, 2011), moreover differences in the levels of total phenolics and in antioxidant activities were observed among different rabbiteye blueberry cultivars during the first days of storage in CA and in the same study fungal development was minimized by CA storage (Schotsmas et al., 2007). Little is known so far about the effects of long-term storage on blueberry. In this study we investigated the effects of two different conditions of controlled atmosphere on two late season blueberry cultivars (‘Legacy’ and ‘Brigitta’) by measuring quality parameters during long-term storage. We measured titratable acidity, soluble solids content, water loss as well as changes in ascorbic acid level, total phenolics, total flavonoids and total anthocyanins. We individuated the most prevalent classes of anthocyanins in blueberry and reported the changes in respective levels, measured by HPLC. Moreover, we measured the levels of malondyaldeide (MDA) expressed as thiobarbituric acid reacting species (TBARS) as a biochemical index of lipid peroxidation during storage.

6.2. Material and methods

6.2.1. Fruit material

Two late season blueberry cultivars, 'Brigitta' and 'Legacy' were used in this experiment. The berries were harvested at full maturity (100% of the surface dark blue colored) from an experimental field in Berbenno (SO), Italy, at 650 mt. altitude.

6.2.2. Storage conditions

Blueberries were transported to the laboratory, sorted into 200 g punnets and stored at 0°C and 90% R.H., either in natural atmosphere (control) or in two different controlled atmospheres:

CA1 and CA3: 10 kPa CO_2, 4 kPa O_2

CA2 and CA4: 9 kPa CO_2, 1 kPa O_2

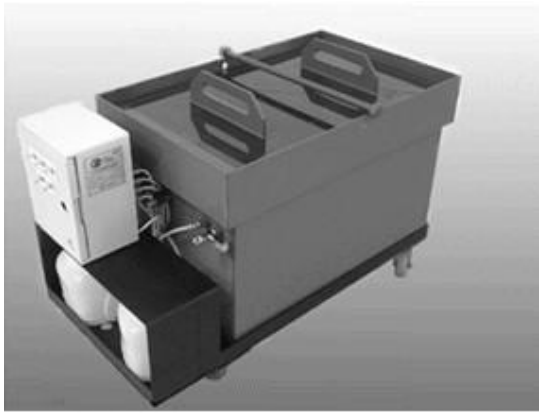


Fig. 25: Cabinet used for the CA storage experiments.

Both of the controlled atmosphere conditions were replicated in double in two different cabinets (Fig.25).

Gas concentrations were monitored and managed through a GAC 5000 device with a gas analyzer (Fruit Control Equipments s.r.l., Locate Triulzi, Italy). Different gas mixtures were generated by mixing flows of air, CO_2 and O_2-free N_2.

Analyses were performed immediately after harvest as well as at nine storage time points along storage, for both of the cultivars and for all the different storage conditions (Tab.8).

Tab. 8: Time points for the two different cultivars.

Time point	'Legacy ' (days of storage)	'Brigitta ' (days of storage)
T1	14	12
T2	28	33
T3	42	47
T4	56	61
T5	71	76
T6	89	94
T7	105	110
T8	127	132
T9	142	147

For each time point a visual evaluation was made, then the weight loss, the percentage of damaged berries, and the percentage of dry matter of fresh berries were measured. Other berries from the same sampling were stored at -80°C for successive analyses. Analyses of weight loss and decay were performed on samples from both replicates for each conditions (CA1 CA2 CA3 CA4) while other analyses were made on samples from CA1 and CA2.

6.2.3. Weight loss

Punnets were weighed at the beginning of the storage and at each time point, the result were expressed as percentage.

6.2.4. Decay

Berries which showed shrivel or fungal spoilage were considered damaged and eliminated. The amount of damaged berries was expressed as percentage on the total in each punnet.

6.2.5. Dry matter

Dry matter content was measured by weighting the berries before and after completely drying in oven at 60 °C and the result was expressed as percentage of dry matter.

6.2.6. Total soluble solids (TSS) determination

Total soluble solids, expressed as °Brix, were determined by a hand refractometer (Atago mod., N1, Tokyo, Japan) on juice obtained from squeezing 5 g of berries.

6.2.7. Total titratable acidity (TTA) determination

The total titratable acidity (TTA) was determined on 5 g of berries following homogenization with an equal weight of water for 5 min. The homogenate was titrated to pH 8.3 with 0.1 N NaOH with a Crison automatic titrator (Crison Instruments SpA, Carpi (Modena)), and TTA was calculated and expressed as milliequivalents per 100 grams of fresh weight.

6.2.8. Determination of total phenols, and total flavonoids

For spectrophotometric analysis, 5 g of homogenized berries were extracted with 25 or 50 mL of acidified methanol (1% HCl), by mixing for one hour, then centrifuged at 10,000×*g* for 10 min at 15°C. Total phenolic content was determined according to the Folin-Ciocalteau method

(Waterhouse, 2005). One milliliter of Folin-Ciocalteau reagent, 5 mL of distilled water and 2 mL of 20% Na_2CO_3 were added to 0,1mL of extract in a 20 mL volumetric flask and immediately diluted to the final volume with distilled water. The optical density, after 90 minutes, was measured at 700 nm on a UV–Vis spectrophotometer (Jasco model 7800, Tokyo, Japan). Results were expressed as milligrams of gallic acid per gram of dry matter. Total flavonoids were evaluated spectrophotometrically at 280 nm. A catechin standard curve was set and results were reported as milligrams of catechin per gram of dry matter (Iriti et al., 2005).

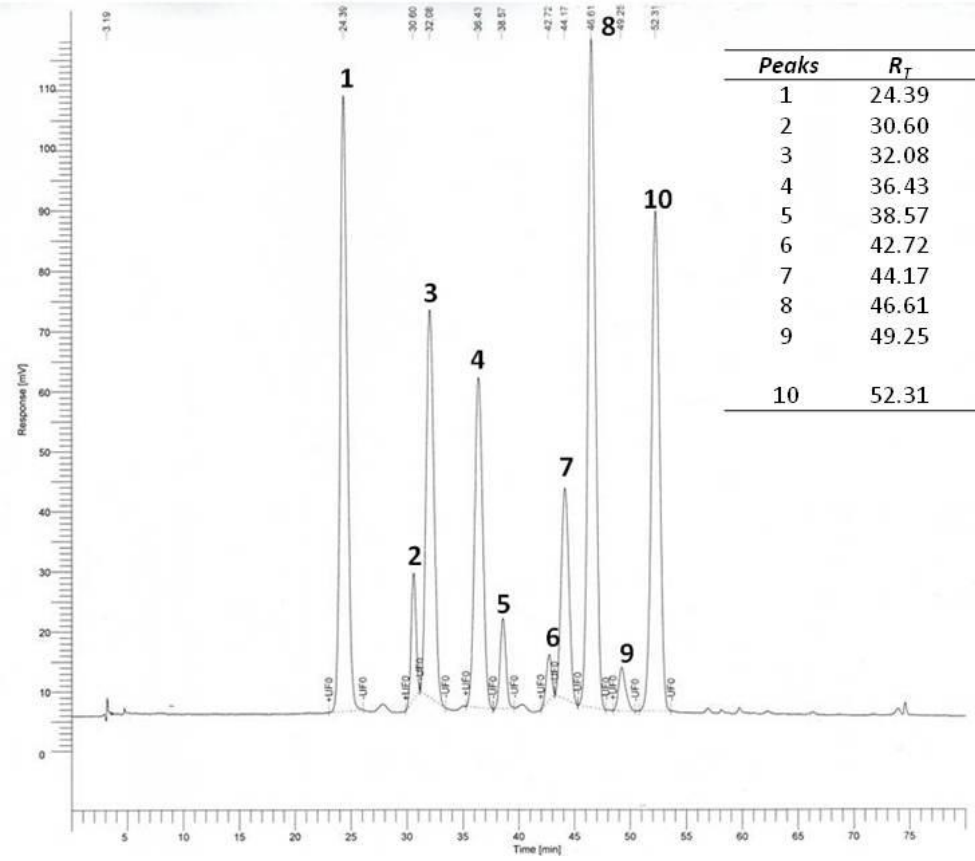
6.2.9. Anthocyanins extraction and determination

Frozen samples (20 g) were grounded in a Waring Blender and extracted two times in a acetone/water solution (70:30). Each sample was evaporated in a vacuum centrifuge at 20°C for 90 minutes and resuspended in 2% methanol and 5% formic acid.

Anthocyanins were identified by LC-DAD-MS and HPLC analyses. Pigments were separated on a Symmetry column (5 µm; 250x4.6 mm), Waters. Anthocyanins (20 µL injected) were eluted with a gradient of 2% HCOOH (mobile phase A) and CH_3CN:CH_3OH:H_2O:HCOOH (20:20:58:2) (mobile phase B). The applied gradient was:

Time (min)	%A	%B
0	80	20
30	65	35
40	55	45
50	45	55
60	35	65
70	20	80
80	10	90
83	80	20

The mass spectrometer was a Perkin Elmer series 200. Anthocyanins present in the extract were evaluated qualitatively by measuring the molecular weight $[M]^+$ and product ions obtained in MS. The structural studies were conducted using the software QuanOptimize. Anthocyanins content were quantified as cyaniding-3-glucoside and total concentration of each representative subclass compound was calculated from a calibration curve. Results are expressed as micrograms per gram of dry matter. Figure 26 shows a typical chromatogram from our samples and a list of compounds we individuated and the relative retention times.



