Effect of plant growth regulator applications on phenolic quality of red grape berry skin and wine Vitis vinifera L., cvs Cabernet Sauvignon and Carmenère

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Dedication

I would like to dedicate this thesis to all those I love.

… at the core of all the troubles we face today is our very ignorance of knowing.

(Maturana and Varela, 1987)

Acknowledgments

This thesis has been developed in the core of the research team of the Faculty of Agronomy and Forestry Engineering of the *Pontificia Universidad Católica de Chile*. The research project was conducted under the supervision of Dr. Edmundo Bordeu and Dr. Laurence Geny, and is part of a cotutelle program between *Pontificia Universidad Católica de Chile* and *Université of Bordeaux*.

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Content

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Thesis Project Abstract

Effect of plant growth regulator applications on phenolic quality of red grape berry skin and wine *Vitis vinifera* L., cvs Cabernet Sauvignon and Carménère. Álvaro S. González Rojas. Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile.

Phenolic composition strongly determines red wine quality: color, taste, texture and most health benefits. Vineyard environmental conditions modulate endogenous hormonal balance and gene expression which control the flavonoid biosynthetic pathway leading to final grape phenolic composition. Even when the effects of plant growth regulator applications on grape endogenous hormonal balance and quality have been studied, the effect of these substances on wine composition and quality is poorly documented. The treatment of wine grapes with plant growth regulators is a potential tool in order to modify red wine phenolic composition and quality.

This thesis project describes six experiments on plant growth regulator applications on developing grapes of *Vitis vinifera* L., cvs Cabernet Sauvignon and Carménère. Abscisic acid, Indole-3-acetic acid and 2-chloroethylphosphonic acid were applied in different phenological stages, doses and environmental conditions: Maipo and Cachapoal regions in Chile and Bordeaux region in France, commercial and experimental vineyards and plants in containers. The effect on changes in the internal hormonal content, expression of flavonoid biosynthetic and regulatory

genes and grape quality, in particular grape skin phenolic composition were examined. In addition, winemaking was performed in order to assess the effect of treatments on wine chemical and phenolic composition and on wine aroma and texture attributes judged by a sensory panel.

Resumen del Proyecto de Tesis

Efecto de la aplicación de reguladores de crecimiento vegetal sobre la calidad fenólica de la piel de uva y el vino tinto *Vitis vinifera* L., cvs Cabernet Sauvignon y Carménère. Álvaro S. González Rojas. Facultad de Agronomía e Ingeniería Forestal. Pontificia Universidad Católica de Chile.

La composición fenólica del vino tinto determina fuertemente su calidad: color, gusto, textura y la mayor parte de los beneficios para la salud. Las condiciones ambientales de la viña modulan el equilibrio hormonal endógeno y la expresión de genes que controlan la vía de síntesis de los compuestos flavonoides, determinando la composición fenólica final de la uva. Aun cuando han sido estudiados los efectos de las aplicaciones de reguladores de crecimiento vegetal sobre el equilibrio hormonal endógeno y calidad de la uva, los efectos de estas sustancias sobre la composición y calidad del vino están pobremente documentados. El tratamiento de las uvas para vinificación con reguladores de crecimiento vegetal es una herramienta potencial a fin de modificar la calidad de las uvas y el vino tinto.

Este proyecto de tesis describe seis experimentos de aplicaciones de regulador de crecimiento vegetal en uvas en desarrollo de *Vitis vinifera* L. cultivares Cabernet Sauvignon y Carménère. Ácido abscísico, ácido indole-3-acetico y ácido 2-cloroetilfosfonico fueron aplicados en diversos estados de desarrollo fenológico de la uva, dosis y condiciones ambientales: regiones de Maipo y Cachapoal en Chile

y región de Burdeos en Francia, en viñedos comerciales y experimentales y en plantas en contenedores. Se examinó el efecto de estos tratamientos sobre el contenido interno de hormonas, sobre la expresión de genes estructurales y reguladores de la síntesis de compuestos flavonoides y sobre la calidad de las uvas, en particular la composición fenólica de su piel. Además, se llevaron a cabo vinificaciones de las uvas tratadas a fin de determinar el efecto de los tratamientos sobre la composición química y fenólica del vino, así como sobre atributos del aroma y la textura de los vinos, juzgados por un panel de evaluación sensorial.

Résumé du Projet de Thèse

Effet de l'application des régulateurs de croissance végétale sur la qualité phénolique de la pellicule du raisin et du vin rouge *Vitis vinifera* L., cépages Cabernet Sauvignon et Carménère. Álvaro S. González Rojas. Facultad de Agronomía e Ingeniería Forestal. Pontificia Universidad Católica de Chile.

La composition phénolique du vin rouge détermine fortement sa qualité: couleur, goût, texture et la plupart des bienfaits pour la santé. Les conditions ambiantes de la vigne modulent l'équilibre hormonal endogène et l'expression de gènes qui contrôlent la voie de synthèse des composés flavonoïdes, en déterminant la composition phénolique finale du raisin. Même s'ils ont été étudiés, les effets des applications des régulateurs de croissance végétale sur l'équilibre hormonal endogène et la qualité du raisin, les effets de ces substances sur la composition et la qualité du vin sont pauvrement documentés. Le traitement des raisins destinés à la vinification avec des régulateurs de croissance végétale est un outil potentiel pour modifier la qualité des raisins et du vin rouge.

Ce projet de thèse a pour objectif d'étudier l'impact d'applications de régulateur de croissance végétale sur la composition phénolique des raisins de *Vitis vinifera* L. cépages Cabernet Sauvignon et Carménère. L'acide abscissique, l'acide indole-3-acétique et l'acide 2-chloroethilphosphonique ont été appliqués à divers stades phénologiques du raisin, doses et conditions environnementales: Les essais ont été menés à Maipo et Cachapoal au Chili et à Bordeaux en France, dans des

vignobles commerciaux et expérimentaux ainsi que sur des plantes cultivées en pots. Il a été examiné l'effet de ces traitements sur le contenu interne d'hormones, sur l'expression de gènes structuraux et régulateurs de la synthèse de composés flavonoides et sur la qualité des raisins, en particulier la composition phénolique de sa pellicule. De plus, des vinifications ont été réalisées à partir de raisins traités pour déterminer l'effet des traitements sur la composition chimique et phénolique du vin, ainsi que sur des attributs qualitatifs tels que les arômes et la texture des vins, jugés par un panel d'évaluation sensorielle.

Chapter 1

Introduction

Grape berry development is a key issue for grape growers and winemakers, determining productivity and quality of grapes and wine. However, even though the importance of this issue for the industry and the considerable research work that has been done, recent research in the field show that this process and its control remains controversial and poorly understood (Davies and Böttcher, 2009).

This development comprises the coordination of a large number of events. Vineyard environmental conditions and cultural practices, such as temperature, radiation, irrigation, source/sink ratio and others, affect the chemical composition of grape and wine (Jackson and Lombard, 1993, Matus *et al.*, 2009, Downey *et al.*, 2006, Ristic *et al.*, 2007, Berli *et al.*, 2008, Pastore *et al.*, 2011) and several scientific reports have shown a relation between some of these factors and the hormonal balance of the grape berry (Yamane *et al.*, 2006, Baigorri *et al.*, 2001, Deluc *et al.*, 2009, Berli *et al.*, 2010). A more complete comprehension of the developmental berry hormonal balance could provide a more complete understanding of these phenomena and its impacts on product quality.

Moreover, as a way to alter berry hormonal balance, the application of plant growth regulators (PGRs) could be an interesting industrial practice for the management and improvement of grape and wine quality. The use of Abscisic Acid

(ABA) as an agrochemical has become feasible recently due to lower production costs. Improved synthesis procedures make it easier to obtain (+)-S-abscisic acid (S-ABA), the natural active enantiomer, with potential use in viticulture, as seen in several patent applications (Venburg *et al.*, 2008a, Venburg *et al.*, 2009, Venburg *et al.*, 2008b) and some reports (Peppi *et al.*, 2006, Villalobos, 2011, Lurie *et al.*, 2009).

One of the most important grape and wine quality factors affected by environmental conditions, cultural practices, hormonal balance and PGR treatments is the phenolic composition (Jeong *et al.*, 2004, Chervin *et al.*, 2009, Lacampagne *et al.*, 2009, Gagné *et al.*, 2011, Davies *et al.*, 1997, Koyama *et al.*, 2009, Delgado *et al.*, 2004). So PGRs could be an interesting tool to improve grape and wine phenolic composition.

1.1 Phenolic Quality of Red Wine

Phenolic compounds (PC) represent some of the major quality factors of red wine, determining projected price (Kassara and Kennedy, 2011, Guerrero *et al.*, 2009). Among the different classes of PC, there are compounds responsible for the color (intensity and stability), mouth-feel, astringency, bitter taste and wine antioxidant properties, beneficial to human health. Quality of wine is related to the amount and types of PC present in the berries at harvest time, but on the other hand is related to the extractability of these compounds to the wine solution (Sacchi *et al.*, 2005).

The study of PC during grape development is of great importance from a scientific and technological point of view. In particular the identification and

determination of their concentrations and the sensory importance that they might have alone and in interaction with other compounds (Kennedy *et al.*, 2006).

1.2 The Grape Berry

To obtain high quality red wines, grape ripeness is a key issue. However, in addition to the traditional flesh ripeness (sugar concentration, titratable acidity and pH of the grape juice), skin and seed ripeness (PC status) must be taken into account (Kontoudakis *et al*., 2010). Nowadays, this usually leads to the harvest of overripen grapes that leads to high alcohol wines. Therefore, any method that improves or advances phenolic maturity is of great interest in order to achieve an equilibrium between traditional ripeness and phenolic ripeness.

1.2.1. Grape Berry Anatomy

In a simple way, berries are fleshy fruits formed from a single ovary. The anatomy of a grape berry is constituted by (1) the skin or exocarp, containing pigments, flavan-3-oles and proanthocyanidins (PAs); (2) the pulp or flesh, structured by mesocarp and endocarp, containing sugars, organic acids, hydroxycinnamic acids and no pigments, and finally; (3) the seed, containing flavan-3-oles and PAs.

The skin or berry dermal system was characterized by Cholet (2001), Considine and Knox (1979) and Park (1995), is made up of two types of tissues, (1) the epidermis: a layer of clear cells on the outside of the berry, covered by cuticle and waxes and (2) the hypodermis, located beneath the epidermis. It is composed of approximately six layers (depending on the variety) of collenchyma

cells. The flesh is composed of three tissues: the external mesocarp, the vascular tissue (xylem, phloem and vascular parenchyma) and the internal endocarp (figure 1). The seed coat or “testa” originates from the outer integument of the ovule and has two layers of different tissues from the point of view of the PC. In *veraison*, the inner cell layers lignify, while the external cells retain their thin primary cell wall and only the cell wall of the outermost layer presents a thickening giving rise to the cuticle on the surface of the seed. The thin-walled cells (between the cuticle and the inner lignified cell layers) are of great relevance, since they contain almost all of the seed soluble PC. The traditional browning of seeds after *veraison* are the consequence of a tannin and flavan-3-ol oxidation process that takes place in these thin-walled cells and not of a process of lignification and has been proposed as an indicator of overall berry ripeness (Ristic and Iland, 2005). The seed coat contains two to five times more flavan-3-oles monomers and PAs when compared with the endosperm (Thorngate and Singleton, 1994). Seed tannins are more polymerized in the cell walls than in the inner fractions of the cell and their composition and polymerization is not affected by the water status of the plants during maturation (Geny *et al.*, 2003).

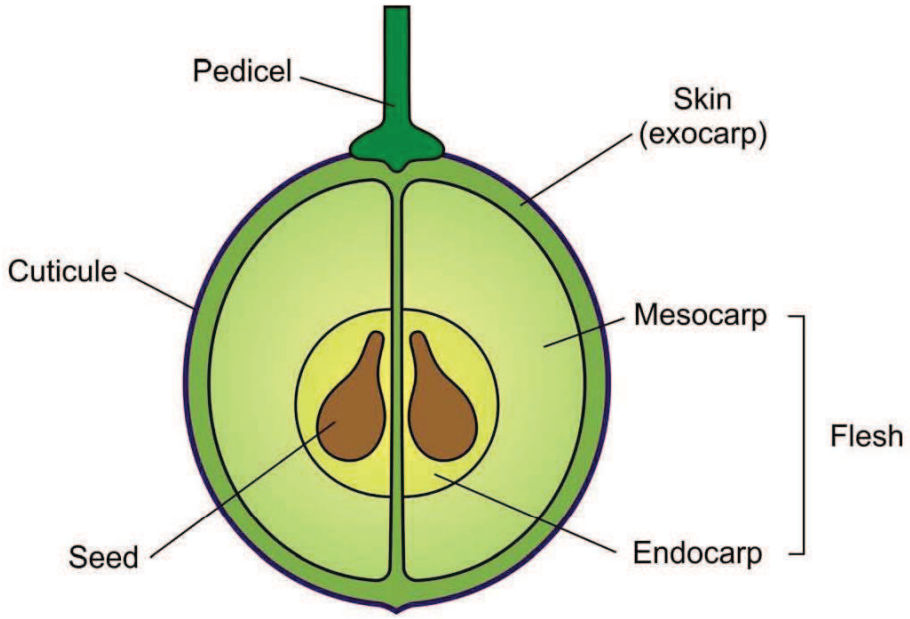


Figure 1. Simple illustration of the grape berry anatomy (Conde *et al.*, 2007).

1.2.2. Grape Berry Development

Berry development is described by two sigmoidal curves (or what is the same, a double-sigmoidal curve), separated by a more stable phase between the two (figure 2). Three berry growth stages are described:

(1) Stage I. Prime sigmoidal berry growth period. In this stage pericarp and seed cell number increases and the seed approaches its final size. At this stage a short period of cell division and subsequent cell expansion takes place which is the main responsible for the increase in berry size (Harris *et al.*, 1968). Organic acids (mainly malate and tartrate), tannins and hydroxycinnamates accumulate during this phenological period.