Peaks	R_T	Anthocyanins
1	24.39	Delphinidin-3-galactoside
2	30.60	Cyanidin-3-galctoside
3	32.08	Delphinidin-3-arabinoside
4	36.43	Petunidin-3-galactoside
5	38.57	Cyanidin-3-arabinoside
6	42.72	Peonidin-3-galactoside
7	44.17	Petunidin-3-arabinoside
8	46.61	Malvidin-3-galactoside
9	49.25	Malvidin-3-glucoside + Peonidin-3-arabinoside
10	52.31	Malvidin-3-arabinoside

Fig. 26: Chromatogram of analytical HPLC of Highbush blueberry anthocyanins.

6.2.10. Determination of AsA content

For ascorbate analysis, 7.5 g of blueberries were homogenized in a mortar with 10 mL of cold 6% (w/v) metaphosphoric acid and centrifuged at 10,000 × *g* at 4 ◦C. The supernatant was transferred into a 25-mL volumetric flask at 4 ◦C. The pellet obtained by centrifugation was washed with 8 mL of cold metaphosphoric acid solution and centrifuged. The supernatants were combined and cold 6% metaphosphoric acid was added to a final volume of 25 mL. After filtration through 0.2 µm nylon filter, a 10 µL sample aliquot was injected onto an Inertsil ODS-3 (5 µm; 4.6mm×250mm) GL Science column at 20 ◦C attached to a Series 200 LC pump (PerkinElmer, Norwalk, CT, USA). The column was eluted with 0.02 M-orthophosphoric acid at a flowrate of 0.7 mL/min and ascorbic acid was monitored at 254 nm with a UV-975 intelligent UV–vis detector (Jasco model 7800, Tokyo, Japan). Peaks were converted to concentrations by using the dilution of stock ascorbic acid to construct a standard curve. Chromatographic data were stored and processed with a Perkin Elmer TotalChrom 6.3 data processor (PerkinElmer, Norwalk, CT, USA) (Sinelli et al., 2008).

6.2.11. TBARS (Thiobarbituric Acid Reactive Substances) determination

Five grams of pulp were used for the extraction. Samples were homogenized in 25 mL of 5% TCA then centrifuged at 4 ° C at a speed of 10,000 *x g* for 30 minutes. For the determination of TBARS the extract was added to a solution of 15% TCA and 0.5% TBA and added of water to a final volume of 3 mL. Samples were then mixed with a vortex and heated at 95°C for 15 minutes in a water bath, rapidly cooled and centrifuged at 4,000 *x g* for 15 minutes to precipitate solid residues. The samples were then analyzed in a spectrophotometer (Jasco model 7800, Tokyo, Japan) at three different wavelengths, 532, 600 and 440 nm. The absorbance at 532 nm was determined and this value was subtracted from the absorbance at 600 nm (as an index of non-specific turbidity). The value obtained was purged from the absorbance at 440 nm due to sucrose. The concentration of TBARS expressed as MDA equivalents (nmol / g FW), was calculated according to the (Du and Bramlage, 1992):

{[(A532-A600) - [(A440-A600) (8.4 / 147)]} / 157000] 106

8.4 = ε of sucrose at 532 nm
147 = ε of sucrose at 440 nm
157000 = ε of MDA at 532 nm

6.2.12. Statistical analysis

Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA). Significant differences were calculated by Duncan's mean test. Differences at $p \leq 0.05$ were considered as significant.

6.3. Results

6.3.1. Weight loss

Weight losses during fruit storage are mainly due to water evaporation. This phenomena are influenced by many factors such as the ratio between area and volume of fruit. Storage in controlled atmosphere can be effective in retarding the weight losses thanks to high relative humidity conditions applied. Table 9 shows the percentage of weight loss in 'Brigitta' stored in two conditions of controlled atmosphere and in the control. The most evident losses were registered in the control samples (C), which showed a weight loss of 42% after 147 days of storage. Samples stored in controlled atmosphere were characterized by a lower weight loss rate at all sampling times. At the end of storage the percentage of weight loss found in all the conditions of controlled

atmosphere was definitely lower compared with the one registered in samples stored in natural atmosphere. The samples stored in CA1 were characterized by a weight loss of about 4.3%, while those stored in CA2 had a lower weight loss at the end of storage (about 3 %).

Tab. 9: Weight loss (%) 'Brigitta'. Values represents means *±SD.*

Storage time (days)	CONTROL	CA1	CA3	CA2	CA4
0	0	0	0	0	0
12	**3,85** *±0,40*	**0,48** *±0,55*	**0,42** *±0,11*	**0,13** *±0,01*	**0,51** *±0,06*
33	**10,22** *±0,08*	**0,53** *±0,29*	**1,40** *±0,07*	**0,40** *±0,42*	**0,76** *±0,08*
47	**11,16** *±13,60*	**1,33** *±0,66*	**1,86** *±0,05*	**0,72** *±0,21*	**1,04** *±0,76*
61	**20,60** *±10,56*	**2,88** *±0,00*	**1,98** *±0,13*	**0,86** *±0,10*	**2,80** *±0,27*
76	**24,32** *±0,52*	**2,66** *±0,99*	**2,41** *±0,02*	**1,29** *±0,06*	**2,15** *±0,04*
94	**38,30** *±3,07*	**1,88** *±0,63*	**3,54** *±0,30*	**1,58** *±0,19*	**3,04** *±0,22*
110	**35,22** *±1,19*	**2,72** *±0,19*	**3,61** *±0,18*	**1,67** *±0,08*	**2,88** *±0,10*
132	**38,40** *±1,58*	**3,47** *±1,23*	**4,31** *±0,41*	**3,00** *±0,25*	**4,87** *±0,60*
147	**42,54** *±0,31*	**4,33** *±0,61*	**4,54** *±0,57*	**2,98** *±0,22*	**3,48** *±0,32*

With regard to the cultivar 'Legacy' samples stored at 0° C in natural atmosphere showed greater weight loss compared with blueberries stored under CA, as shown in Table 10.

The lower weight loss was observed in the condition CA2, (with losses of about of 4 %) while berries stored in other conditions showed higher losses.

Compared to 'Brigitta', 'Legacy' showed slightly higher weight losses, both in control (48,41% for 'Legacy' and 42,54% for 'Brigitta') and in controlled atmosphere (8,35% for 'Legacy' and 4,54% 'Brigitta').

Tab. 10: Weight loss (%) 'Legacy'. Values represents means *±SD.*

Storage time (days)	CONTROL	CA1	CA3	CA2	CA4
0	0	0	0	0	0
14	**4,63** *±0,02*	**0,47** *±0,01*	**0,5** *±0,02*	**0,38** *±0,09*	**0,54** *±0,15*
28	**9,87** *±0,37*	**1,01** *±0,17*	**1,49** *±0,15*	**1,05** *±0,08*	**0,84** *±0,11*
42	**14,32** *±0,32*	**1,22** *±0,07*	**2,04** *±0,24*	**1,82** *±0,28*	**3,29** *±0,45*
56	**19,08** *±0,74*	**1,4** *±0,53*	**2,99** *±0,76*	**1,97** *±0,58*	**4,57** *±2,27*
71	**22,77** *±2,14*	**2,09** *±0,11*	**2,7** *±0,40*	**2,22** *±0,08*	**3,75** *±0,64*
89	**29,63** *±0,76*	**2,16** *±0,88*	**3,94** *±0,33*	**2,18** *±0,10*	**6,22** *±1,68*
105	**30,37** *±7,83*	**2,87** *±1,37*	**4,76** *±0,07*	**2,49** *±0,52*	**7,63** *±1,36*
127	**42,96** *±0,20*	**4,68** *±2,35*	**6,59** *±0,08*	**4,06** *±0,75*	**8,14** *±0,81*
142	**48,41** *±1,96*	**4,56** *±1,68*	**6,22** *±0,49*	**3,71** *±0,25*	**8,35** *±0,67*

6.3.2. Decay

At each time point the punnets were visually inspected for the appearance of blemishes and fungal development. Decay was minimal up to 47 days. In all storage conditions, the percentage of damaged berries of the variety 'Brigitta' reaches 50% (estimated as the percentage over which the produce is not marketable anymore) at the 147th day of storage. Table 11 shows that during the first two months of storage (till 61 days) the number of damaged berries increased slowly, then a rapid deterioration of the quality was observed.

Tab. 11: Damaged berries (%) 'Brigitta'. Values represents means *±SD.*

Storage time (days)	CONTROL	CA1	CA3	CA2	CA4
0	0	0	0	0	0
33	**7,04** *±0,93*	**3,87** *±1,98*	**3,29** *±3,10*	**1,00** *±0,41*	**4,23** *±2,65*
47	**4,71** *±1,56*	**4,98** *±0,98*	**5,46** *±3,16*	-	**2,21** *±1,70*
61	**12,29** *±5,31*	**6,15** *±0,68*	**6,79** *±0,38*	**3,43** *±2,05*	**4,96** *±3,73*
76	**14,28** *±2,31*	**8,31** *±0,63*	**2,94** *±1,02*	**5,88** *±2,16*	**20,61** *±17,35*
94	**21,28** *±8,8*	**11,44** *±0,66*	**8,28** *±3,18*	**2,25** *±0,18*	**12,82** *±4,44*
110	**19,27** *±1,68*	**18,35** *±0,9*	**11,99** *±4,8*	**12,25** *±2,63*	**22,78** *±3,93*
132	**35,97** *±5,28*	**31,01** *±1,65*	**36,04** *±1,91*	**44,38** *±0,09*	**46,63** *±8,49*
147	**51,86** *±1,37*	**46,52** *±1,9*	**48,17** *±0,3*	**53,57** *±1,72*	**50,23** *±3,09*

Also with regard to the cultivar 'Legacy' (Tab. 12) the control reaches 50% of damaged berries after 142 days of storage, we observe a linear increase in decay incidence starting from the 71st day until the end of the experiment. At end of storage no significant differences were observed among samples stored in natural conditions (control) and in controlled atmosphere, with the only exception of the samples in CA4 condition where the degradation occurred quickly.

Tab.12: Damaged berries (%) 'Legacy'. Values represents means *±SD.*

Storage time (days)	CONTROL	CA1	CA3	CA2	CA4
0	0	0	0	0	0
14	**1,06** *±0,05*	**2,14** *±1,69*	-	-	**1,46** *±0,71*
28	**4,75** *±1,44*	**2,00** *±1,42*	**1,74** *±0,69*	**3,48** *±1,03*	**0,53** *±0,74*
42	**6,29** *±2,35*	**5,80** *±4,19*	**3,59** *±1,76*	**0,42** *±0,06*	**3,54** *±2,21*
56	**7,00** *±1,89*	**7,29** *±3,16*	**7,76** *±0,19*	**10,87** *±8,56*	**14,61** *±6,71*
71	**8,08** *±0,95*	**8,94** *±0,73*	**8,49** *±2,58*	**8,95** *±2,68*	**20,35** *±4,97*
89	**12,16** *±1,16*	**27,97** *±20,27*	**21,02** *±10,44*	**9,53** *±6,03*	**38,21** *±4,82*
105	**11,97** *±1,08*	**17,14** *±1,18*	**28,72** *±7,72*	**24,67** *±13,34*	**34,95** *±1,08*
127	**40,13** *±7,33*	**36,8** *±0,61*	**46,66** *±5,81*	**45,33** *±6,07*	**46,69** *±6,49*
142	**50,06** *±4,31*	**51,09** *±4,43*	**50,69** *±1,79*	**49,42** *±3,23*	**47,74** *±5,61*

6.3.3. Dry matter

The percentage of dry matter was generally lower in samples stored in controlled atmosphere in both cultivars. This result is in accordance with what showed from the analysis of weight losses.

In 'Brigitta' (Fig. 27) the values measured in the early days of storage were similar for all the conditions. Starting from 47th day, levels in the fruits stored in natural atmosphere increased from 12.5% to 14.7% and remained stable until the end of storage (14.5%).

At the same time point (at the end of storage) levels in CA1 and CA2 dropped reaching values around 11.5%, without substantial differences between the two different conditions of controlled atmosphere.

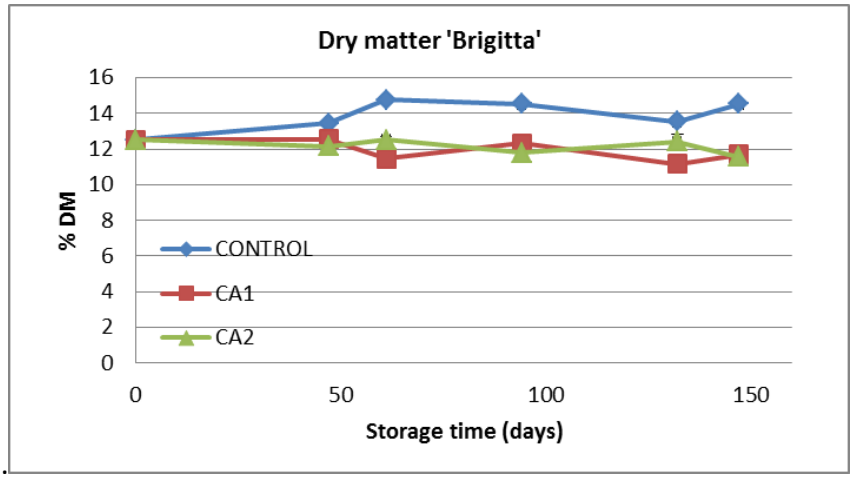


Fig. 27: Dry matter (%) 'Brigitta'. Values represent means ± SD (*n =3*)

A similar trend was observed in 'Legacy ' (Fig. 28), with only a slight decrease in the samples stored in CA1 and CA2. In fact, after 89 days of storage a decrease was observed and from an initial value of 14.6%, at the end of storage the values were around 13%. On the other hand fruits stored in natural atmosphere reached the maximum amount of dry matter at the end of storage (16.1%).

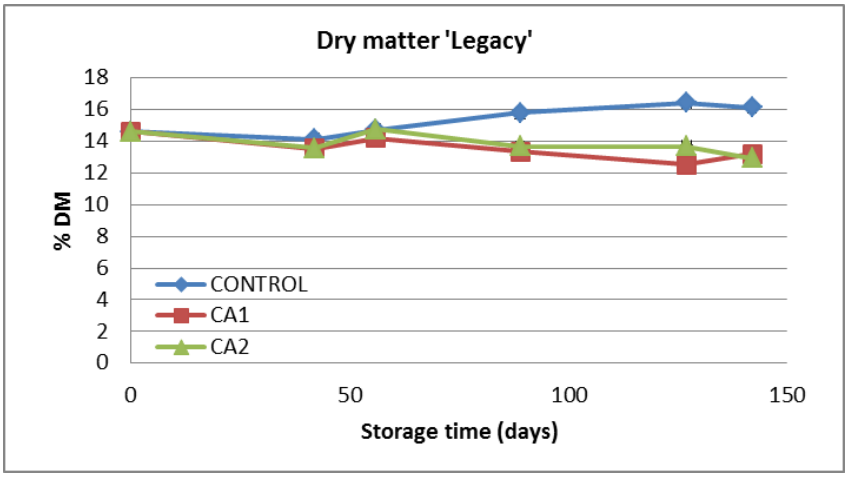


Fig. 28: Dry matter (%) 'Legacy'. Values represent means ± SD (*n =3*).

6.3.4. Total soluble solids (TSS) determination

At harvest, the soluble solid content, expressed as °Brix was similar among the two cultivars (11.1 in 'Brigitta' and 11.2 in 'Legacy'). In 'Brigitta' (Fig. 29) as well as 'Legacy' (Fig. 30), berries stored in natural conditions (control) showed levels of TSS higher than those measured in berries stored in CA, but different trends were observed comparing the two cultivars. In fact in 'Brigitta', values measured in control samples were generally higher than those in CA1 and CA2 for most of the storage period, with the only exception of samples analyzed after 132 days of storage in which the values among the three different conditions were comparable.

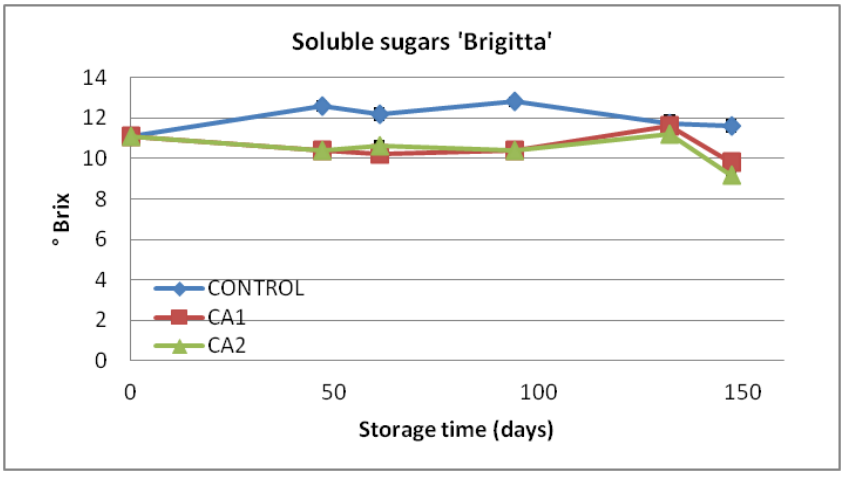


Fig. 29: Total soluble solids 'Brigitta'. Values represent means ± SD (*n =3*).

No substantial differences among different storage conditions were observed In 'Legacy' at the beginning of the trial (until 42 days). The control showed a stronger decrease after 56 days of storage compared with values registered in controlled atmosphere, but along the storage, the levels were constant until the end of the trial, and were comparable to those measured at harvest time. On the other hand, in CA1 and CA2 a gradual decline was observed and minimum values were reached at the end of storage. No substantial differences between different conditions of controlled atmosphere (CA1 and CA2) were observed.