(2) Stage II. It is defined as the lag phase, characterized by the absence of berry size change. The seed embryo is completely developed and the seed coat

gets harder. At the end of this stage the berry begins to soften, lose chlorophyll and begins to accumulate sugars just prior to véraison (the onset of ripening).

(3) Stage III. The beginning of this second sigmoidal berry growth period is marked by *veraison*. This is the berry ripening stage, moment in which sugars accumulate. It is characterized by berry softening, cell expansion, sugar accumulation (mostly glucose and fructose), reduction of organic acid levels and anthocyanins accumulation. Several flavor and aroma compounds are synthetized at the end of this stage (Coombe, 1992, Coombe, 1976). In terms of the transcriptional program of the berry, there is an increase in primary and secondary metabolism, hormones synthesis and transportation and an increase of cell wall enzymes expression (Deluc *et al.*, 2007, Goes da Silva *et al.*, 2005). In particular, two of the most significant pectin-degrading enzymes that are implied in berry softening: pectin methylesterase (PME; EC 3.1.1.11) and polygalacturonase (Hurst *et al.*) show differences in activity (*i.e*: PME) and expression (*i.e*: VvPME1 and VvPG1) during this stage (Deytieux-Belleau *et al.*, 2008).

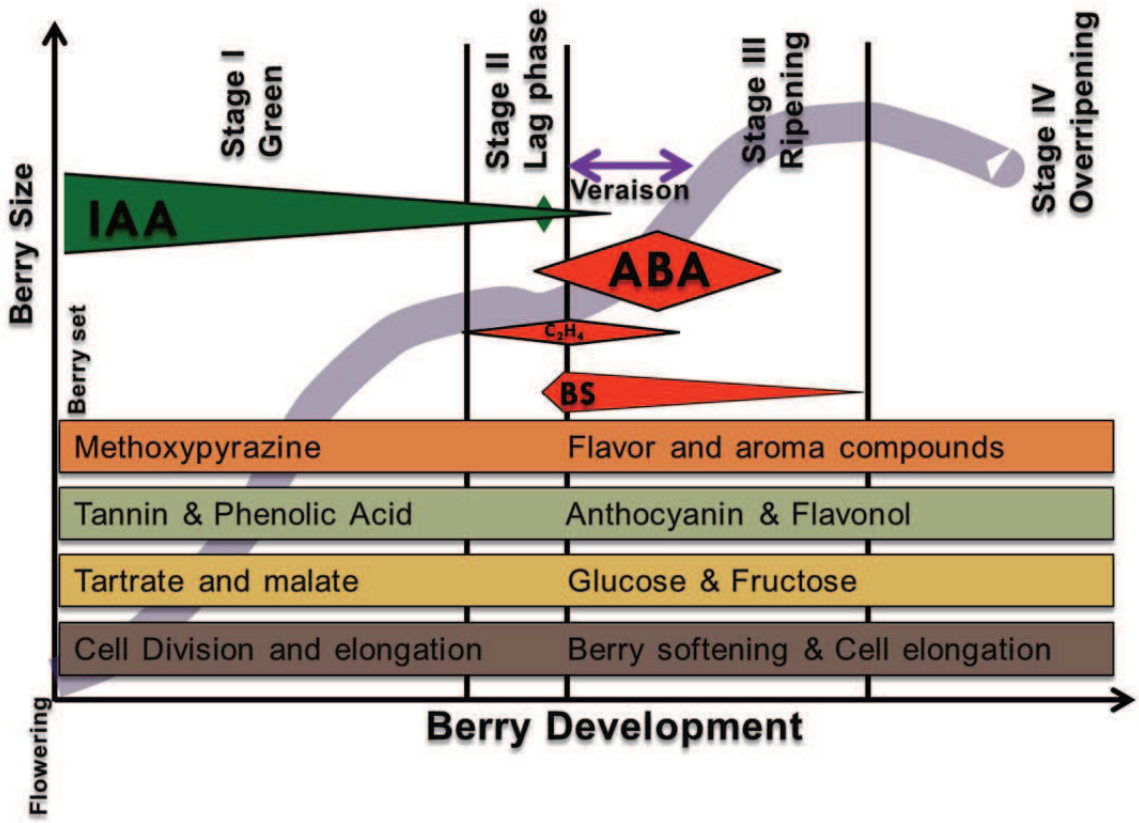


Figure 2. Grape berry growth, developmental stages, relevant hormones and quality composition as described by Coombe (1992).

Development of the pericarp in cv. Gewürstraminer was studied by Hardie *et al.* (1996). Table 1 shows that mesocarp cells have a strong elongation between 14 and 126 days after anthesis, reaching an average radial cell width of 68 μm. During the same period of time, the size of hypodermal cells is maintained or decrease reaching an average radial cell width of 5.5 μm. On the other hand, cell wall thickness increases in hypodermal cells, reaching an average of 1.9 μm, while mesocarp cells maintain an average cell wall thickness of 0.17 μm.

Table 1. Cell radial width and cell wall thickness in hypodermis and internal mesocarp cells from 14 to 126 days after anthesis in *Vitis vinifera* L. berries (Hardie *et al.*, 1996).

	Hypodermis		Inner mesocarp	
	14 DAA[1]	126 DAA	14 DAA	126 DAA
Radial width (µm) +/- s.d.[2]	7.0 (+/- 0.5)	5.4 (+/- 0.6)	17.8 (+/- 1.5)	68.0 (+/- 4.5)
Cell-wall thickness (µm) +/- s.d.	0.20(+/-<0.1)	1.90 (+/- 0.2)	0.17(+/- <0.1)	0.17(+/- <0.1)

[1] DAA: days after anthesis
[2] s.d: standard deviation

To summarize, during division and expansion stages, the cells of the hypodermis do not increase in size, staying small, however their walls get thicker, while the cells of the inner mesocarp strongly increase in size and maintain relatively thin walls. At harvest date, the mesocarp cells are 9.7 times larger (75 times larger in volume) and hypodermal cell walls are 10 times thicker. Mesocarp cell walls become 59% thinner, being at harvest date 20 times thinner than hypodermal cell walls.

The importance of this phenomenon is that phenolic composition of mesocarp and hypodermal cells is dissimilar at harvest date. Vacuolar PC of pericarp cells experiment large changes while PC of mesocarp cells decreases as berry develop to end in nearly no PC content at harvest ripeness (Park, 1995, Hardie *et al.*, 1996, Considine and Knox, 1979). Cholet *et al*. (2002) with histochemical observations in early development, have verified that berries accumulate flavonoids only under the cuticle, gallic acid derivatives bind to condensed tannins in peripheral cells, seeds and the septum fissure and caffeic

acid derivatives in the vascular bundles. The differences in cell width and in particular in cell wall thickness are crucial to understand how PC are extracted during winemaking (Sacchi *et al.*, 2005).

1.3 Hormonal Regulation of Grape Berry Development

Grape berry ripening occurs under hormonal control (Chervin *et al.*, 2004, Symons *et al.*, 2006, Davies and Böttcher, 2009) and therefore a close relation between the PC accumulation and the hormonal status has been envisaged (Lacampagne *et al.*, 2009, Böttcher *et al.*, 2010) as a way to improve grape and wine quality (Kennedy *et al.*, 2006).

From a physiological point of view, fruit have been divided into two groups: (1) climacteric fruit that display a peak in respiratory activity and an increase in ethylene concentration and (2) non-climacteric fruits that don't display a peak in respiratory activity and seem to be more independent from ethylene but react with other plant hormones (Tucker, 1993). Some evidence shows that climacteric and non-climacteric fruits could in fact share similar ripening metabolic pathways (Barry and Giovannoni, 2007).

The fruit of the grapevine (*Vitis vinifera* L.) is considered non-climacteric, therefore ripening would occur independently of the presence of ethylene (Giovannoni, 2001). Grape ripening hormonal signaling is a complex process that is activated at *veraison*: the expression of genes related to the metabolism of ABA, auxins, ethylene and brassinosteroids (BS) were shown to be significantly modified at the onset of ripening, as is shown in figure 2 (Broquedis, 1983, Pilati *et al.*, 2007, Symons *et al.*, 2006).

Ethylene peak at *veraison* is very low in the tissues of the berry (Davies and Böttcher, 2009). However, these tissues have a functional ethylene synthesis pathway, which is triggered just before *veraison*, the moment when a transient increase in internal ethylene content is evidenced (Chervin *et al.*, 2004). Even if changes in the ethylene gas levels during grape berry ripening are small compared to climacteric fruits, the physiological response to ethylene may be modulated more by changes in the sensitivity of perception during development (Davies and Böttcher, 2009). This production of ethylene appears to be critical in inducing some changes during the development of the berry, such as an increase in diameter, sucrose and anthocyanin accumulation and a decrease in acidity (Chervin *et al.*, 2004, Chervin *et al.*, 2008). In other non-climacteric fruit, the strawberry, it was also reported an increase in ethylene and furthermore a small rise in respiration at ripening (Iannetta *et al.*, 2006), supporting those who believe that the classic physiological division of climacteric and non-climacteric fruit is less clear.

ABA is an essential plant hormone that regulates plant responses to environmental abiotic stress and seed maturation and germination (Leung and Giraudat, 1998, Finkelstein and Rock, 2002). ABA is a 15-carbon molecule (figure 3) with two interconvertible isomers (*cis* and *trans*) and two non-interconvertible enantiomers, (+)-S-ABA and (-)-R-ABA. Both enantiomers are responsible for long term responses like maturation and seed dormancy, but it has also been reported that they induce different responses on the plant metabolism (Huang *et al.*, 2007). Natural occurring ABA is in the (+)-S-*cis* form (Venburg *et al.*, 2008a), the only one implied in fast responses (Taiz and Zeiger, 2010, Zaharia *et al.*, 2005). ABA is transported systemically through the plant in its conjugate form of glucose ester of

ABA (ABA-GE; figure 3) which acts as a perfect long-distance signal (Jiang and Hartung, 2008) and can be stored in the vacuole or in the apoplastic space (Dietz *et al.*, 2000).

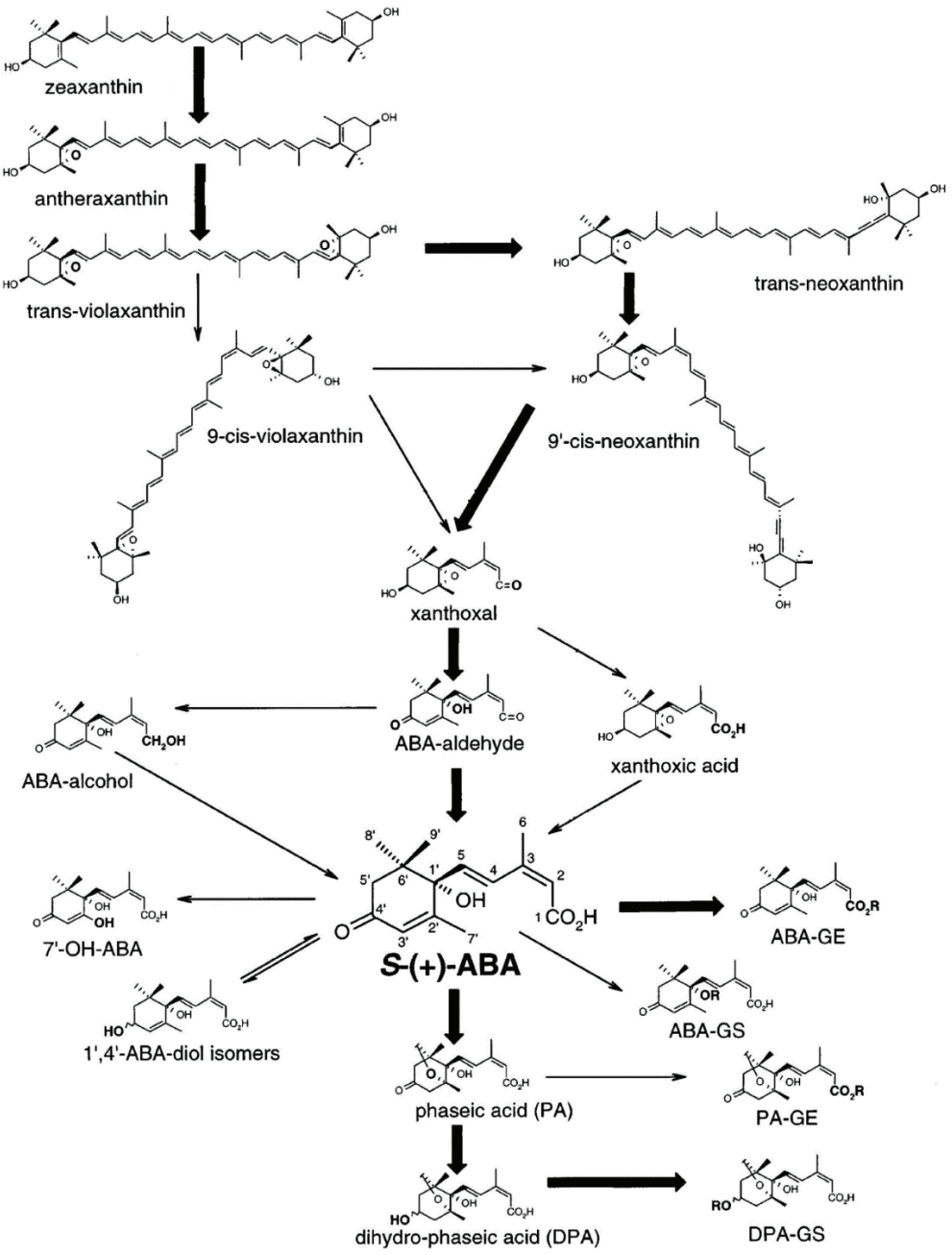


Figure 3. Biosynthetic pathway and chemical formulas of ABA and other related forms. Adapted from Finkelstein and Roch (2002).