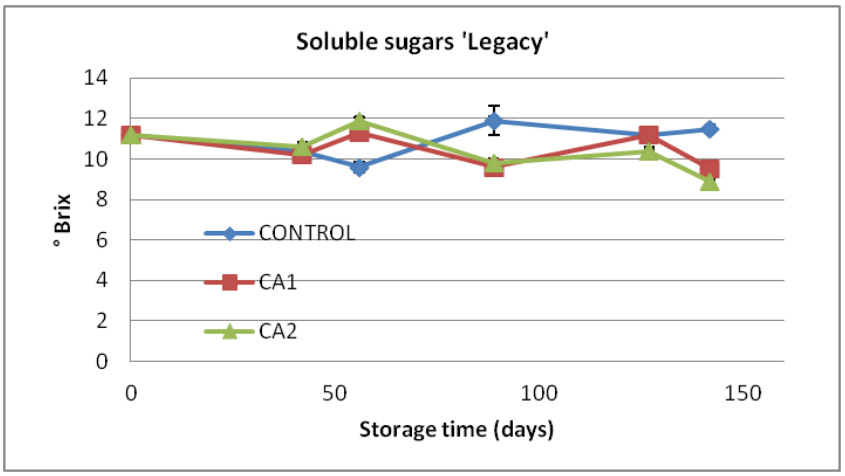


Fig. 30: Total soluble solids 'Legacy'. Values represent means ± SD (*n =3*).

6.3.5. Total titratable acidity (TTA)

The acidity values measured for the two cultivars throughout the storage period are shown in Figures 31 and 32. At the beginning of the storage 'Brigitta' showed of 11.6 meq/100 g fw. These values tended to increase along storage showing however an irregular trend, characterized by a slight initial drop, and followed by a decisive increase after 61 days. At the end of the experiment, berries stored in controlled atmosphere showed much higher values compared with those measured at harvest. The maximum values were reached in blueberries stored in natural atmosphere (control) after 132 days while at the end of storage values were comparable with those measured at harvest time.

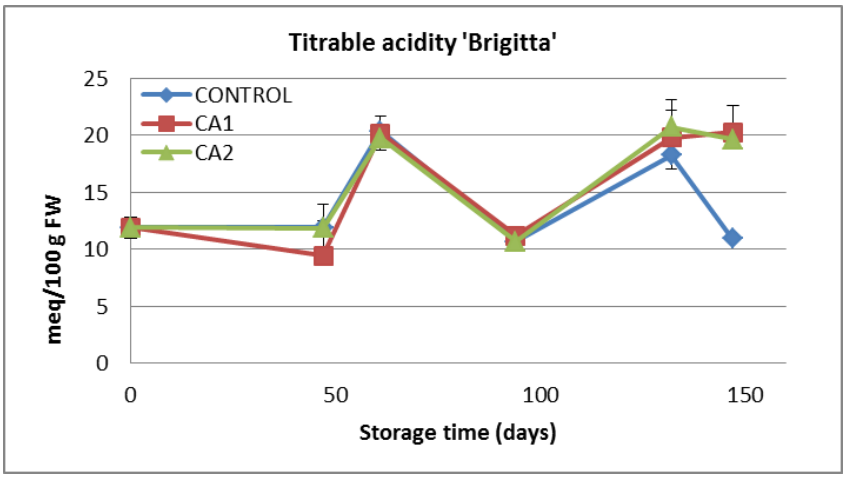


Fig. 31: Total titratable acidity 'Brigitta' (meq/100 g FW). Values represent means ± SD (*n =3*).

In 'Legacy' at harvest the values of acidity were about the half of those found in 'Brigitta' (5.6 meq/100 g FW). However at the end of storage, after 142 days the values were only slightly lower than those measured in 'Brigitta'. Also in 'Legacy' the gradual increase in acidity was characterized by an irregular pattern, as seen in 'Brigitta'. In general there were no significant differences between the three different storage conditions. The only exception was observed in samples stored in CA1 which showed a marked increase in acidity values after 127 days, followed by a decline recorded at the succesive sampling in which values were similar with those measured in control and CA2.

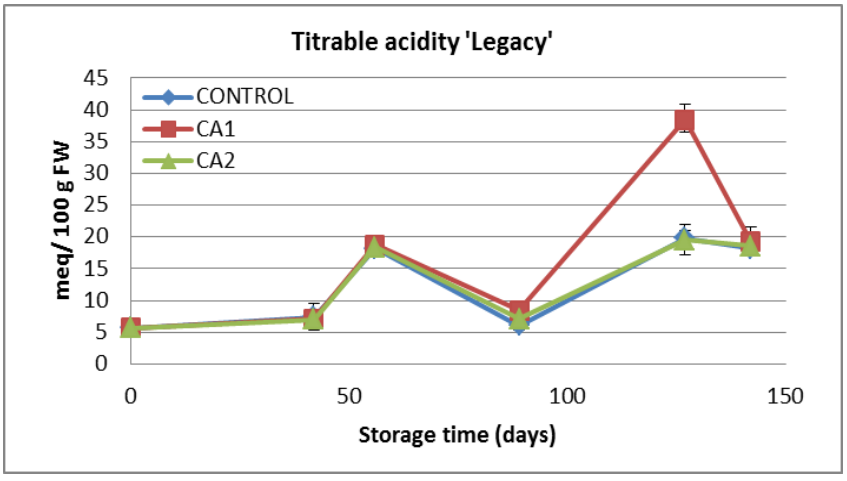


Fig. 32: Total titratable acidity 'Legacy' (meq/100 g FW).Values represent means ± SD (*n =3*).

6.3.6. Total polyphenols

The levels of total polyphenols at harvest were found to be significantly higher in 'Legacy' (21,06 mg gallic acid/g DW) compared with 'Brigitta' (12,98 mg gallic acid/g DW). The changes in polyphenol content were different among the two cultivars studied. In 'Brigitta' (Fig. 33), after 47 days of conservation, there were slight differences and the higher levels were measured in

samples stored in controlled atmosphere (CA1 and CA2). Then there was no evidence of marked differences among the three different conditions of storage that we applied. At the last two samplings, after 132 and 147 days of storage, berries in natural atmosphere (control) showed higher levels, reaching a maximum (19.52 mg gallic acid/g DW) after 132 days of storage.

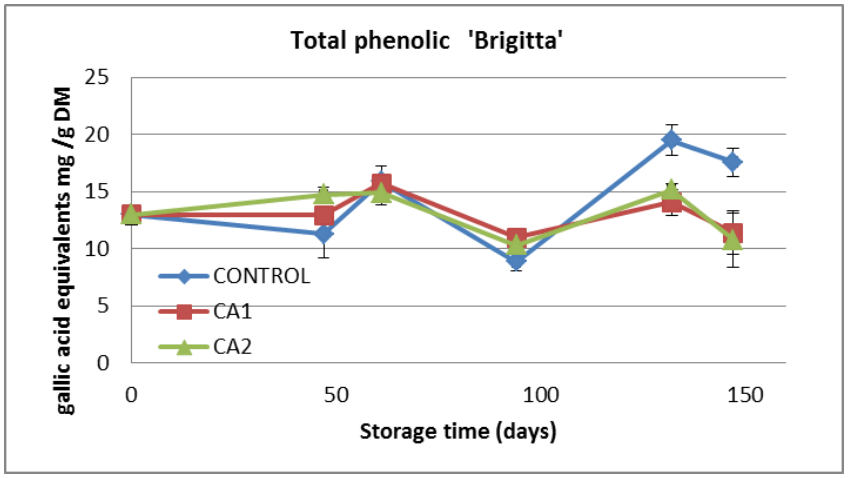


Fig. 33: Total phenolics 'Brigitta'. Values represent means ± SD (*n =4*).

In 'Legacy' the content of total polyphenols underwent significant changes throughout the storage period (Fig. 34). After 42 days the highest levels were reached in samples stored in CA2 storage condition. However, these values, decreased dramatically after 56 days and remained stable around 15 mg of gallic acid / g DW up to 127 days of storage, showing only a slight increase at the end of the experiment (18.52 mg gallic acid/g DW). However, fruit stored in CA1 and control showed an opposite behaviour, with a strong increase of levels after 56 days. In subsequent sampling the samples stored in natural atmosphere showed only a gradual decline followed by a slight increase at the end of storage. Berries stored in CA1 showed a decrement after 89 days, followed by a peak (25.4 mg gallic acid / g DW) recorded at 127 days of storage. At the end of the experiment the values were similar to those measured after 42 and 89 days (16.3 mg gallic acid/g DW) and were comparable with those found in CA2.

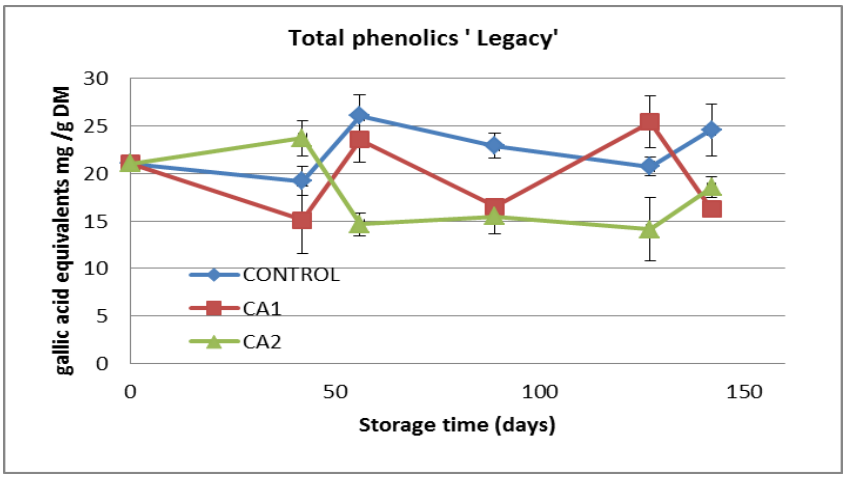


Fig. 34: Total phenolics 'Legacy'. Values represent means ± SD (*n = 4*).

6.3.7. Total flavonoids

At harvest time 'Legacy' (Fig. 36) was found to be a richer source of flavonoids than 'Brigitta' (Fig. 35), with values of about 18 mg catechin/g DW compared with 11 mg catechin/g DW measured in 'Brigitta'.

In 'Brigitta' after 47 days of storage the highest values were measured in berries stored in CA2, then these values decreased reaching a minimum after 94 days (7.79 mg catechin/g DW). Blueberries stored in CA1 maintained a stable trend, with contents around 12 mg catechin/g DW, showing a decrease only at the latest stages of storage, after 132 days. At the end of storage, after 147 days the content in the fruits stored in CA1 and CA2 was similar (about 9 mg catechin/g DW). 'Brigitta' stored under natural atmosphere (control) accumulated the highest levels of flavonoids at the end of storage (16.6 mg catechin/g DW) showing, however, an irregular trend over the considered storage period.

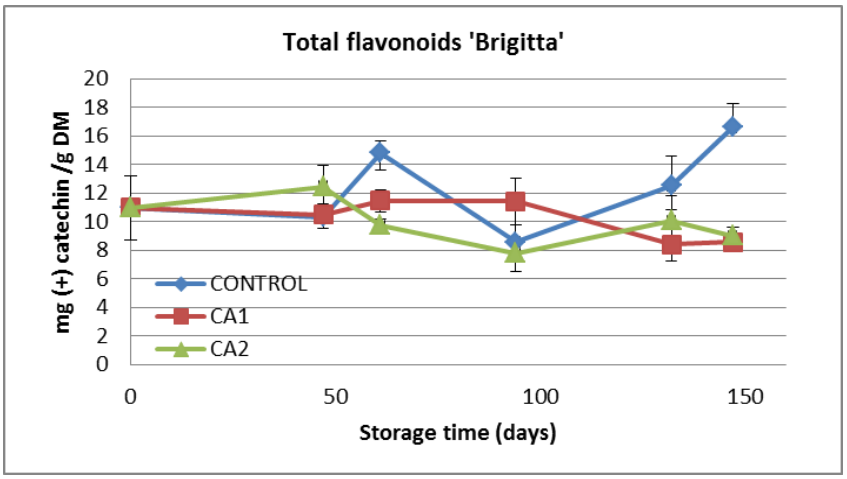


Fig. 35: Total flavonoids 'Brigitta'. Values represent means ± SD (*n = 3*).

In 'Legacy' only minor differences between different treatments were observed. Berries stored in CA2 showed the highest levels. However, a peak in the accumulation of flavonoids (29.14 mg catechin/g DW) was measured at the end of storage (142 days) in fruits stored in natural atmosphere.

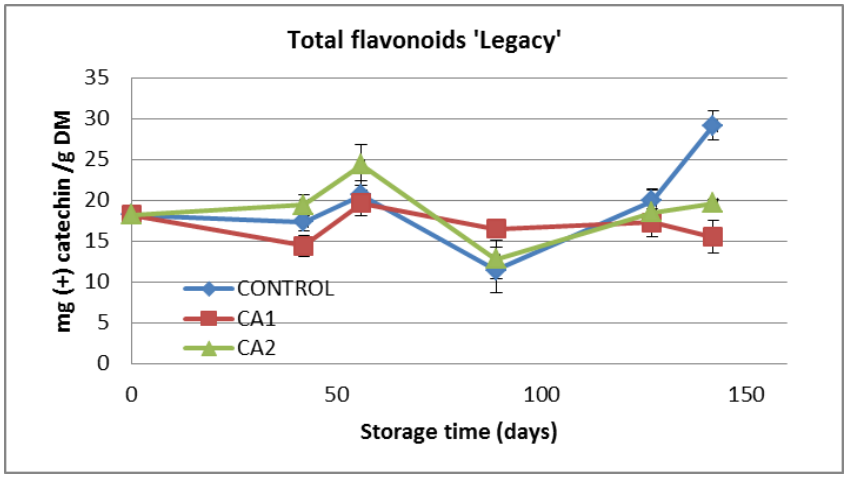


Fig. 36: Total flavonoids 'Legacy'. Values represent means ± SD (*n = 3*).

6.3.8. Total anthocyanins

The anthocyanin content is reported as sum of all the singular peaks individuated in the HPLC chromatograms.

At harvest the content was more than 1,5 times higher in 'Legacy' compared with 'Brigitta'. The changes that we observed in anthocyanin levels along storage were quite different, revealing differences among the two cultivars.

During the first days of storage in 'Brigitta' (Fig. 37) there was a general increase in the anthocyanin concentration. In the initial period of storage the highest levels were detected in samples stored in CA2 condition. From the 61th day of storage the values slightly decreased in samples stored in CA while remain quite stable in those stored in natural atmosphere.

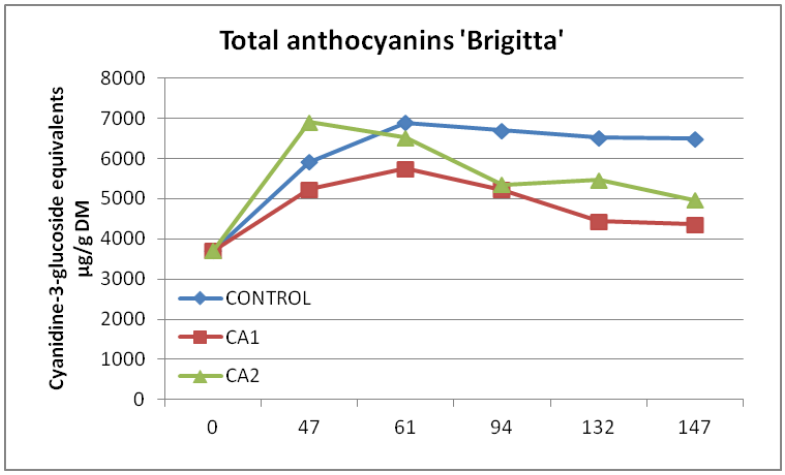


Fig. 37: Total Anthocyanins 'Brigitta'. Values are the sum of particular anthocyanins identified by HPLC expressed as Cyanidin-3-galctoside equivalents (µg/g DM).

During the first two months of storage there was an evident positive effect of the controlled atmosphere on the anthocyanin content on berries from the cultivar 'Legacy' (Fig. 38). In fact while the samples stored in natural atmosphere showed a rapid decrement in the content, berries stored in CA1 and CA2 conditions showed a drastic increment during the first days of storage. From that time point until the end of the trial levels decreased in all the different CA conditions, reaching a stable level similar to the one measured at harvest for the samples stored in controlled atmosphere and slower for those conserved in natural atmosphere.

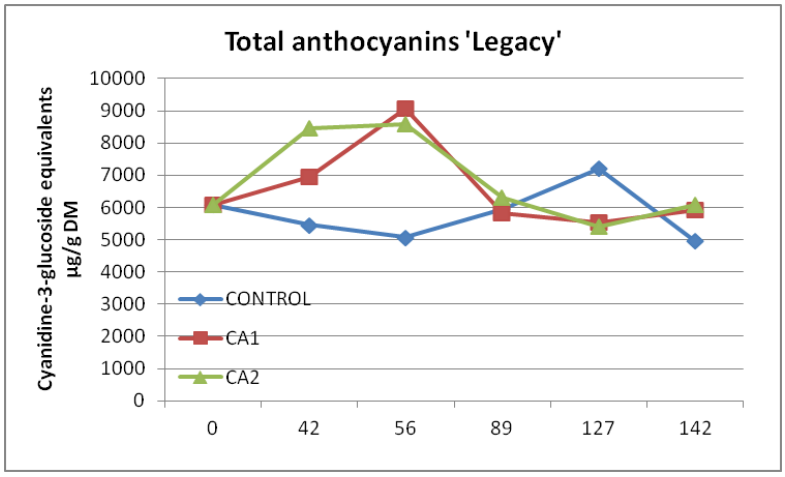


Fig. 38: Total Anthocyanins 'Legacy'. Values are the sum of particular anthocyanins identified by HPLC expressed as Cyanidin-3-galctoside equivalents (µg/g DM).

6.3.9. Anthocyanin profiling

In this experiment we detected 10 different anthocyanins from blueberry by separation of pigments using HPLC technique. The compounds we found and their prevalence expressed as percentage are resumed in table 13.

Tab. 83: Anthocyanins found in Blueberry.
Percentage indicates the prevalence of each molecule among the total amount.

ANTHOCYANIN	BRIGITTA %	LEGACY%
Delphinidin-3-galactoside	17	15
Cyanidin-3-galctoside	3	4
Delphinidin-3-arabinoside	11	7
Petunidin-3-galactoside	13	13
Cyanidin-3-arabinoside	2	2
Peonidin-3-galactoside	1	2
Petunidin-3-arabinoside	7	5
Malvidin-3-galactoside	28	34
Malvidin-3-glucoside + Peonidin-3-arabinoside	1	2
Malvidin-3-arabinoside	16	16

Malvidin-3-galactoside, Delphinidin-3-galactoside Malvidin-3-arabinoside, Petunidin-3-galactoside, Delphinidin-3-arabinoside resulted as the most prevalent anthocyanins found in both of the cultivar we analyzed in this work.

The storage conditions similarly affected the quantitative changes of particular anthocyanins and total anthocyanins.