ABA is likely to be the key hormone during the onset of ripening stage in non-climacteric fruits (Coombe and Hale, 1973, Davies *et al.*, 1997, Wheeler *et al.*, 2009, Deytieux-Belleau *et al.*, 2007, Düring *et al.*, 1978). A peak of endogenous ABA can be observed in berries at *veraison*, reaching a maximum concentration at the time of 80% colored berries, suggesting a key role of this hormone in the ripening phase (Gagne *et al.*, 2006a, Deytieux-Belleau *et al.*, 2007, Wheeler *et al.*, 2009). The concentrations of endogenous ABA can be highly variable and very different concentrations have been described in two seasons for the same vineyard, being earlier and higher in warm and dry years (Gagne *et al.*, 2006a). Endogenous free ABA is not uniformly distributed among the different berry tissues. Prior to the onset of ripening, ABA is present in a larger proportion in the berry flesh, thereafter flesh ABA levels decrease progressively to finally find at harvest a greater ABA proportion in the berry skin. Grape seeds also display higher concentrations of ABA compared with the flesh (Wheeler *et al.*, 2009), suggesting some transport of ABA from the flesh to the skin during development (Deytieux-Belleau *et al.*, 2007) or from the seeds to the flesh and the skin. However, the source of the ABA accumulated in berries is not clear, and there are reports that support the idea that leaves synthetize most of the ABA for subsequent transport via phloem (Antolin *et al.*, 2003), while others suggest that berry ABA levels are not always dependent on leaf ABA levels. It is improbable that berry ABA levels could be due to mobilization of glycosylated GE-ABA and so the berries (skin or seeds) may have the potential to synthetize ABA *in situ* (Cawthon and Morris, 1982, Deytieux-Belleau *et al.*, 2007, Okamoto *et al.*, 2004, Wheeler *et al.*, 2009). The recent report of ATP-binding cassette (ABC) transporter genes in *Arabidopsis*

thaliana encoding protein transporters that export or import ABA through the plasma membrane (figure 4), gives new insights on the mechanisms of regulation of ABA intercellular transportation and the active control of this process (Kuromori and Shinozaki, 2010).

Lund *et al.* (2008) have demonstrated through real-time quantitative polymerase chain reaction (RTQ-PCR) analysis, a positive regulation of 9-cis-epoxycarotenoid dioxygenase (VvNCED2) gene and a putative orthologue of an ABA receptor (VvGCR2) in berry seeds and pericarp, with the beginning of ripening and suggest a role of these genes in ABA signaling during maturation. ABA may enhance sink strength for carbohydrate allocation in cereals and other species (Brenner, 1987, Travaglia *et al.*, 2007).

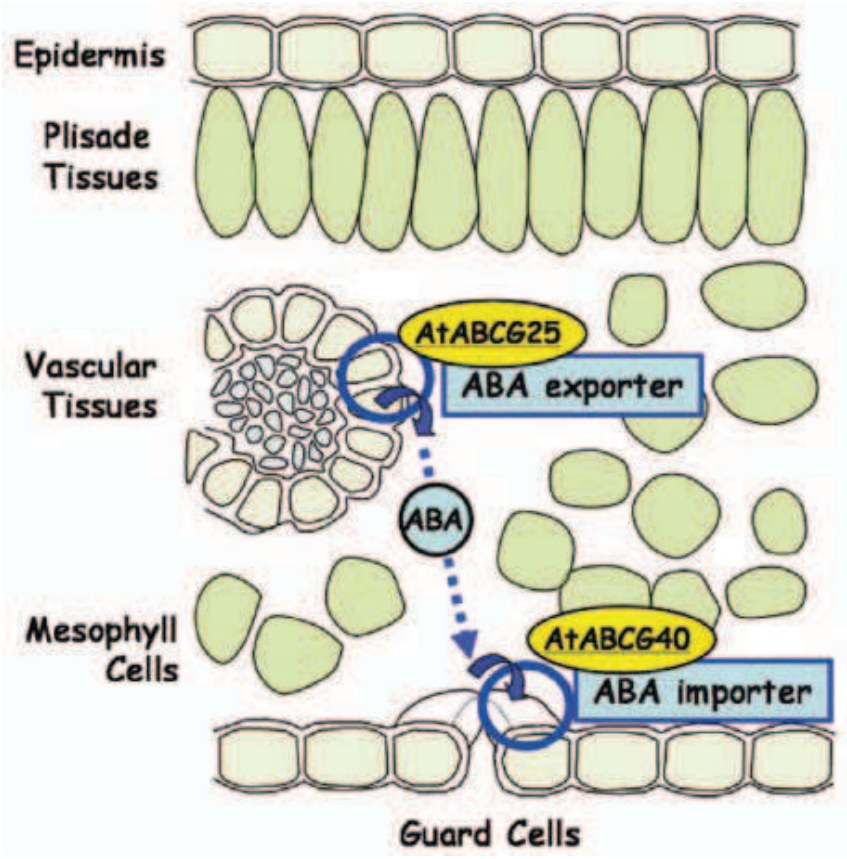


Figure 4. Illustration of the hypothetical ABA intercellular transportation (Kuromori and Shinozaki, 2010).

Cell culture experiments in Cabernet Sauvignon have shown that applied 2-chloroethylphosphonic acid (CEPA, also called Ethephon), an ethylene-releasing compound, as a single inducer failed to induce expression of phenylalanine ammonia-lyase (PAL) or triggered anthocyanin production (Faurie *et al*., 2009), while exogenous ABA treatment increased the cell ABA content and induced both the structural and regulatory genes involved in anthocyanin production, suggesting that ABA (and not ethylene) initiates anthocyanin production (Gagné *et al*., 2010). Nevertheless, Sun *et al*. (2010) demonstrated that endogenous ethylene induces the transcription of NCED1 that encodes 9-cis-epoxycarotenoid dioxygenase

(NCED) an enzyme that catalyzes the first step of ABA biosynthesis from carotenoids in chloroplasts, suggesting that ABA and ethylene are interplaying for the control of the onset of ripening. ABA and CEPA could be acting by different mechanisms but in the same direction and there may be a positive interaction between ABA and ethylene in the expression of some genes associated to PC synthesis like the UDP glucose-flavonoid 3-*O*-glucosyltransferase (UFGT) gene (Chervin *et al.*, 2009).

Grape ABA levels are modulated by different environmental factors like water stress, UV-B radiation and temperature (Yamane *et al.*, 2006, Quiroga *et al.*, 2009, Berli *et al.*, 2010). Even if water stress induce higher ABA levels and impacts positively grape quality, it impacts negatively on yield per plant (Castellarin *et al.*, 2007). Quiroga *et al*. (2009) demonstrated that ABA does not affect yield per plant, so the effect on yield is attributed to other effects of water stress different from ABA levels. Antolin *et al*. (2003) found that xylem and berry ABA concentration were correlated in non-irrigated grapevines. On the other hand, high temperature (30°C) reduced endogenous ABA concentration (Tomana *et al.*, 1979). After a temperature treatment in the ripening stage, concentration of ABA in berry skins was 1.6 times higher at 20°C than at 30°C (Yamane *et al.*, 2006). The results obtained by Berli *et al*. (2010) suggest that grape leaf defense system against UV-B includes the participation of ABA downstream in the signaling pathway.

Indole-3-acetic acid (IAA) has been described as a plant hormone that promotes cell elongation in the first stages of berry development and can have a negative effect on maturation by delaying *veraison* and diminishing coloration by reduction of the anthocyanin concentration in some non-climacteric fruits such as

strawberries and grapevines (Given *et al*., 1988, Ban *et al*., 2003, Deytieux-Belleau *et al*., 2007, Böttcher *et al*., 2010). IAA is present in large quantities at early developmental stages of the berries and then decrease until maturity. There is a small and fast increase of this hormone around 50% color-change berries at *veraison* (Deytieux-Belleau *et al.*, 2007), that could be indicating a collaboration between ABA and IAA for the control of ripening, impacting in an opposite way on the expression of genes involved in the control of the ripening process (Davies *et al.*, 1997, Deytieux-Belleau *et al.*, 2007).

With regard to other hormones, Symons *et al*. (2006), observed a dramatic increase of the bioactive brassinosteroid (BS) castasterone during the onset of ripening, as well as an increase in the levels of expression of two putative homologous genes of BSs biosynthetic pathway. This evidence would indicate that changes in endogenous levels of these hormones could influence the ripening development.

It is important to notice that plant hormones interact between them and with other factors. This fact does not agree with a linear visualization of hormone signaling, where one hormone controls a single aspect of plant growth and development. The cross-talk between hormones makes the understanding of grape ripening hormonal control even more complex (Gazzarrini and McCourt, 2003).

1.4 Phenolic Compounds of Grapes and Wine

1.4.1 Characterization of the Major Phenolic Compounds of Grape and Wine

PC are products of the secondary metabolism of the plant, a chemically heterogeneous family of compounds, which are characterized by the presence of a phenol group (an aromatic ring with a functional hydroxyl group). In plants, these compounds comply with various functions, such as defense against herbivores and pathogens, mechanical support, attracting pollinators, seed dispersal and absorption of harmful ultraviolet radiation, among others (Taiz and Zeiger, 2010).

The concentrations of the different PC present in the wines are different for red, rosé and white wines. The red wines generally have much higher concentrations of total PC in relation to the white wine. This is mainly due to the content of PAs and anthocyanins, which are not found in the white wine and a higher content of hydroxycinnamic acids, flavan-3-oles and other non-flavonoids (Singleton, 1992).

Due to the similarity of the structures, most of the grape- and wine-related PC can be classified into three groups: (1) C6-C1: the group of the hydroxybenzoic acids, *i.e*: gallic acid, salicylic acid and ellagic acid; (2) C6-C3: the group of the hydroxycinnamic acids, *i.e*: cinnamic acid, p-coumaric acid, caffeic acid, coutaric acid, caftaric acid and fertaric acid and; (3) C6-C3-C6: the group of flavonoids (figure 5). This latter group includes the anthocyanins, responsible for the color of red wine; the flavan-3-oles, associated mainly with the bitter taste; the PAs or condensed tannin, corresponding to polymers of flavan-3-oles and responsible for

the sensation of astringency; and the flavonols known for its antioxidant effect (Margalit, 1997).

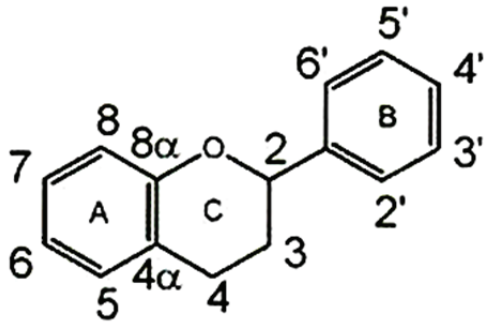


Figure 5. Structure and carbon numbering of flavonoids (Kennedy *et al.*, 2006)

In grapes and wine, hydroxycinnamates are esterified with tartaric acid (Margalit, 1997). They are present in red and white wines in similar concentrations and being the most abundant PC in white wine (table 2). Due to the low ratio between skin and flesh in the berries, the hydroxycinnamic acids of grape must and wine are mainly derived from the flesh.

Table 2. Classes, concentration and impact of major wine phenolic compounds.

		RED WINE ($mg\ L^{-1}$)	WHITE WINE ($mg\ L^{-1}$)	IMPACTS ON WINE	CLASS MEMBERS
NON-FLAVONOID	Hydroxybenzoic acids	50-100	10-15	Bitter taste	Gallic acid
	Hydroxy-cinnamates	50-200	120-200	Browning, bitter taste and precursor of volatile phenols	*p*-coumaric acid (esterified with tartaric acid)
	Stibenes	1.5-5	tr.	Antioxidant, anti-inflammatory and anti-carcinogenic	Resveratrol
FLAVONOIDS	Anthocyanins	20-500	-	Color properties of red wine	Cyanidin-3-G Petunidin-3-G Malvidin-3G
	Flavonols	10-50	tr.	Antioxidant and cofactor of co-pigmentation	Myricetin-3G Quercetin-3G
	Flavan-3-ols	150-200	15-25	Bitter taste and some association with astringency	(+)-Catechin (-)-Epicatechin
	Proanthocyanidins	450-900	0-10	Astringency and color stability (polymeric pigments)	

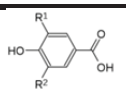
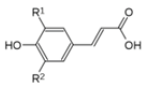
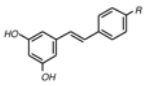
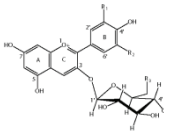
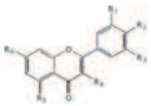
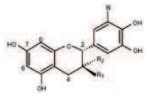
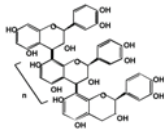
Adapted from Frankel *et al*. (1995), Okuda and Yokotsuka (1996), Singleton (1992), Vivas *et al*. (2003).
tr: Trace amounts.

Anthocyanins accumulate in the vacuoles of thick walled cells of the hypodermis in the skin. The anthocyanidins found in grapes (Vitis spp.) are delphinidin, cyanidin, petunidin, peonidin and malvidin, that can be found in the form of glycoside (monoglucoside or diglucoside), acylated (acetylglucoside, coumaroylglucoside and in lesser extent caffeoylglucoside) or not acylated derivatives (Fournand *et al.*, 2006). In the case of *Vitis vinifera*, they are only in monoglucoside (Boulton *et al.*, 1996) form and in particular in the cv. Pinot noir, they are only nonacylated anthocyanins, so it is only possible to find five of the 20 anthocyanins present in the rest of cultivars (Mazza *et al.*, 1999). Along with other

PC and non-PC, during the winemaking process, anthocyanins extraction from the solid phase of the cap (grape skins and seeds) to the liquid phase of the must/wine takes place (Canals *et al.*, 2008, Ribéreau-Gayon *et al.*, 2006). Usually there is a maximum extraction of these compounds before the end of the fermentation. The color of wine is affected by the balance of the anthocyanin derivatives in the wine solution. The factors that affect this balance are mainly the pH and the concentration of sulfur dioxide. In the wine solution, most of the anthocyanins are present as the non-colored hemiacetal form and less than 10% are in the form of flavylium ion with color (Fulcrand *et al.*, 2004, Kontoudakis *et al.*, 2011). In addition to the above, wine color is affected by co-pigmentation phenomena, through which the anthocyanins combine with other PC, forming associations that allows the stabilization of wine color (Boulton, 2001).

Flavonols are found in the forms of glucosides, galactosides and glucuronides. Their accumulation depends on grapes light exposure (Price *et al.*, 1995, Spayd *et al.*, 2002, Matus *et al.*, 2009) and they are usually found in lower concentrations than all previous PC classes (table 3).

Table 3. Classes, molecular structure and concentration of major PC in wine grape tissues.

	CLASS	MOLECULAR STRUCTURE	SKIN	SEED (mg Kg^{-1})	FLESH	PROPERTIES KNOWN
NON-FLAVONOIDS	Hydroxybenzoic acids (C6-C1)		-	40-460	-	Hydrolysable tannins
	Hydroxycinnamates (C6-C3)		5-50	-	16-430	Oxidation, browning, volatile phenol formation, copigmentation
	Stibenes (C6-C2-C6)		5-10	-	-	Response to fungi attack and high UV light exposure
FLAVONOIDS (C6-C3-C6)	Anthocyanins		200-5000	-	-	Color properties
	Flavonols		20-95	-	-	Plant UV protection and antioxidant
	Flavan-3-ols		14-66	50-1000	tr.	building blocks for proanthocyanidins
	Proanthocyanidins		20-750	1250-1700	-	Protection from herbivore predation

Adapted from Jeandet *et al.* (1991), Okuda and Yokotsuka (1996), Singleton *et al*. (1986) and Vivas *et al*. (2003).

Flavan-3-ol monomers location at the cellular level, has been related to the cell walls of the outer integument of the seed (Cadot *et al.*, 2006a). Factors that lead to its extraction in winemaking are maceration time, temperature and ethanol concentration (Sacchi *et al.*, 2005). The flavan-3-ols molecules present two stereo centers. The most abundant monomers in grape and wine are in 2,3-*trans* and 2,3-*cis* configurations, they are (+)-catechin and (-)-epicatechin respectively. However,

it is found also (-)-epigallocatechin and a form esterified with gallic acid, (-)-epicatechin gallate (Margalit, 1997, Ribéreau-Gayon *et al.*, 2006). In the seeds, the presence of (-)-epigallocatechin has not been detected and there is a high proportion of (-)-epicatechin gallate monomers, which usually cannot be found in the skins (Cheynier, 2005). This feature is useful for determining the origin of wine tannins: the proportions of grape skin to grape seed tannins (Peyrot Des Gachons and Kennedy, 2003).

PAs or condensed tannins are the consequence of flavan-3-oles polymerization. They are to a greater extent found in the internal cells of the seed coat soft parenchyma (between the cuticle and the hard seed coat) and in the inner cell layers of the seed integument. Secondarily, they are found in the vacuoles of the hypoderm skin cells and in the tissues of the grape cluster stem (Cadot *et al.*, 2006b). The PAs can be classified according to its B ring subunit substitution (figure 5): Those that possess an hydrogen atom radical at carbon 5' receive the name of procyanidins, such as (+)-catechin and (-)-epicatechin and those that possess an hydroxyl group radical at carbon 5' receive the name of prodelphinidins, such as (-)-gallocatechin and (-)-epigallocatechin. On the other hand, there are subunits esterified with gallic acid, such as (-)-epicatechin gallate. Of all chemically possible forms, the main four subunits founds in grapes and wine are: (+)-catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin gallate (Margalit, 1997). In skin tissue, (-)-epicatechin and (-)-epigallocatechin are present in significant proportions, in particular as extension subunits and terminal subunits respectively and (-)-epigallocatechin-3-gallate is absent or present in very small amounts (Gagne *et al.*, 2006b).

The condensed tannins are extracted to the solution of the must/wine during the processes of pre-fermentative, fermentative and post-fermentative macerations (Sacchi *et al.*, 2005). Its relevance is due to: (1) they have the primary responsibility in the astringency sensation of red wine, tactile sensation generated by the interaction of these compounds with glycoproteins from saliva (McRae and Kennedy, 2011) and (2) they combine with anthocyanins forming stable colored tannins, also called polymeric pigments (Adams and Harbertson, 1999).

1.4.2. Phenolic Compound Biosynthesis

Two metabolic pathways, the shikimic acid pathway and the malonic acid pathway, are involved in the origin of the phenylpropanoid pathway, which is responsible for the biosynthesis of the PC (figure 6). The first pathway is present in plants, fungi and bacteria, but absent in animals and converts simple carbohydrates derived from the glycolysis and the pentose phosphate pathway in aromatic amino acids, among these phenylalanine, which is transformed in cinnamic acid in a reaction catalyzed by the PAL, a key enzyme. These pathways mainly originate the synthesis of proteins, lignins and PC (Taiz and Zeiger, 2010). The phenylpropanoid pathway takes place in the cytoplasm associated to the endoplasmic reticulum (Poustka *et al.*, 2007).

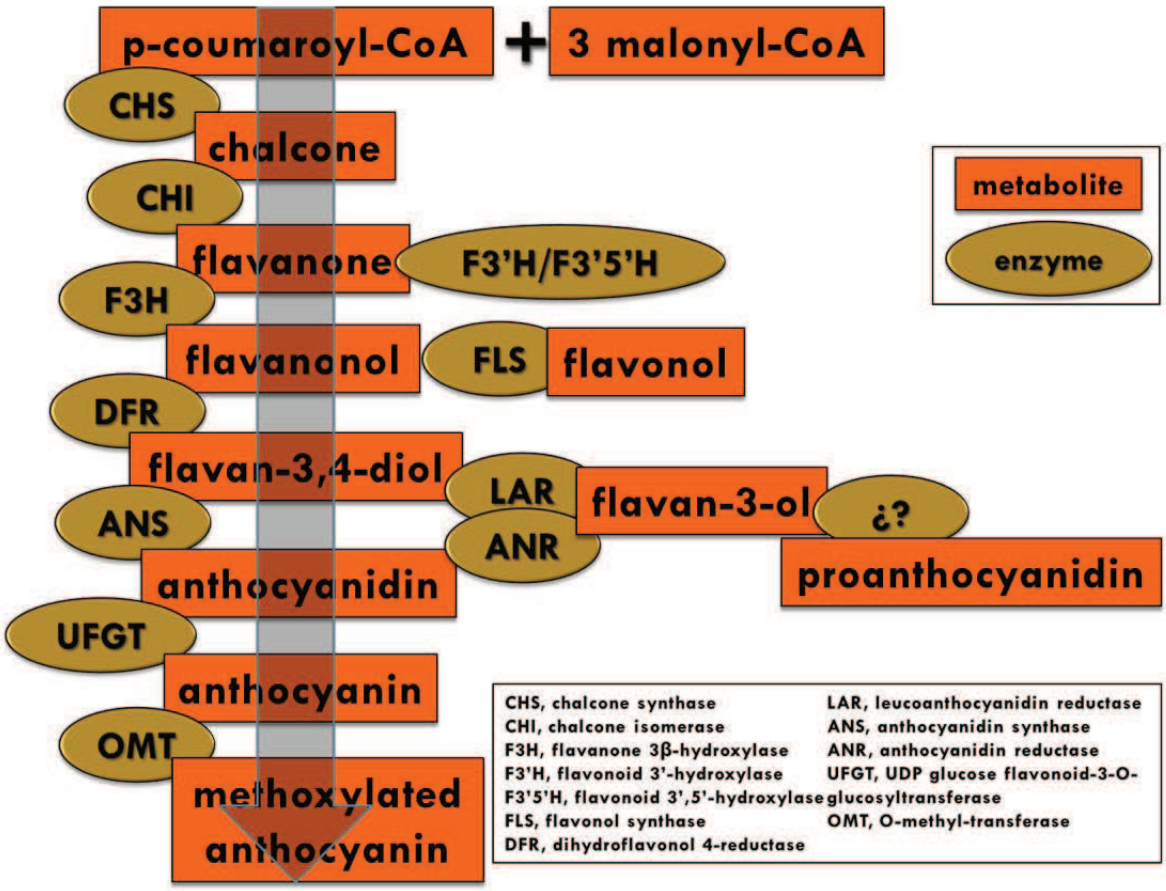


Figure 6. Phenylpropanoid pathway and enzymes involved

Cells that do not produce phenylpropanoids, proteins, or do not lignificate, place a minimal amount of carbon in the shikimic acid pathway. Cells that lignificate place a high percentage of carbon in that pathway. Finally, cells that accumulate PC do place a percentage of carbon according to the amount of PC accumulated in the metabolic pathway. The berry cells are characterized by low protein content and high phenylpropanoids content, so it is believed that this pathway is matched to the production of PC (Adams, 2006).

Flavonoid biosynthesis begins with the reaction catalyzed by the enzyme chalcone synthase (CHS), point of union of shikimic acid and malonic acid pathways. If the CoA ester used as reactant by this enzyme is *p*-coumaroyl-CoA, then this should lead as a final product to pelargonidin, an anthocyanin that presents only one hydroxyl group in the B ring (a monohydroxylated anthocyanin). The fact that it is possible to find in grapes other flavonoids with only one hydroxyl group in the B ring, as kaempferol and dihidrokaempferol, supports the idea that *p*-coumaroyl-CoA is in fact used as reactant at least in part by the enzyme. However, it has been observed that the grape berries do not accumulate pelargonidin. This fact could be explained by the presence of very effective hydroxylase enzymes that transform almost all monohydroxylated to dihydroxylated and trihydroxylated flavonoids which are present in abundance in the grape berry (Boss *et al.*, 1996a).

In the tissues of the skin, the phenylpropanoids as phenylalanine and the free and CoA esters of cinnamic acids are found in low concentrations. By contrast, the anthocyanins behave as terminal biosynthesis products with low degradation, which favors its accumulation in these tissues. The rest of the products suffer accumulation and declination. In all cases studied, it is better known how is the production and less known how and why they decline (Adams, 2006).

The discovery of the enzyme anthocyanidin reductase (ANR), first described by Xie *et al*. (2003), revealed that an anthocyanidin is intermediary in the route of synthesis of (-)-epicatechin, the most abundant PA unit. This intermediary allows changing the stereo configuration generated by the chalcone isomerase (CHI) enzyme. In this way, (+)-catechin (2,3-*trans* stereo configuration) can be directly

synthesized from flavan-3,4-diol by the leucoanthocyanidin reductase (LAR) enzyme, but (-)-epicatechin should pass through the intermediary cyanidin.

Boss *et al*. (1996a) quantified anthocyanins by high-performance liquid chromatography (HPLC) in cv. Syrah during maturation, leaving in evidence that, as in the other PC classes, one member of the class is the dominant, in this case malvidin-3-glucoside and its acylated forms. This trend can be checked in the table 4, which shows the results of a study of flavonoid content in the hypodermic cells of the DeChaunac hybrid, carried out by Moskowitz and Hrazdina (1981).

Table 4. Phenolic compounds classes concentration and proportion of the class dominant member (Moskowitz and Hrazdina, 1981)

Class	Concentration (mmol g^{-1})	Dominant member	Proportion (%)
Anthocyanins	2.88	Malvidin-3-glucoside	42
Hydroxycinnamates esters	0.98	Caftaric acid	77
Flavonol glycosides	0.19	Quercetin glicoside	93

Finally, the high variability of anthocyanidin concentration in different hypodermic cells, could leave evidence of single-cell control of the biosynthetic process (Moskowitz and Hrazdina, 1981).

1.4.3. Phenylpropanoid Pathway Regulation

Metabolic regulation acts at different levels, some of these are gene transcription, post-transcriptional regulations, gene translation and enzymatic activity, altering the final level of metabolites. The most relevant progress in the comprehension of phenylpropanoid pathway control derives from the study of complex multi gene regulation at the transcriptional level.

Transcription regulation

The main transcription factors (TFs) acting over structural genes of the phenylpropanoid pathway are from three different families (figure 7): R2R3-MYB, basic helix-loop-helix (bHLH, also known as MYC) and tryptophan-aspartic acid repeat (WDR or WD40) proteins (Payne *et al.*, 2000, Baudry *et al.*, 2004). They interact to form a complex that binds specific DNA binding motifs found in the promoter region of the structural genes. The analysis of the promoter sequence of structural genes of the phenylpropanoid pathway reveals the existence of different DNA binding motifs, these elements allow the induction or repression of the genes in response to different factors, between them sucrose, light, calcium, and other TFs (Gollop *et al.*, 2001).

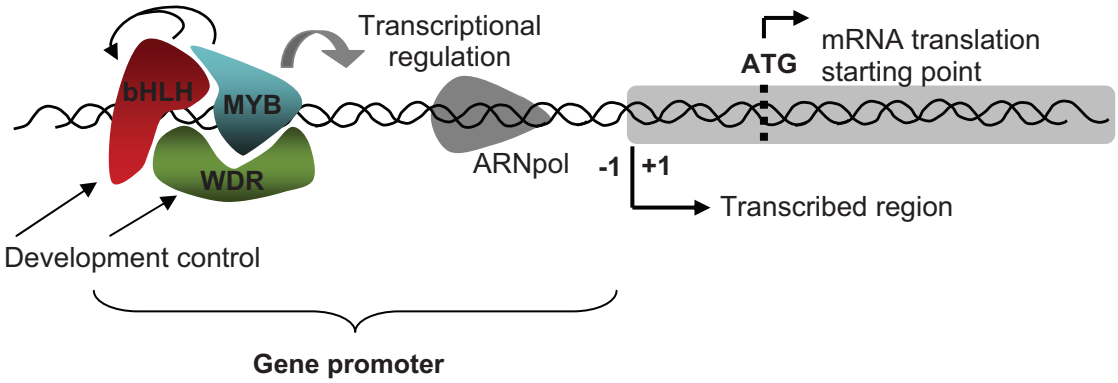


Figure 7. MYB-bHLH-WDR transcription factor complex regulation. Adapted from Matus (2008).