Considering the changes in the anthocyanin profile during storage, a general increment was observed after about 2 months of storage (61 days for 'Brigitta' and 56 days for 'Legacy') for all the different conditions applied and then at the successive dates of analysis there was a general decrease of pigments content in berries. However differences were observed between controlled atmosphere and common cold storage. In general, comparing the two different conditions of controlled atmosphere, the highest levels of the prevalent pigments were registered in berries stored in CA2 condition (9 kPa CO_2 and 1 kPa O_2). In 'Brigitta' Malvidin-3-arabinoside (Tab.15) and secondly Delphinidin-3-arabinoside (Tab.14) showed high levels in berries stored under natural atmosphere, especially at the end of storage. On the other hand Delphinidin-3-galactoside (Tab. 14) and Petunidin-3-galactoside (Tab. 14) appeared generally to be more abundant in berries stored under one of the controlled atmosphere condition compared with berries under natural cold storage.

Tab. 9: Content of particular anthocyanins (Delphinidin-3-galactoside, Cyanidin-3-galactoside, Delphinidin-3-arabinoside, Petunidin-3-galctoside, Cyanidin-3-arabinoside) expressed as Cyanidin-3-galctoside equivalents (µg/g DM) in 'Brigitta' depending on the gaseous composition of atmosphere and storage time. Values represent means ± SD (*n = 3*).

Storage Time (days)	Condition of storage	Delphinidin-3-galactoside		Cyanidin-3-galactoside		Delphinidin-3-arabinoside		Petunidin-3-galctoside		Cyanidin-3-arabinoside	
0	**AT HARVEST**	**483,7**	*±2,7*	**97,8**	*±1,1*	**312,2**	*±1,9*	**515,5**	*±1,6*	**65,2**	*±0,6*
47	**CONTROL**	**1012,2**	*±0,1*	**156,7**	*±0,9*	**663,7**	*±1,0*	**775,4**	*±0,9*	**115,4**	*±0,3*
47	**CA1**	**1034,7**	*±2,5*	**130,6**	*±0,7*	**649,2**	*±1,7*	**699,2**	*±2,3*	**88,0**	*±0,4*
47	**CA2**	**1351,6**	*±2,5*	**219,6**	*±1,1*	**834,7**	*±2,1*	**998,5**	*±3,2*	**141,6**	*±0,1*
61	**CONTROL**	**1037,3**	*±0,4*	**196,5**	*±0,9*	**656,4**	*±0,2*	**923,7**	*±2,8*	**131,3**	*±0,0*
61	**CA1**	**851,1**	*±1,6*	**147,3**	*±1,4*	**562,2**	*±0,1*	**766,4**	*±2,3*	**97,6**	*±1,8*
61	**CA2**	**1198,3**	*±2,7*	**144,1**	*±1,5*	**682,3**	*±2,5*	**942,9**	*±2,7*	**105,4**	*±0,3*
94	**CONTROL**	**1186,6**	*±2,6*	**161,1**	*±1,3*	**784,1**	*±2,2*	**834,1**	*±3,5*	**125,7**	*±1,9*
94	**CA1**	**1039,0**	*±1,7*	**171,9**	*±0,8*	**647,6**	*±0,8*	**755,2**	*±2,8*	**108,7**	*±0,1*
94	**CA2**	**849,8**	*±2,1*	**176,6**	*±1,9*	**520,8**	*±2,1*	**703,9**	*±4,4*	**111,8**	*±0,2*
132	**CONTROL**	**1123,2**	*±8,7*	**176,5**	*±0,8*	**792,1**	*±1,2*	**757,3**	*±2,2*	**149,1**	*±0,6*
132	**CA1**	**785,3**	*±0,4*	**136,8**	*±3,6*	**478,0**	*±8,3*	**619,3**	*±1,8*	**89,4**	*±2,3*
132	**CA2**	**1287,5**	*±8,2*	**372,8**	*±1,9*	**520,0**	*±4,8*	**1049,3**	*±4,2*	**177,2**	*±2,4*
147	**CONTROL**	**1238,5**	*±10,7*	**178,9**	*±1,6*	**875,7**	*±7,2*	**779,2**	*±4,1*	**153,7**	*±1,8*
147	**CA1**	**811,5**	*±2,8*	**137,2**	*±0,3*	**491,9**	*±2,2*	**617,5**	*±0,1*	**86,1**	*±0,1*
147	**CA2**	**933,4**	*±0,8*	**153,2**	*±0,6*	**582,2**	*±1,0*	**721,1**	*±0,5*	**99,4**	*±0,8*

Tab. 10: Content of particular anthocyanins (Peonidin-3-galactoside, Petunidin-3-arabinoside, Malvidin-3-galactoside, Malvidin-3-glucoside + Peonidin-3-arabinoside, Malvidin-3-arabinoside) expressed as Cyanidin-3-galctoside equivalents (µg/g DM) in 'Brigitta' depending on the gaseous composition of atmosphere and storage time. Values represent means ± SD (*n = 3*).

Storage Time (days)	Condition of storage	Peonidin-3-galactoside		Petunidin-3-arabinoside		Malvidin-3-galactoside		Malvidin-3-glucoside + Peonidin-3-arabinoside		Malvidin-3-arabinoside	
0	**AT HARVEST**	**41,3**	±1,2	**276,7**	±0,5	**1180,4**	±2,9	**53,3**	±0,7	**673,3**	±1,3
47	**CONTROL**	**56,0**	±0,8	**437,0**	±1,6	**1571,0**	±1,7	**79,4**	±0,7	**1051,1**	±2,4
47	**CA1**	**43,8**	±0,8	**375,1**	±1,0	**1384,8**	±4,5	**70,9**	±1,8	**757,4**	±1,1
47	**CA2**	**59,1**	±0,4	**533,9**	±1,8	**1715,8**	±2,1	**77,7**	±0,3	**972,6**	±5,6
61	**CONTROL**	**72,6**	±0,2	**499,5**	±0,9	**1982,6**	±1,0	**94,0**	±0,2	**1289,4**	±3,9
61	**CA1**	**53,0**	±0,2	**436,4**	±0,5	**1721,3**	±4,3	**72,5**	±0,0	**1032,7**	±3,4
61	**CA2**	**54,4**	±1,5	**469,8**	±5,3	**1914,3**	±37,0	**70,4**	±1,9	**939,0**	±6,7
94	**CONTROL**	**61,8**	±1,4	**472,2**	±0,4	**1780,5**	±76,6	**85,6**	±1,4	**1210,0**	±0,9
94	**CA1**	**51,8**	±0,8	**393,5**	±0,3	**1397,0**	±1,4	**64,1**	±0,1	**766,1**	±2,1
94	**CA2**	**65,3**	±0,4	**369,1**	±2,1	**1555,7**	±5,3	**71,9**	±0,7	**922,3**	±3,8
132	**CONTROL**	**68,5**	±0,2	**474,8**	±0,3	**1683,0**	±120	**86,4**	±0,3	**1208,4**	±3,7
132	**CA1**	**33,6**	±2,4	**327,1**	±3,0	**1306,0**	±11,0	**53,5**	±4,6	**611,6**	±0,5
132	**CA2**	**134,4**	±4,2	**367,0**	±0,4	**2958,0**	±19,5	**115,3**	±0,5	**1147,6**	±7,5
147	**CONTROL**	**70,1**	±0,7	**483,5**	±4,5	**1449,2**	±10,3	**90,4**	±0,3	**1167,4**	±7,2
147	**CA1**	**43,0**	±0,3	**321,5**	±0,6	**1158,6**	±0,1	**51,9**	±1,1	**642,9**	±0,8
147	**CA2**	**44,7**	±0,4	**385,9**	±0,1	**1261,8**	±0,9	**55,8**	±0,8	**724,4**	±1,4

Tab. 11: Content of particular anthocyanins (Delphinidin-3-galactoside, Cyanidin-3-galactoside, Delphinidin-3-arabinoside, Petunidin-3-galctoside, Cyanidin-3-arabinoside) expressed as Cyanidin-3-galctoside equivalents (µg/g DM) in 'Legacy' depending on the gaseous composition of atmosphere and storage time. Values represent means ± SD (*n = 3*).