According to the similarities with de MYB orthologues in *Arabidopsis thaliana*, Matus *et al*. (2008) have classified 108 putative MYB family members in *Vitis vinifera*. The MYB genes that have been identified in grapevines are shown in table 5. The bHLH type TF MYC1 protein together with MYB and WDR type TFs form a complex that bind DNA. Hichri *et al.* (2010) have reported this protein to interact with MYB5a, MYB5b, MYBA1/A2, and MYBPA1 proteins, promoting anthocyanin accumulation in grape cells. MYB factors have been described to target some specific structural genes: MYBA1 and MYBA2, that are expressed only from *veraison*, would acts over UFGT; MYBPA1, that have been detected in flowers, skin and seeds, would acts over LAR1, ANR, CHI, flavonoid-3'5'-hydroxylases (F3'5'H) and leucoanthocyanidin oxidase (LDOX); and MYBF1 would acts over flavonol synthase (FLS) gene (Kobayashi *et al.*, 2002, Bogs *et al.*, 2007, Czemmel *et al.*, 2009). MYB5a and MYB5b show expression at the first stage of berry development and they are related to synthesis of tannins (Deluc *et al.*, 2006, Deluc *et al.*, 2008).

Table 5. MYB, bHLH, and WDR transcription factor proteins involved in the regulation of the phenylpropanoid pathway in grapevine. Adapted from Hichri *et al*. (2011).

TF family	Protein	Function	Reference
bHLH	VvMYC1	Promotion of anthocyanin accumulation in grape cells	Hichri *et al*. (2010)
MYB	VvMYBA	Regulation of anthocyanin accumulation	Kobayashi *et al*. (2002)
	VvMYBPA1	Induction of proanthocyanidin synthesis	Bogs *et al*. (2007)
	VvMYBPA2	Induction of proanthocyanidin synthesis	Terrier *et al*. (2009)
	VvMYB5a	Regulation of phenylpropanoid accumulation	Deluc *et al*. (2006)
	VvMYB5b	Regulation of phenylpropanoid accumulation	Deluc *et al*. (2008)
	VvMYBF1	Regulation of flavonol accumulation	Matus *et al.* (2008) and Czemmel *et al*. (2009)
WDR	VvWDR1	Contributes to the accumulation of anthocyanins	Matus *et al.* (2010)

The transcription regulation is also controlled by epigenetic mechanisms. This kind of regulations has been described for the phenylpropanoid pathway in maize (Hernandez *et al.*, 2007).

Enzymes activity

After gene transcription and translation, the activity of an enzyme can modify the metabolite amount found and the phenotypic expression of a gene. De Vetten *et al.* (1999) have shown that a particular cytochrome is essential to improve the

activity of the F3’5’H enzyme, that seems to act as an electron donor to the NADPH:cytochrome P450 reductase related with cytochrome P450 proteins. After this report, Brugliera *et al.* (2000) reported that when the petunia F3’5’H and cytochrome b5 genes are introduced together, maximal activity and phenotypic expression of F3’5’H was found, showing the importance of this enzyme activity. On the other hand, Saslowsky and Winkel-Shirley (2001) have shown how specific mutations on the CHS enzyme reduce its activity, destabilizing the protein or interfering with dimerization. Gagne *et al*. (2009) studied LAR and ANR activity and they found that both enzymes are activated early, facilitating precocious tannin biosynthesis.

As is shown in figure 8, phenylpropanoid pathway regulation is complex and allows a regulated genes expression in function of the time, the phenology, the specific tissue, the environmental conditions, the hormones, etc.

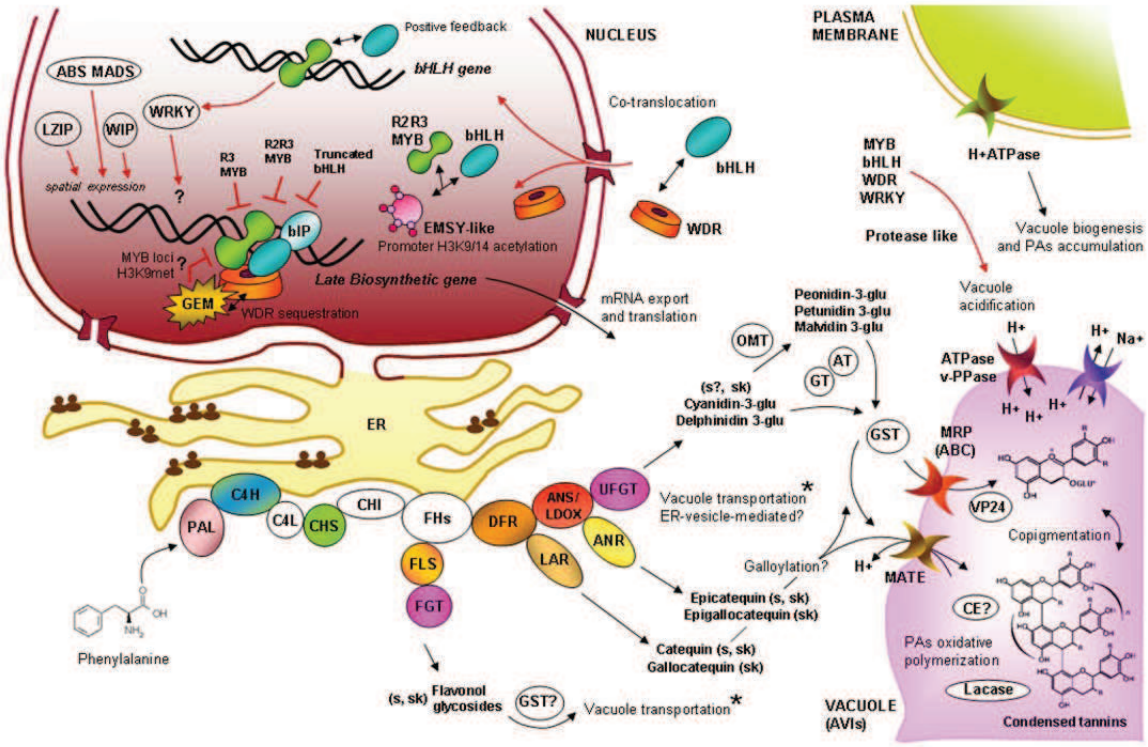


Figure 8

Overview model of the phenylpropanoid pathway and its regulation. Grape anthocyanins, flavonols and flavan-3-ol monomers are differentially synthesized in berry skin (sk) and seeds(s). Asterisks show alternative pathways for tagging and ER-vacuole transport mechanisms. Biosynthetic enzymes which appear under color have been proved to be regulated at least by one of the MYB, bHLH or WDR regulators. PAL: phenylalanine ammonium liase, C4H: cinamate 4 hydroxylase, C4L: cinamate 4 liase, CHS: chalcone synthase, CHI: chalcone isomerase FHs: flavonoid hydroxylases (F3H, F3'H, F3'5'H), FLS: flavonol synthase, FGT: flavonol glucosyl-transferase, DFR: dihydroflavonol reductase, ANS/LDOX: anthocyanin synthase/ leucoanthocyanidin oxidase, LAR: leucoanthocyanidin reductase, ANR: anthocyanidin reductase, UFGT: UDP-glucose flavonoid 3-*O*-glucosyltransferase, OMT: O-methyl-transferase, AT: acyltransferase , GT: glucose transferase (e.g., 3-glucoside rhamnosyltransferase), GST: glutathione-S-transferase, MATE: multidrug and toxic compound extrusion transporter, ABC MRP: ATP-binding cassette multidrug resistance-associated protein, VP24: vacuolar 24-kDa protein possibly involved in glutathione removal, CE?: hypothetical condensing enzyme necessary for flavan-3-ol polymerization, GEM: GL2 expression modulator (Matus, 2008)

1.4.4. Polymerization of Proanthocyanidins

The PA polymerization is carried out by inter flavan-3-ol monomer unions. These unions normally are between carbons four and eight (C4-C8) and to a lesser extent between carbons four and six (C4-C6) (Margalit, 1997).

In enology, PAs or tannin quality is a concept related to the kind of roughness these polymers are able to produce, both in intensity and in other possible sensory characteristics. Chemically, PA quality is determined at first instance by the degree of polymerization of these polymers, which usually varies between three and 83 subunits (McRae and Kennedy, 2011). Secondarily, PA quality would also be affected by the amount of gallic acid esters.

Due to the existence of different subunit constituent and the possibility of forming polymers of varying subunits number, one can calculate that there are tens or hundreds of thousands of unique species, which are impossible to identify with accuracy with the current analytical techniques, including reverse phase HPLC (Adams and Harbertson, 1999). For measuring PAs degree of polymerization, HPLC after acid-catalyzed cleavage methodology is used. In general, skin PAs have a greater degree of polymerization than seed ones (Kennedy *et al.*, 2001).

The way that interflavan C4-C6 and C4-C8 bonds establishes, the place of the cell where this process occurs and the way they are transported through the tonoplast (in the case biosynthesis does not occur in the vacuole, but in the cytoplasm, the plastid or another organelle), to the vacuole, place of final storage, are still open questions. It has been observed that plasma membrane H^+-ATPase *Arabidopsis thaliana* mutants may not be able to cumulate tannin in the vacuoles of endothelial cells of the seed coat (Baxter *et al.*, 2005). This fact gives standing to

believe that PAs biosynthesis would occur outside the vacuole and that there is an active transport system involved.

1.4.5. Phenolic Compound Evolution during Grape Development

The changes observed in PC composition during grape development show an independent and separated regulation in the different tissues of the grape berry: the hypodermis in the berry skin, the mesocarp in the berry flesh and the thin walled cells of the seed coat.

It is necessary to take into consideration that PC concentration will vary with changes in berry volume, decreasing when the berry is growing during the first and third stages of maturation and increasing when the berry is shrinking by dehydration. It is for this reason that reporting of information in a "per berry" base is of great relevance to display separately the effects of changes in volume with respect to PC net biosynthesis.

Phenolic Compound Evolution in the Berry Skin

During maturation, PAs join to the inside tonoplast proteins and to the cell wall polysaccharides via glycosidic bonds (Amrani-Joutei *et al.*, 1994). The PAs in the hypodermal tissue of the skin appear early in the berry development and after *veraison* they vary little or present small decreases in a "per berry" basis (Adams and Harbertson, 1999, Jordao *et al.*, 2001, Fournand *et al.*, 2006), matching at harvest date the amount of PAs found in the seed (Harbertson *et al.*, 2002).

With regard to the mean degree of polymerization (mDP), it seems to increase to some extent with maturity (Fournand *et al.*, 2006). A study carried out

in cv. Syrah by Kennedy *et al*. (2001), showed that mDP varies from 7.3 subunits three weeks after berry set to 11.3 subunits at ripening and to 27 subunits at harvest, coupled with an increase in the proportion of (-)-epigallocatechin extension subunits and an increase in the level of anthocyanins associated with PAs. Gagne *et al*. (2006b) suggested that tannin polymerization in the skins takes place near the cell walls and is aggregated in the vacuole during berry development.

In the skin there are monomeric and polymeric pigments (Harbertson *et al.*, 2003). With regard to the first ones, mainly anthocyanins, its presence has been detected two to three weeks prior to *veraison* (Darne, 1988), however their accumulation becomes visible in red cultivars typically in *veraison*, being one of the most obvious facts in the vineyard. This accumulation occurs together with the accumulation of sugars in the flesh and reaches a maximum around harvest date (Kennedy *et al.*, 2002) or approximately when the flesh reach the higher accumulation of sugars to then fall slightly or remain stable (Fournand *et al.*, 2006).

Two classes of polymeric pigments have been observed in the skin: small polymeric pigments (SPP) that do not precipitate with proteins and large polymeric pigments (LPP) that do so. The grapes generally contain very little polymeric pigment as compared with the wine, being almost all SPP and little LPP. These results suggest that most of the LPP found in wine, is formed during winemaking (Harbertson *et al.*, 2003). With the use of mass spectroscopy, it has been confirmed the existence of anthocyanin oligomers in berry skin extracts (Vidal *et al.*, 2004).

Phenolic Compound Evolution in the Berry Flesh

The flesh of the majority of the wine grape varieties contains mostly hydroxycinnamates and does not contain pigments or PAs. Hydroxycinnamates accumulation takes place prior to *veraison*, reaching a maximum at that time, then there is a small decrease in their content, to remain relatively constant during maturation (Romeyer *et al.*, 1983). The total concentration of hydroxycinnamates at harvest time is very variable and depends on the cultivar, without a different behavior between red and white cultivars (Singleton *et al.*, 1986). It is unknown how these PC vary with respect to the “terroir” and vineyard cultural practices.

Phenolic Compound Evolution in the Berry Seed

Initially, seeds are green, thick and they have a flexible coat. Starting the *veraison* they become brown, dehydrated and the coat hardens (Kennedy *et al.*, 2000b). It is because of this feature that seed coat appearance and color have been proposed as good indicators of ripeness in wine grapes (Ristic and Iland, 2005). The soluble PC fraction of the seeds is mainly composed of PAs and to a lesser extent of flavan-3-oles monomers: (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate (Kennedy *et al.*, 2000a).

The accumulation of PAs in the seed is carried out in the first ripening stage together with the gain of berry fresh weight, peaking in a period of time covered between three weeks prior to *veraison* and at the *veraison*, when it is achieved seed lignification (Cadot *et al.*, 2006a). During this previous time period, the number of flavan-3-ol monomers increases 5 fold, revealing that PAs and flavan-3-ols monomers accumulate at different times (Kennedy *et al.*, 2000b).

After *veraison*, in the ripening period, parallel to the accumulation of sugars and degradation of malic acid in the flesh, Adams and Harbertson (1999) and Harbeston *et al*. (2002) described a reduction in the amount of PAs (parallel to the phenomenon of browning and drying of the seeds), which can occur in a rapid or slow manner according to the season (Jordao *et al.*, 2001) to then maintain relatively constant quantities during the four weeks prior to harvest (De Freitas *et al.*, 2000, De Freitas *et al.*, 1998). Pastor Del Rio and Kennedy (2006) reported in cv. Pinot noir that maturity does not have a significant effect on the total PA content in wine, but the proportion of seed-derived extracted PAs consistently increased as ripening advanced. During maturation of the grape berries, the flavan-3-ol content fell in the seeds whereas PA level increased. This suggests an interrelationship between these compounds (Romeyer *et al.*, 1986). Downey *et al*. (2003) showed that the decline of PAs after *veraison* may be due to a covalent linkage of this tannins to the insoluble matrix of the seed. Jordao *et al*. (2001) noted that the level of oligomeric PAs decreases during maturation, while monomers grow during the early stages of berry development, followed by a sharp drop and stabilization. Kennedy *et al*. (2000b), based on documented evidence, has proposed that the observed changes in PC after *veraison* could be explained by oxidation events. Thus, there are authors that on the basis of different analytical techniques, have been reported increases or decreases in the total tannins after *veraison* (De Freitas *et al.*, 2000).

The evolution of seed mDP measured by reverse phase HPLC, is unclear and values from 8.29 units at *veraison*, to 5.63 units at ripening and to 11-15 units at over-ripening have been reported (Kennedy *et al.*, 2000b). However, it has been

reported increases and decreases of mDP at this time, as well as changes in the PA subunits composition (Kennedy *et al.*, 2001, Kennedy *et al.*, 2002). These contradictory reports could be the consequence of different polymerization processes in the cell wall and in the cell vacuole. Geny *et al*. (2003) reported a superior increase of tannin mDP in a cell wall fraction when it was compared to the mDP of a cell internal fraction during ripening stage.

The evolution of seed PAs during berry development has a direct impact on the wine quality. From the total grape PC, 44% is found in seeds (Singleton and Esau, 1969), mainly PAs. Seed derived PAs impact red wine astringency: higher seed PA content have as a consequence a more astringent wine (Pastor del Rio and Kennedy, 2006).

1.5 Plant Growth Regulators Application and Grape Quality

1.5.1 2-Chloroethylphosphonic Acid

CEPA is an ethylene releasing compound (figure 9). In the tissues of the plant at pH above 4.5 it breaks down in ethylene, chloride and phosphate (Maynard and Swan, 1963). The treatment of grapes with CEPA, has been extensively studied (Szyjewicz *et al.*, 1984). It has been demonstrated that CEPA treatments significantly increased endogenous ethylene content (El-Kereamy *et al.*, 2003).

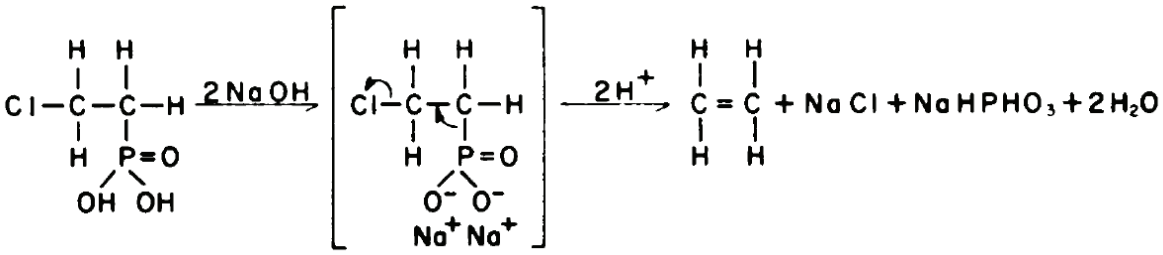


Figure 9. Breakdown of CEPA into ethylene in the presence of a base (Warner and Leopold, 1969).

Ethylene and CEPA treatments around *veraison* time induce an increase in berry diameter (Amiri *et al.*, 2009) that could be explained by an increase in sap intake and modifications of the cell wall structure, allowing cell elongation (Chervin *et al.*, 2008), nevertheless some reports show no effect of CEPA treatment at *veraison* on berry weight (El-Kereamy *et al.*, 2003). CEPA treatment in cv. Flame seedless decreases berry firmness (Peppi *et al.*, 2006). A generally controversial subject is the effect of CEPA treatments on the total solid soluble (TSS) content, showing increments and diminutions of this parameter (Szyjewicz *et al.*, 1984, Delgado *et al.*, 2004, Amiri *et al.*, 2009, Eynard, 1975). CEPA treatments at *veraison* increase grape color and the content of anthocyanin (table 6) and seem to increase primarily the end product anthocyanins peonidin and malvidin (El-Kereamy *et al.*, 2003, Human and Bindon, 2008, Amiri *et al.*, 2009, Szyjewicz *et al.*, 1984, Powers *et al.*, 1980, Tira-Umphon *et al.*, 2007, Roubelakis-Angelakis and Kliewer, 1986).

Microarray experiments have shown that the treatment of berries with ethylene significantly affect the concentration of 73 gene transcripts (from 14.562

tested genes) at the onset of ripening stage (Chervin *et al.*, 2008). CEPA treatments are able to stimulate expression of some genes implied in flavonoids and anthocyanins biosynthesis pathway, increasing the transcript levels of CHS and flavanone 3β-hydroxylase (F3H) genes and to lesser extent anthocyanidin synthase (ANS) and UFGT genes, not affecting transcript levels of the dihydroflavonol 4-reductase (DFR) gene (El-Kereamy *et al.*, 2003). Moreover, treatment with 1-methylcyclopropene (MCP, a specific inhibitor of ethylene receptors) inhibited UFGT transcript accumulation (Chervin *et al.*, 2009). These results contribute to the hypothesis that the ethylene signal is likely a regulator of UFGT expression in grapes and this stimulation has shown to be independent from the MYBA1 transcription factor (Tira-Umphon *et al.*, 2007).

CEPA treatments may induce an acceleration of the normal peroxidase activity that could be responsible for increasing the IAA degradation, thereby rendering the tissue sensitive to ethylene and enhancing ripening (Szyjewicz *et al*., 1984).

1.5.2. Abscisic Acid

Exogenous ABA treatments have shown to be able to increase the internal ABA levels (Deytieux-Belleau *et al*., 2007), to accelerate the beginning of ripening and to decrease the firmness of the berry (Peppi and Fidelibus, 2008, Deytieux-Belleau *et al.*, 2007, Gagne *et al.*, 2006a, Peppi *et al.*, 2008, Lacampagne *et al.*, 2009). ABA treatments at *veraison* and later showed no effect on berry weight (Sandhu *et al.*, 2011).

ABA treatments at *veraison* (10 and 75 % color-change) seem to increase TSS (Omran, 2011). ABA treatments may be able to hasten the initiation of sugar accumulation when applied early (before *veraison*) but cannot enhance it once ripening has already commenced (Davies and Böttcher, 2009). Nevertheless, there are reports of experiments on muscadine grapes that showed no effect of ABA treatments at *veraison* and later on TSS and pH (Sandhu *et al.*, 2011, Lurie *et al.*, 2009).

As is shown in table 6, ABA treatments have induced higher grape skin coloration and a greater accumulation of anthocyanins (Peppi *et al.*, 2008, Jeong *et al.*, 2004, Omran, 2011, Ban *et al.*, 2003, Gagne *et al.*, 2006a, Deytieux-Belleau *et al.*, 2007, Villalobos, 2011, Hiratsuka *et al.*, 2001). Anthocyanin concentration of the treated grapes evidence changes as soon as a week after the treatment (Peppi *et al.*, 2008).

ABA treatment 14 days after anthesis (DAA) have shown to be able to reduce the tannin content (Lacampagne et al., 2009) while applied days before *veraison* have shown to be able to increase the tannin and (-)-epicatechin contents and reduce (+)-catechin content (Villalobos, 2011). Flavonols seems to be transiently induced by ABA treatment and finally repressed to the end of ripening (Sandhu *et al.*, 2011, Villalobos, 2011). Other PC that seems to be transiently increased by ABA is the ellagic acid. As a consequence of improved PC accumulation, there is an increased antioxidant capacity of grape skins (Sandhu *et al.*, 2011).

Higher accumulation of anthocyanin and other PC as a consequence of the ABA treatments could be explained by the effect on structural and regulatory gene

expression. The ABA treatment has shown the ability to modify transcript accumulation of several structural genes, including PAL, CHS, CHI, DFR, F3H, LDOX, LAR2, ANR and UFGT (Ban *et al*., 2003, Jeong *et al*., 2004, Lacampagne *et al*., 2009). Some of these genes are controlled by transcriptional regulators MYBA1 and MYB4A, also regulated by ABA treatment (Ban *et al.*, 2003, Jeong *et al.*, 2004, Villalobos, 2011). However, there seems to be a threshold of ABA for activation of the PAL over which there would be no additional effect (Gagne *et al.*, 2006a). ABA treatment at 14 DAA decreases the LAR2 and ANR activity, thereby reducing the tannin content (Lacampagne *et al.*, 2009) and ABA treatment days before *veraison* increased LAR2 expression (Villalobos, 2011). Additionally to the study of structural and regulatory gene transcript accumulation, proteomic studies have shown that ABA treatments act through the over- or under-expression of the same pool of proteins involved in the ripening process, where anthocyanin biosynthesis enzymes are up-regulated (Giribaldi *et al*., 2010).

It was noted in cv. Flame seedless that the more effective dose of ABA treatment at *veraison* was the highest (300 mg L^{-1}), while all doses between 75 and 300 mg L^{-1} were superior to the CEPA treatments with respect to anthocyanin accumulation (Peppi *et al.*, 2006). However, the most efficient ABA application time and concentration are cultivar dependent (Boss *et al.*, 1996a, Boss *et al.*, 1996b, Jeong *et al.*, 2004) and there are less information in wine grapes varieties and no reports on cv. Carménère, an important variety in the Chilean viticulture. Venburg *et al*. (2009) showed that combined S-ABA and CEPA treatments have a synergistic effect on grape color and wine sensory characteristics improvement.

1.5.3. Auxins

The treatment of grapes with auxinic growth regulators like benzothiazole-2-oxyacetic acid (BTOA), IAA (the most abundant auxin) or naphthaleneacetic acid (NAA), causes a delay in the beginning of the berry ripening (Deytieux-Belleau *et al.*, 2007, Davies *et al.*, 1997, Böttcher *et al.*, 2010), presenting a delay in various parameters of ripening as berry weight, TSS, anthocyanins, berry firmness and in the commonly observed ABA peak accumulation at *veraison*, suggesting a possible co-involvement of ABA and auxins in controlling the ripening process (Davies *et al.*, 1997, Deytieux-Belleau *et al.*, 2007, Böttcher *et al.*, 2010). Böttcher *et al*. (2010) found that pre-*veraison* NAA treatment enlarged berry size at harvest and increased the synchronicity of berry TSS accumulation. It was also noted a change in the expression of genes involved in the process of maturation as a vacuolar invertase, CHS, UFGT and quitinasa genes (Davies *et al.*, 1997). It has been reported that pre-*veraison* treatment with the auxinic PGR BTOA caused an increase in grape ethylene concentration in a lesser extent than CEPA does (Coombe and Hale, 1973, Weaver and Singh, 1978) while CEPA treatment have shown to decrease the auxin IAA content presumibly by an acceleration of the normal peroxidase activity that could be responsible for increasing IAA degradation, thereby rendering the tissue sensitive to ethylene and enhancing ripening (Szyjewicz *et al.*, 1984).

1.5.4. Other Plant Growth Regulators

Exogenous application of the BS epi-brasinolide significantly accelerated the ripening of berries, while the exogenous application of brasinazole (a BS biosynthesis inhibitor) significantly delayed maturation (Symons *et al.*, 2006).

The effect of field applications of N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU, a synthetic cytokinin) results in a strong increase in berry and bunch size, increase of bunch compaction and a linear ratio between berry firmness and CPPU concentration (Zabadal and Bukovac, 2006). On the other hand, this treatment reduces the berry skin color and promoted berry set and development. Peppi and Fidelibus (2008) recommended the simultaneous use of CPPU and ABA, which do not interact and have benefits together in terms of improving the grape color and maintain berry firmness in table grapes.

Grape treatment with salicylic acid (SA) can delay or inhibit ripening when applied two to three weeks prior to véraison (Kraeva *et al.*, 1998). SA is known as an ABA antagonist and can be involved in the berry ripening control.

The grapes treatment with methyl jasmonate at véraison significantly promoted the production of resveratrol and ε-viniferin and the ripening of berries (Vezzulli *et al.*, 2007).

However, research in this field shows that the hormonal control of grape berry development remains controversial (Davies and Böttcher, 2009).

Table 6. Effect of PGR treatments on grape anthocyanin accumulation, color, gene expression and others effects.