Storage Time (days)	Condition of storage	Delphinidin-3-galactoside		Cyanidin-3-galactoside		Delphinidin-3-arabinoside		Petunidin-3-galctoside		Cyanidin-3-arabinoside	
0	**AT HARVEST**	**889,9**	*±3,2*	**238,0**	*±0,9*	**389,5**	*±0,6*	**823,7**	*±2,6*	**120,0**	*±0,5*
42	**CONTROL**	**774,0**	*±2,3*	**210,9**	*±0,2*	**362,1**	*±1,6*	**691,2**	*±1,5*	**114,3**	*±0,2*
42	**CA1**	**914,0**	*±2,5*	**214,4**	*±1,5*	**416,9**	*±0,1*	**722,2**	*±2,2*	**110,9**	*±0,7*
42	**CA2**	**1494,8**	*±7,1*	**322,0**	*±4,1*	**661,5**	*±4,9*	**1182,3**	*±5,2*	**168,1**	*±0,1*
56	**CONTROL**	**669,6**	*±0,5*	**238,0**	*±0,2*	**308,0**	*±0,9*	**620,1**	*±1,6*	**129,6**	*±0,2*
56	**CA1**	**1300,7**	*±22,5*	**315,7**	*±2,5*	**598,4**	*±13,3*	**1170,5**	*±21,1*	**159,4**	*±0,1*
56	**CA2**	**1171,6**	*±3,2*	**282,2**	*±0,4*	**460,4**	*±0,8*	**1160,3**	*±2,2*	**144,3**	*±0,8*
89	**CONTROL**	**942,8**	*±8,0*	**224,8**	*±3,3*	**425,0**	*±5,9*	**789,6**	*±5,8*	**119,7**	*±1,0*
89	**CA1**	**894,5**	*±5,4*	**273,1**	*±0,8*	**404,0**	*±2,2*	**794,9**	*±4,5*	**138,7**	*±0,8*
89	**CA2**	**989,6**	*±11,1*	**250,7**	*±1,4*	**462,9**	*±3,9*	**856,9**	*±8,3*	**127,2**	*±1,7*
127	**CONTROL**	**1318,1**	*±5,0*	**347,7**	*±3,0*	**578,8**	*±5,4*	**1063,9**	*±7,1*	**181,3**	*±0,8*
127	**CA1**	**658,7**	*±0,7*	**283,1**	*±0,5*	**273,0**	*±0,8*	**692,2**	*±0,5*	**136,4**	*±0,1*
127	**CA2**	**923,5**	*±6,6*	**118,8**	*±2,2*	**561,5**	*±7,0*	**706,3**	*±8,9*	**81,8**	*±1,4*
142	**CONTROL**	**769,2**	*±20,7*	**208,5**	*±10,3*	**314,7**	*±11,7*	**618,5**	*±13,3*	**107,0**	*±3,9*
142	**CA1**	**1002,6**	*±1,9*	**261,6**	*±1,1*	**447,5**	*±1,1*	**816,9**	*±2,6*	**133,7**	*±0,7*
142	**CA2**	**924,7**	*±1,8*	**340,1**	*±0,1*	**385,2**	*±0,7*	**851,7**	*±1,7*	**161,8**	*±0,2*

Tab. 127: Content of particolar anthocyanins (Peonidin-3-galactoside, Petunidin-3-arabinoside, Malvidin-3-galactoside, Malvidin-3-glucoside + Peonidin-3-arabinoside, Malvidin-3-arabinoside) expressed as Cyanidin-3-galctoside equivalents (µg/g DM) in 'Legacy' depending on the gaseous composition of atmosphere and storage time. Values represent means ± SD (*n = 3*).

Storage Time (days)	Condition of storage	Peonidin-3-galactoside		Petunidin-3-arabinoside		Malvidin-3-galactoside		Malvidin-3-glucoside + Peonidin-3-arabinoside		Malvidin-3-arabinoside	
0	**AT HARVEST**	**108,9**	±1,1	**316,3**	±0,5	**2099,1**	±3,5	**98,5**	±0,9	**1012,2**	±3,7
42	**CONTROL**	**97,6**	±1,4	**287,1**	±0,3	**1811,0**	±7,9	**93,6**	±1,1	**995,7**	±1,8
42	**CA1**	**85,4**	±0,1	**296,7**	±0,3	**1651,0**	±5,7	**80,5**	±0,5	**870,6**	±2,1
42	**CA2**	**127,2**	±1,1	**475,1**	±3,1	**2581,4**	±20,8	**121,9**	±1,5	**1331,8**	±7,7
56	**CONTROL**	**98,6**	±0,1	**260,5**	±0,5	**1641,7**	±3,8	**90,5**	±0,7	**1015,3**	±0,8
56	**CA1**	**139,0**	±4,0	**436,6**	±11,3	**3153,9**	±199,8	**115,3**	±7,1	**1405,3**	±31,4
56	**CA2**	**137,6**	±1,7	**406,4**	±1,4	**3318,7**	±24,8	**124,1**	±0,4	**1353,7**	±7,5
89	**CONTROL**	**103,2**	±0,9	**305,7**	±2,9	**1911,0**	±11,5	**94,4**	±0,5	**991,4**	±9,5
89	**CA1**	**104,4**	±1,2	**313,0**	±1,6	**1868,2**	±10,5	**91,8**	±0,0	**961,7**	±4,7
89	**CA2**	**100,5**	±0,6	**352,1**	±3,3	**1967,4**	±19,7	**92,8**	±0,1	**1028,9**	±12,5
127	**CONTROL**	**145,3**	±0,5	**411,8**	±0,7	**3010,3**	±11,2	**129,8**	±1,1	**1372,7**	±9,9
127	**CA1**	**104,0**	±1,4	**268,4**	±0,4	**1995,7**	±32,9	**89,9**	±1,0	**979,3**	±3,5
127	**CA2**	**44,0**	±0,1	**379,5**	±3,0	**1722,1**	±18,9	**59,0**	±2,0	**789,4**	±6,1
142	**CONTROL**	**84,6**	±2,6	**242,9**	±0,5	**1697,6**	±15,4	**74,9**	±0,3	**776,5**	±18,6
142	**CA1**	**99,4**	±0,7	**317,0**	±1,3	**1825,9**	±0,7	**86,8**	±0,2	**911,6**	±5,7
142	**CA2**	**111,3**	±1,1	**324,8**	±1,0	**1913,0**	±3,9	**95,5**	±0,6	**996,1**	±4,1

Different storage conditions affected the levels of anthocyanins also in 'Legacy'. All the most prevalent anthocyanins showed highest levels in berries stored under controlled atmosphere, with only few exceptions (Tab. 16 and 17). CA2 resulted as the controlled atmosphere condition which more frequently determinates the highest content of pigments. However, comparing with 'Brigitta', in samples from the cultivar 'Legacy' the differences between storage conditions were less marked.

6.3.10. AsA content

Due to its high lability, ascorbic acid levels were monitored for a shorter period (94 days) in comparison with that considered for the analysis of phenolic compounds and quality attributes.

Moreover, the cultivar 'Legacy' resulted to be a very poor source of ascorbic acid, with levels in certain cases undetectable, thus data measured from this cultivar are not reported in the present work.

The ascorbic acid content in 'Brigitta' is illustrated in Figure 39. Fruits stored in natural atmosphere (control) showed an initial increase in content along the first 12 days of storage, with values higher than those measured in samples from CA conditions, then a gradual decline occurred and, after 94 days, ascorbate levels were similar to those measured at harvest.

In berries stored in CA1 and CA2 the ascorbic acid content increased during the first 12 days of storage, although levels were lower than the ones measured in the control. No relevant differences between the two different conditions of CA were observed.

In samples stored in controlled atmosphere, the levels of ascorbic acid were more stable and generally higher than those registered in natural atmosphere (control), especially in samples from CA2 condition. Highest levels were measured during the first two months of storage. After 76 days of cold storage, the AsA levels were hardly detectable in all the different storage conditions.

Fruits collected at that time point presented very low contents of ascorbic acid and showed a very strong decrease compared to the previous samplings.

At the end of the experiment, AsA levels remained low in samples from CA2, while slightly increased in those from CA1 and control.

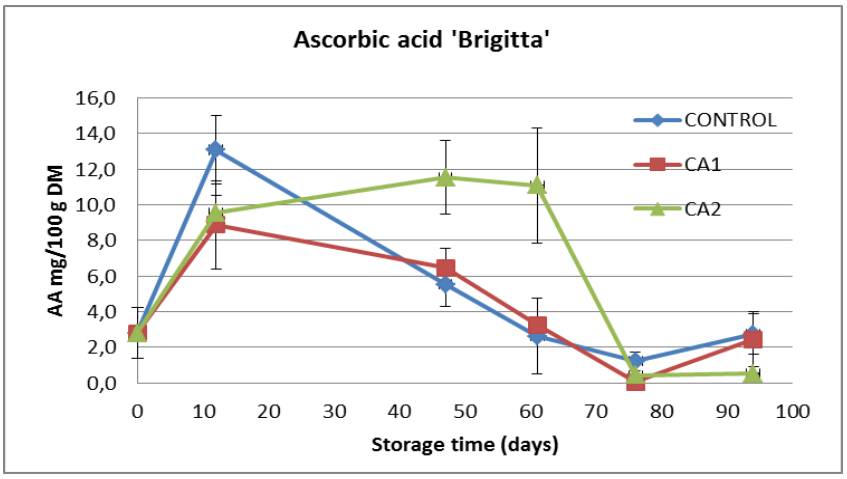


Fig. 39: Ascorbic acid content 'Brigitta'. Values represent means ± SD (*n = 3*).

6.3.11. TBARS (Thiobarbituric Acid Reactive Substances)

The measurement of TBARS (Thiobarbituric Acid Reactive Substances) represents a useful assay for screening and monitoring lipid peroxidation, as an indicator of oxidative stress in fruits and vegetables.

Figure 40 shows the changes in the concentration of TBARS expressed as MDA equivalents (nmol / g DM) during cold storage of blueberries form the cultivar 'Brigitta'.

In the middle of storage period, TBARS levels were higher in fruit stored in natural atmosphere (control), indicating a more pronounced state of senescence.

After an initial decrement, samples stored under natural atmosphere showed the highest levels of peroxidation until 94 days of storage. The levels of TBARS determined in the fruits stored in CA1 showed a similar trend, even if lower, than those in the control. On the other hand the content of TBARS in samples stored in CA2 condition, did not vary throughout the storage, and showed the lowest values duringduring the same period, indicating a smaller level of oxidative stress and a slowdown in the process of senescence in fruits stored in that condition.This effect was more pronounced in the intermediate period of storage.