PGR	EFFECT ON ANTHOCYANINS & COLOR	EFFECT ON GENE EXPRESION	OTHER EFFECTS	SOURCE
ABA	+ (overtake)	+ MYBA1, PAL, CHS, CHI, DFR and UFGT and HT1	+ endogenous ABA, sugar, pH, softening. - Acidity	Ban *et al*. 2003, Dan y Lee 2004, Gagne *et al*. 2006; Hiratsuka *et al*. 200; Jeong *et al*. 2004; Matsushima *et al*. 1989; Peppi *et al*. 2006
Ethylene	+	+	+ aroma. - Acidity, auxins (Variable effect)	Roubelakis-Angelakis and Kliewer 1986
CEPA	+	+ CHS, F3H, LDOX, UFGT,	+ endogenous ethylene	El-Kereamy *et al*. 2003
MCP	-	- SUC11 and SUC12	+ acidity - Sucrose & berry size	Chervin *et al*. 2006; Mailhac and Chervin, 2006
Auxins (2,4 D, IAA)	- (delay)	- (delay) PAL, CHS, CHI, DFR Y UFGT		Ban *et al*. 2003; Davies *et al*. 1997; Deytieux-Belleau *et al*. 2007; Jeong *et al*. 2004
Brassinosteroids	+		associated with ethylene in tomato	Symons *et al*. 2006; Vardhini and Rao 2002
Brassinazole	-		- sugar accumulation	Symons et al. 2006
Jasmonic acid	+			Belhadj *et al*., 2007
GA_3 / CPPU	- (Dilution?)		+ berry size	Dan and Lee 2004

1.5.5. Early Plant Growth Regulator Treatments

Early ABA treatments have shown the ability to control ripening and flavonoid biosynthesis, reducing tannin content of green berries but increasing it at *veraison* (Gagne *et al.*, 2006a, Lacampagne *et al.*, 2009). Davies and Böttcher (2009) hypothesized that ABA treatments may have the ability to hasten the initiation of sugar accumulation when applied before *veraison*, but cannot enhance it once ripening has already began. On the other hand, CEPA is considered to be a promoter or inhibitor of ripening depending on the phenological stage of the berries at treatment time (Hale *et al.*, 1970). Early treatments seem to delay ripening, reducing TSS and pH (Szyjewicz and Kliewer, 1983) while treatments just before véraison seem to promote ripening (Coombe and Hale, 1973). There are only a few experiences on early PGR treatments, and their effects on grapes and wine quality are somehow inconsistent.

1.6 Plant Growth Regulators Application and Wine Quality

Literature on the effects of PGR applications on wine composition and sensory characteristics is poor and somehow contradictory.

Wine from CEPA-treated grapes of cv. Pinot noir (a poorly colored wine grape variety) during two seasons were 47 to 60% superior in color after 6 to 15 months of bottle aging (Powers *et al.*, 1980). Wines from CEPA-treated grapes increase total polyphenol and anthocyanin, enhancing wine coloration and color stability (Szyjewicz *et al.*, 1984). Delgado *et al*. (2004) showed that the CEPA-treated grapes lead to increases in wine alcohol content. While some reports show no influence of CEPA on sensory characteristics (Szyjewicz *et al.*, 1984), other

reports show that the taste panel judged wines from CEPA-treated grapes better than control wines (Powers *et al.*, 1980).

Venburg *et al.* (2009) showed a greater accumulation of anthocyanins in wines from the CEPA-plus-ABA-treated grapes. Other reports showed that ABA treatments altered wine color value (Delgado *et al*., 2004). PGR treatments could have different effects on the expression or activity of the F3'5'H and flavonoid-3'-hydroxylase (F3'H) genes (Castellarin *et al.*, 2006) and this could have an effect on the color of grapes and wine as dihydroxylated anthocyanins produce predominantly orange hues while trihydroxylated ones confer red-purple hues (Heredia *et al*., 1998). Even if Böttcher *et al.* (2010) found small changes in wine volatile compounds, they did not find significant differences in sensory properties between small-scale wine lots made from control and NAA-treated fruit.

1.7 Exposition of the Problem and Hypotheses

Grapes and wine quality

PC biosynthesis and accumulation program in wine grape berries takes place at specific times of the berry development process and in specific berry tissues. The study of these features is a key aspect to determine the optimal harvest time and extraction techniques to use during winemaking.

Some environmental factors can negatively impact PC biosynthesis, in particular anthocyanin and color accumulation. The importance of the temperature during berry development is well known, as are the detrimental effect of excessively high temperatures (above 35-40ºC), the positive effect of low night temperature condition and the effect of temperature on anthocyanin degradation

(Bergqvist *et al.*, 2001, Spayd *et al.*, 2002, Adams, 1973). From a molecular point of view, Mori *et al*. (2007) have shown that high temperatures limit anthocyanin biosynthesis and there are reports presenting results that support an induction of phenylpropanoid pathway genes under low night temperature condition (Mori *et al.*, 2005, Yamane *et al.*, 2006). The effect of temperature on grapes and wine quality is highly determined once the vineyard is established, so any cultural practice that allows to resolve this problem and improve grapes quality under these conditions are desirable, specially in a global warming scenario.

The application of exogenous PGRs can induce modifications in the hormonal balance of grape tissues, affecting the normal development of the grapes and modifying several grape and wine quality parameters, especially when environmental conditions are not favorable.

Regulation and authorization of CEPA and ABA utilization

The application of CEPA is world-wide authorized for some cultivated species. In Chile, the application was authorized since March 13, 2006 in table grape, tomato, walnuts, bell pepper, lemon and orange by the “Servicio Agrícola y Ganadero (SAG)”. The application of ABA in Chile is authorized by the SAG since November 25, 2009 for table grapes cv. Crimson seedless. In the USA ABA is authorized since April 19, 2010 by the “United States Environmental Protection Agency” and the “California Department of Pesticide Regulation” for table grapes.

Hypotheses

Exogenous treatments with promoters of ripening ABA and/or CEPA at *veraison* are able to positively influence grape and wine characteristics; specifically enhancing grape berry skin and wine flavonoid content, reducing grape total soluble solids and hence wine alcohol content and reducing wine herbaceous aroma. Treatment shortly after fruit set may promote higher effects than at *veraison* and treatment with the inhibitor of ripening IAA at *veraison* is able to induce opposite effects to those of promoters of ripening.

General objective

The objective of this study is to determine the effect of PGR treatments after fruit set and at *veraison* on the accumulation of flavonoid compounds in grapes and wines cvs Cabernet Sauvignon and Carménère.

Specific objectives

1. To determine the effect of IAA, S-ABA and CEPA treatments at *veraison* on the internal hormonal balance of berry skins and flavonoid content of grape skins and wines cv. Cabernet Sauvignon.
2. To determine the effect of S-ABA and CEPA treatments early in development and at *veraison* on flavonoid biosynthetic and regulatory gene expression and flavonoid content in grape skins and wines cv. Carménère.

3. To determine the effect of low-dose ABA and CEPA treatments performed early in development and at *veraison* in two different environmental conditions as are Bordeaux (France) and Maipo (Chile), on the accumulation of flavonoid compounds in berry skins and wine from cv. Cabernet Sauvignon treated grapes.

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Chapter 2

Materials and Methods

Plant Material and Sample Collection

Cachapoal and Maipo Cabernet Sauvignon 2009

The grape samples were collected from a commercial vineyard experiment (CVE) and a plants-in-70-L containers experiment (PCE), located at the Colchagua Valley (34.28ºS 71.27ºW) and the Maipo Valley (33.50ºS 70.62ºW) of Chile, respectively, during 2009 (table 1). The plants of *Vitis vinifera* L. Cv. Cabernet Sauvignon were planted as a traditional north/south vertical trellis with spur pruning. All the plants had rooted on their own and were between 12 and 15 years old and the CVE planting density was 2,667 vines per hectare.

The CVE layout consisted of three completely randomized 16-plant replicates and the PCE layout consisted of three completely randomized 12-bunch replicate blocks. Thirty random 5-berry bunch fragment samples were collected from the CVE layout at three phenological stages. In the PCE layout, 60 random berry samples per replicate were collected at five phenological stages.

Cachapoal Carmenère 2008 and 2010

The grape samples were collected from a commercial vineyard experiment, located at the Cachapoal Valley of Chile (34.28ºS, 71.27ºW) during 2007/2008 and 2009/2010 seasons (table 1). The plants of *Vitis vinifera* L. cv. Carmenère were conducted as a traditional north-west/south-east vertical trellis with cane pruning. Vines were rooted on their own, 12 years old and planting density was 2,667 vines per hectare.

The experiment layout consisted of three completely randomized 16-plant replicates. Thirty random 5-berry bunch fragment samples were collected per each replicate at three and seven phenological stages in 2007/2008 and 2009/2010 respectively.

Bordeaux Cabernet Sauvignon 2009 and Maipo Cabernet Sauvignon 2010

The grape samples were collected from an experimental vineyard located at Bordeaux, France (44.78ºN, 0.56ºW) during 2009 and a commercial vineyard experiment, located at the Maipo Valley of Chile (33.63ºS, 70.65ºW) during season 2009-2010 (table 1). The Bordeaux and Maipo plants of *Vitis vinifera* L. cv. Cabernet Sauvignon were conducted as a traditional east/west vertical trellis with cane pruning and a traditional north/south vertical trellis with spur pruning, respectively. Bordeaux plants were grafted onto rootstock 101-14, were 39 years old and planting density was 7,576 vines per hectare (1.2 x 1.1 m). Maipo plants

had rooted on their own, were 12 years old and planting density was 4,000 vines per hectare (2.5 x 1.0 m).

The Bordeaux experiment layout consisted of three completely randomized 3-plant-replicate blocks and the Maipo experiment layout consisted of three completely randomized 6-plant-replicates. Twenty random 5-berry bunch fragment samples per replicate were collected from each experiment layout at eight phenological stages.

Table 1. Characterization of plant material used in the different experiments.

Region	**Maipo**	**Colchagua**	**Colchagua**	**Bordeaux**	**Maipo**
Variety	**Cabernet Sauvignon**	**Cabernet Sauvignon**	**Carmenère**	**Cabernet Sauvignon**	**Cabernet Sauvignon**
Year	**2009**	**2009**	**2008 & 2010**	**2009**	**2010**
Vineyard type	Plants in 70 L containers	Commercial vineyard	Commercial vineyard	Experimental vineyard	Commercial vineyard
Localization	33.30ºS 70.37ºW	34.17ºS 71.16ºW	34.18ºS 71.16ºW	44.47ºN 0.35ºW	33.38ºS 70.39ºW
Trellising	N/S vertical	N/S vertical	NW/SE vertical	E/W vertical	N/S vertical
Pruning	Spur	Spur	Cane	Cane	Spur
Rootstock	No	No	No	101-14	No
Irrigation	Drip	Drip	Drip	No	Drip
Plantation year	2007 (transplantation)	1994	1996	1974	1998
Age (years)		15	12-14	35	12
Plant density		2,667	2,667	7,576	4,000
Experimental Design	Randomized block	Completely randomized	Completely randomized	Randomized block	Completely randomized
Experimental Unit	12 bunches	16 plants	16 plants	2-3 plants	6 plants

Experimental Treatments

In Cachapoal Carmenère 2008 and 2010, and Maipo and Cachapoal Cabernet Sauvignon 2009 experiments, S-ABA, the naturally-occurring enantiomer was used and in Bordeaux Cabernet Sauvignon 2009 and Maipo Cabernet Sauvignon 2010 experiments synthetic ABA, a racemic mixture of (+)-S-ABA and (-)-R-ABA was used. These two enantiomer molecules can be different in inducing gene expression and physiological responses (Zaharia et al., 2005). PGR concentrations and application time for all experiments are detailed in table 2. ABA and CEPA mass concentrations used in Bordeaux 2009 and Maipo 2010 experiments (53 and 29 mg L^{-1} respectively), are equivalent to a molar concentration of 2 x 10^{-4} mol L^{-1}.

Table 2. Experiment PGR concentrations (mg L^{-1}) and application time relative to anthesis and *veraison*.

Region and year	Variety	Application time	VBC-30051	ABA	IAA	Sierra	Ethrel®
Cachapoal 2009	Cabernet Sauvignon	4 DAV[1]	400		265		480
Maipo 2009	Cabernet Sauvignon	4 DAV	400		265		480
Cachapoal 2008	Carmenère	-6 DAV	200 & 400				240 & 480
Cachapoal 2010	Carmenère	10 DAA[2] 1 DAV	400 400				480 480
Bordeaux 2009	Cabernet Sauvignon	10 DAA 2 DAV		53 53		29 29	
Maipo 2010	Cabernet Sauvignon	10 DAA 0 DAV		53 53			29 29

[1]Days after *veraison*, with *veraison* defined as one per-cent color change in the grapes.
[2]Day after anthesis, with anthesis defined as 80% cap fall.

The treatment involved applying the PGR solution containing 0.1 % Tween80 as a wetting agent on the treated grapes, or water containing the same Tween80 concentration on the control grapes. Complete information of used products is detailed in table 3.

Table 3. Commercial plant growth regulators used in the experiments

	VBC-30051	**ABA**	**IAA**	**Sierra®**	**Ethrel® 48 SL**
Concentration	20% p/p	98,5% p/p	98% p/p	18% p/v	48 %p/v
Fabricant	Valent BioSciences Corp. USA.	Sigma, France & Germany.	Sigma-Aldrich, USA.	Bayer CropScience Lyon, France	Bayer CropScience AG, Germany
Active compound	(+)-S-ABA	(±)-S/R-ABA	Indole-3-acetic acid	(2-chloroethyl) phosphonic acid	(2-chloroethyl) phosphonic acid
Formula	$C_{15}H_{20}O_4$	$C_{15}H_{20}O_4$	$C_{10}H_9NO_2$	$C_2H_6ClO_3P$	$C_2H_6ClO_3P$
Molecular weight g mol^{-1}	264,32	264,32	175,18	144.5	144.5

In Cachapoal Cabernet Sauvignon 2009 and Cachapoal Carmenère 2008 and 2010 experiments, all the clusters of the entire plants were treated using an agricultural hand sprayer at a water rate of 1,000 L ha^{-1}. In the plants-in-70-L containers experiment (Maipo Cabernet Sauvignon 2009), all the grapes were treated by complete cluster immersion for one minute in the respective PGR solution. In Bordeaux Cabernet Sauvignon 2009 and Maipo Cabernet Sauvignon 2010 experiments, all and each cluster of entire plants were treated with 10 mL of the respective solution using a hand sprayer. All treatments were performed at sunset to minimize the photodestruction of the ABA.