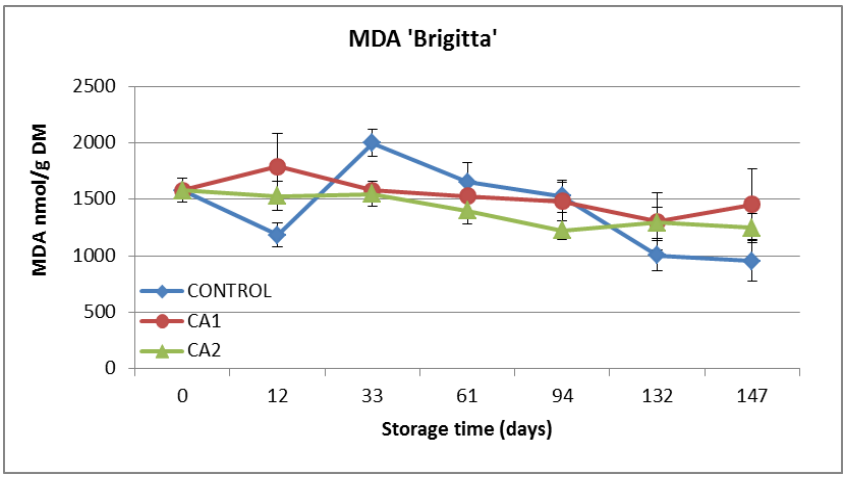


Fig. 40: MDA content in 'Brigitta' expressed as nmol/g DM. Values represent means ± SD (*n = 3*).

6.4. Discussion

The use of cold storage and controlled atmosphere with high CO_2 and low O_2 levels has been recognized to be the most effective technique to preserve the quality of fruits and extend their commercial life.

In this study we set up a trial storing blueberry fruit under different conditions of cold storage, in natural and controlled atmosphere. We evaluated the effects of storage by measuring different quality parameters such as weight loss, decay, soluble sugars content and total titratable acidity. We also measured the changes in levels of phenolic compounds, flavonoids, anthocyanins and ascorbic acid. The experiment was conducted using two late season cultivar, 'Brigitta' and 'Legacy', with the aim to prolong as much as possible the storability of this valuable product and preserve its quality attributes.

We protracted the samplings for a period much longer than the one normally expected for this produce. In general we have focused more attention to what was happening at the beginning of the conservation, in a period of about two months, being the most interesting in terms of commercial and practical applications.

Weight losses were lower in berries stored under controlled atmosphere, with a particular positive effect in those stored under 9 kPa of CO_2 and 1 kPa O_2. Dry matter content was also generally higher in berries stored under controlled atmosphere.

Sampling procedures were quitted when more than 50% of berries resulted damaged because of high shrivel or fungal development. This happened after about 150 days of storage for both of the considered cultivars, without differences due to different storage conditions. However during the

first two months of storage no significative decay incidence was registered in consitance with data which were previously reported (Connor et al., 2002).

We observed a general negative effect of controlled atmosphere on the TSS content. This effect was more evident in 'Brigitta' compared with 'Legacy'. Different behaviour among cultivars was observed also during storage in controlled atmosphere of rabbiteye blueberries (Schotsmans et al., 2007). Our results are different from what found by Smittle and Miller (1998) , which reported a general decrement in sugar content during storage with a positive effect of controlled atmosphere compared with natural conditions. However the changes we report in this study can be considered low, and the sugar amount that we estimated during the whole storage period indicates that fruits had sufficient reserves and substrates of respiratory metabolism.

Berry fruits contain many different organic acids and changes in their amount could influence other compounds stored in the vacuoles such as anthocyanin pigments. During storage organic acids can undergo to changes due to several factors. High CO_2 concentrations could affect the pH and the metabolism of organic acids during storage as reported by Holcroft and Kader (1999). In our study TTA was found to be higher in 'Brigitta' compared with 'Legacy' at harvest. During storage the values were characterized by a general increase and by an irregular pattern. This increase was unexpected because usually a decrement in TTA was reported in berry fruits (Gil et al., 1997; Holcroft, 1998). This phenomena could be explained thinking that not all the organic acid are consumed during storage but some could be accumulated. For example Holdcroft and colleagues (1998) observed an increase in succinic acid levels during storage of strawberries and this was hypothesized to be the result of inhibition of the enzyme succinate dehydrogenase which can take place during storage and be affected by storage conditions.

Total phenolic compounds were measured in two cultivars and differences were observed at harvest as well as during storage. 'Legacy' showed values clearly higher than 'Brigitta' and this data appear to be consistent with those reported in previous studies for the same cultivars (Connor et al., 2002). During storage slight changes were observed in 'Brigitta' while a more irregular pattern has been observed in 'Legacy'. In both cases after the first 42 and 47 days of storage respectively, the highest levels were measured in berries stored under controlled atmosphere (9 kPa of CO_2 and 1 kPa of O_2).

Flavonoids represent a relevant portion of the phenolic compounds pool in berry fruits (Marinova et al., 2005). Flavonoids concentration was not reported to be affected by controlled atmosphere conditions during cold storage of apples (van der Sluis et al., 2001). In a study on cold storage of

strawberry fruits, the amount of flavonols did not change along storage (Cordenunsi et al., 2005) contrarly to what observed for anthocyanins. From our results 'Legacy' was as well a better source of flavonoids as it was for total phenolic compounds. The content of flavonoids has been positively influenced by controlled atmosphere conditions (9 kPa of CO_2 and 1 kPa of O_2) especially at the beginning of storage and it can be hypothesized that the changes were mostly due to an increment in the levels of anthocyanins.

In this work we performed an HPLC analysis in order to identify and study the changes in anthocyanin contents in blueberry fruits belonging to two of the most widespread commercial varieties. We identified 10 anthocyanin molecules and the data appeared to be comparable with those previously reported for blueberry (Krupa and Tomala, 2007; Määttä-Riihinen et al., 2004).

Our results confirmed 'Legacy' as the richest source of all classes of phenolic compounds analyzed, compared with 'Brigitta'. Total anthocyanin content as well as levels of particular anthocyanins were similarly affected by different storage conditions. A general increment in levels was observed during the first days of storage in both the cultivars as reported in previous studies (Connor et al., 2002; Holcroft and Kader, 1999; Krupa and Tomala, 2007). The increment in the anthocyanin levels at the beginning of storage could be explained by a protraction in the activity of secondary metabolism during storage as well as by changes in the molecular structure of some phenolic compounds which could lead to a switch from some classes to others.

During storage, changes in the structure of cellular components and tissues should be also taken into account, thus tissue changes and degradation could influence the extractability of pigments.

Gas composition had a different effect on anthocyanin levels depending on the cultivar. In fact, in 'Brigitta', samples stored in CA2 showed the highest content of anthocyanins while samples stored in CA1 conditions showed results more similar to those from the control. The same differences between CA1 and CA2 were not observed in 'Legacy', in which both of the gas compositions appeared to be effective in increasing the amount of anthocyanins pigments.

Ascorbic acid levels were monitored during storage of blueberries from the cultivar 'Brigitta', in parallel we measured the levels of TBARS as index of lipid peroxidation of cellular membranes. We observed a pronounced increment of AsA levels during the first 12 days of storage. Many factors such as genetic diversity, growing and environmental conditions, and date of harvest could affect the accumulation and biosynthesis of this important nutraceutical component in fruits. Previous studies showed that storage in high CO_2 reduced vitamin C concentrations in many fruits and vegetables (Bangerth, 1997). Storage in controlled atmosphere with high CO_2 levels do not have a

beneficial effect on vitamin C content (Agar et al., 1997). The reduction of vitamin C could be explained by an increase of ascorbate peroxidase (APX) activity stimulated by an increment of ethylene, triggered by CO_2 as reported by Agar and colleagues (1997). Moreover in the same study was hypothesized that and increment in CO_2 levels could inhibit the activity of monodehydroascorbic reductase (MDHAR) and dehydroascorbic reductase (DHAR). The initial increment that we observed in AsA levels is not completely supporting those hypotheses. We measured the amount of AsA, and we did not measure the levels of dehydroascorbate (DHA) thus it is possible to speculate that the increment that we reported is likely due to changes in AsA/DHA ratio and to changes in the activity of the enzymes of the Halliwell –Asada cycle instead of an increase in the biosynthesis. During storage CA2 condition appeared to be the best to control this parameter. In fact AsA levels were higher in samples stored under 9 kPa of CO_2 and 1 kPa of O_2. In parallel, CA2 condition appeared to be effective in maintaining the lowest levels of TBARS. during the storage period. TBARS is so confirmed to be an useful index of product degradation during storage.

In conclusion fruits from tested cultvars demonstrated stability in quality attributes and marketability during the cold storage period. Among the two cultivars, 'Legacy' appeared to be a better source of phenolic compounds but at the same time a very poor source of ascorbic acid. 'Brigitta' showed generally a good attitude to storage that could be likely due to higher acidity values in comparison to 'Legacy'.

Controlled atmosphere was effective in preserving berries from weight losses but had no significative effects on retarding decay. Among the different applied CA conditions, CA2 (9 kPa CO_2 and 1 kPa O_2) was the most effective in reducing weight losses and in maintaining high levels of flavonoids, anthocyanins and ascorbic acid.

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