Skin Phenolic Extractions

Immediately after sampling, berries were frozen *in situ* with liquid nitrogen. The berries were weighed and their skins were hand separated after thawing. The skins were rinsed with distilled-deionized water, frozen again and then lyophilized, before final grinding. In Carmenère 2008 and Cabernet Sauvignon Maipo and Colchagua 2009 experiments, one gram of ground lyophilized skins was extracted (Kennedy and Jones, 2001) in 10 mL of a 2:1 acetone/water solution for one hour in a shaker at room temperature and then centrifuged at 4,000 rpm for six minutes. After centrifugation, the supernatant extract was concentrated under reduced pressure at 35 °C in a rotary evaporator to remove the acetone and then dissolved in a 100 mL model wine solution (12 % v v^{-1} ethanol, pH 3.6 and 0.033M tartaric acid). To minimize oxidation, the solutions were sparged with nitrogen gas. In Cabernet Sauvignon Bordeaux 2009, Carmenère 2010 and Cabernet Sauvignon 2010, one hundred milligrams of ground lyophilized skins were extracted in 10 mL of 50% v v^{-1} aqueous ethanol pH 2.0 solution for one hour in a shaker at room temperature and then centrifuged at 4,000 rpm for six minutes. After centrifugation, the supernatant was sparged with nitrogen gas in order to minimize oxidation and retained at 4ºC until analyzed.

From the homogenized berry pulp, we determined TSS by direct reading in a digital refractometer (Pocket PAL-1, Atago, Japan), the pH using a pH meter (Orion 5-Star, Thermo Scientific, Singapore) and the titratable acidity (TA) using a pH meter and 0.1N NaOH.

ABA and IAA Analysis

Internal “free” ABA was determined following the method described by Antolin *et al.* (2003) and internal IAA was determined according to the method of Abbas *et al.* (2000). For the extraction of both hormones, 500 mg of the ground lyophilized skins of 100 berries were extracted with 100 mL methanol 80 % v v^{-1}, containing BHT (2.6-di-*tert*-butylphenol) as an antioxidant, with continuous stirring at 4°C overnight. After filtration, the extract was concentrated under reduced pressure at 35°C to remove the methanol and adjusted to pH 3.0 (± 0.05). The residue was dissolved in ultrapure water and mixed with polyvinyl poly-pyrrolidone (5 %, w/v) at 4°C for 20 min. After filtration and the adjustment of the pH to 2.5 (± 0.05), the extract was subjected to three consecutive liquid-liquid extractions with diethyl ether (v/v). The organic phases, containing free ABA and IAA, were pooled and dried under reduced pressure. The residue was dissolved in one mL diethyl ether and stored at -80°C until analysis. Prior to HPLC analysis, the extract was dried under nitrogen, dissolved in 300 µL of methanol and filtered through a 0.45 µm membrane.

The extract was automatically injected and processed by an HPLC (Thermo Fisher Scientific, Illkirch, France) equipped with a reverse-phase column (4.6x250 mm Hypersyl® BDS C18, 5 µm). A gradient solvent system was used with methanol as solvent A and 0.1 M H_3PO_4 in ultrapure water as solvent B. The elution program had the following proportions of solvent A: 0-7 min, 60 %; 7-7.5 min, 60-65 %; 7.5-11 min, 65 %; 11-11.5 min, 65-60 %; 11.5-20 min, 60 %. The

flow rate was one mL per minute and the column was kept at room temperature. The detection was performed in combination with UV spectrophotometry for the ABA (λ: 254 nm) and with fluorescence spectrometry for the IAA at 280 and 360 nm excitation and emission wavelengths, respectively. The data acquisition and processing were performed using the Chromquest 4.2 software (Thermo Fisher Scientific, Illkirch, France).

The ABA and IAA calibration curves were established using commercial ±-*cis*,*trans*-ABA (Sigma-Aldrich, Saint Quentin Fallavier, France) and IAA (Sigma-Aldrich, Saint Quentin Fallavier, France) and these curves were used to quantify the endogenous free ABA and IAA in the skin extracts. A sample of the ±-*cis*,*trans*-ABA and IAA external standard was included after every five samples in each HPLC sequence to check both the retention time and the concentration of ABA and IAA. The purity of the PGR peaks was first controlled by mass spectrometry coupled to HPLC to validate the procedure and then independently checked on randomly chosen samples. The extraction and the quantification were repeated three times per sample.

RNA extraction and cDNA synthesis

RNA extraction, cDNA synthesis and quantitative comparison of gene expression were done on the 2010 experiment in samples 1 to 5 (10 to 81 DAA; table 1). Grape skins from 25-to-50-frozen-berries were carefully removed for analysis using razor blades and the tissue was grounded with liquid nitrogen. A

tissue pool mix was done of every three biological replicates. Total RNA was isolated from berry skins according to the procedure of Reid *et al*. (2006), using a CTAB-Spermidine extraction buffer. RNA integrity was verified using denaturing formaldehyde agarose gel electrophoresis with ethidium bromide. A DNAse treatment was performed using RQ1 RNase-Free DNase (Promega, Wisconsin, USA). For cDNA synthesis, one µg of total RNA was reverse transcribed with random hexamer primers in an 18 µl reaction mixture using the SuperScript™ II reverse transcriptase (Invitrogene, California, USA) according to the manufacturer's instructions. The cDNA product purity was checked by PCR amplification and agarose gel electrophoresis, using gene-specific primers, to verify the absence of genomic DNA.

Quantitative comparison of gene expression throughout berry skin development

Relative transcript quantification of isolated genes was performed by real-time RT-PCR, using the Sensimix® SYBR® Kit (Bioline) and the Mx3000P detection system (Stratagene, California, USA) as described in the manufacturer's manual. Amplification of a fragment of the ACTIN1 gene (72 bp) was used as an internal control for the calibration of relative expression (Serrano, unpublished results). PCR conditions, standard quantification curves for each gene (table 2), primer efficiency values and relative gene expression calculations were conducted according to Matus *et al.* (2008). Standard quantification curves with serial dilutions

of PCR products were constructed for each gene to calculate amplification efficiency according to:

$$\mathrm{E} = \left[10^{\left(-\frac{1}{\mathrm{slope}}\right)}\right] - 1$$

This value was then used to obtain an accurate ratio between the expression of the gene of interest (GOI) and the housekeeping gene, using:

$$\frac{(1 + E_{GOI})^{-\Delta Ct}}{(1 + E_{Ubiquitin})^{-\Delta \mathrm{Ct}}} = \frac{(1 + E_{GOI})^{-(\mathrm{Ct\ GOI} - \mathrm{Ct\ GOI\ calibrated})}}{(1 + E_{Ubiquitin})^{-(\mathrm{Ct\ Ubi} - \mathrm{Ct\ Ubi\ calibrated})}}$$

Gene expression levels were normalized to the expression of the first sampling date control sample, in order to obtain a calibrated DCt for each gene. In all cases, R^2 values of standard curves were above 95%. All experiments were performed with three technical replicates. Reaction specificities were tested with melt gradient dissociation curves, electrophoresis gels, and cloning and sequencing of each PCR product.

Glories Method for ripeness assessment

Separated berry samples were collected in the Bordeaux experiment to perform a grape berry ripeness assessment during the two final sampling dates (75 and 101 DAA). Extraction of phenolic compounds of grapes with pH 1 and pH 3.5

buffers was performed according to the method described by Glories (2001). This method of phenolic ripeness allows calculating total phenols, extractable anthocyanidin, total tannin, anthocyanin extractability and the seed tannin contribution index.

Winemaking

Small-scale winemaking was performed in: (1) Carmenère Colchagua 2008 (only for the high doses treatments); (2) Cabernet Sauvignon Colchagua 2009 (commercial vineyard experiment); (3) Cabernet Sauvignon Maipo 2010 and; (4) Carmenère Colchagua 2010, considering all biological replicates for each treatment. The wine was made through a traditional red wine fermentation protocol. The grapes were picked on the commercial harvest date, April 18, 2008 (corresponding to 85 DAV), April 6, 2009 (corresponding to 79 DAV), April 28, 2010 for Carmenère (corresponding to 90 DAV and 150 DAA) and April 23, 2010 for Cabernet Sauvignon (corresponding to 81 DAV and 137 DAA). Twenty-five kilograms of grapes were harvested from each biological replicate of the control and the PGR-treated plants. Fermentation was carried out in plastic 25-L containers in a controlled temperature room with Lalvin EC-1118 selected yeast. Complete spontaneous malolactic fermentation was performed and an adjustment to 30 mg L^{-1} free sulfur dioxide was made in all the wines prior to bottling.

Phenolic Compounds Analysis

The phenolic composition of the grapes and wine was determined using a UV/Vis spectrophotometer model Spectronic Genesys 2 (Milton Roy, Rochester, NY). The total anthocyanins were determined at 520 nm according to the method described by Puissant and Leon (1967), the total phenols were determined by DO280 (Iland *et al.*, 2004) and the total tannin was determined by precipitation with methyl cellulose (Sarneckis *et al.*, 2006). The tannin determination with the 4-(dimethyl-amino)cinnamaldehyde (DMAC) assay, in accordance with the method described by Vivas *et al*. (1994), was performed in the 2008 experiment to estimate a tannin polymerization index (TPI), calculated as the quotient between the total tannin and the DMAC index. All the analyses were performed in duplicate.

Anthocyanins Analysis by HPLC

The chromatographic system for the HPLC-DAD analysis of the anthocyanins consisted of a 1,024 photodiode-array detector model L-2455, a high throughput analysis pump model L-2130, a column oven model L-2350 and an auto sampler model L-2200 (Hitachi LaChrom Elite, Japan). Separation was performed using a Purospher® STAR (Merck, Germany) reverse-phase C_{18} column (250 mm X 4,6 mm i.d., 5µm) at 20ºC. The detection was carried out at 520 nm. The elution gradient consisted of two solvents: Solvent A was water/formic acid 90:10 v/v and solvent B was acetonitrile, following the methodology described

by Fanzone *et al.* (2010). After filtering through a 0.45 µm pore size membrane, a 150 µL aliquot of grape skin extract was injected. Delphinidine-3-glucoside, peonidine-3-glucoside and malvidin-3-glucoside (Extrasynthese, Lyon, France), were used as standards and the other anthocyanins were identified by comparison of the standard retention times.

Wine Sensory Analysis

A 9-to10-person sensory analysis panel was formed consisting of students and staff of the Enology Laboratory of the Faculty of Agricultural Sciences, “Pontificia Universidad Católica de Chile”. All the panel members were familiarized with the method of sensory analysis and aromatic standards during a 2-week training (four sessions). For the vegetal aroma, 3-isobutyl-2-methoxypyrazine and 3-isopropyl-2-methoxypyrazine standards (Centro de Aromas y Sabores, DICTUC, Chile) were used. For the astringency, tannic intensity and bitterness, the method proposed by Delteil (2000) was used. A structured 9-unit linear scale was used. The samples were individually three-digit coded and presented in random order to the panelists. The assessments were made following general requirements for sensory testing conditions. Each wine sample was tasted three times by each panelist.

Statistical Analysis

The results were compared by one-way (for completely randomized experiments) and two-way (for completely randomized block experiments) analysis of variance (ANOVA) and Tukey's HSD multiple comparison procedure, with $P<0.05$ statistical significance between treatments, using Statgraphics Plus (Statistical Graphics Corp., Princeton, NJ, USA).

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Chapter 3

S-Abscisic Acid, 2-Chloroethylphosphonic Acid and Indole-3-Acetic Acid Treatments Modify Grape (*Vitis vinifera* L. cv. Cabernet Sauvignon) Hormonal Balance and Wine Quality

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ABSTRACT

The phenolic composition of red wine strongly determines its quality. Even when the applications of plant growth regulator (PGR) affect grape quality, there is almost no information on the effect of these treatments on the grape's internal hormonal balance and the wine composition and quality. In the present study, changes in the internal hormonal content following the application of (+)-S-abscisic acid (S-ABA), 2-chloroethylphosphonic acid (CEPA) and indole-3-acetic acid (IAA) at *veraison* were examined to determine their effects on Cabernet Sauvignon grapes and wine composition in a plants in containers experiment and in a commercial vineyard experiment. Applied PGRs had a significant effect on the hormonal balance and phenolic composition of grape skins. The S-ABA-treated grapes showed a significantly higher skin internal free abscisic acid concentration in the plants in container experiment and the CEPA-treated grapes showed a reduction in skin internal IAA concentration in the commercial vineyard experiment. Winemaking was performed in the commercial vineyard experiment. Wine's chemical composition was affected by these treatments and an up-to 63 % increase in malvidin-3-glucoside concentration and an up-to 70 % increase in total tannin concentration were found in wines made from the CEPA-treated grapes. The alcohol content was 10.3 % higher (from 12.6 to 13.9 % v v^{-1}) in wines made from the CEPA-treated grapes. No significant differences in the wine sensory attributes (aroma and mouth-feel) between the control and the PGR-treated wines were identified by a sensory panel.

INTRODUCTION

Grape berry ripening occurs under hormonal control (Chervin *et al.*, 2004, Symons *et al.*, 2006, Davies and Böttcher, 2009) and therefore a close relation between the phenolic compound accumulation and the hormonal status has been envisaged (Lacampagne *et al.*, 2009, Böttcher *et al.*, 2010) as a way to improve grape and wine quality (Kennedy *et al.*, 2006).

The fruit of the grapevine (*Vitis vinifera* L.) is considered non-climacteric and therefore its ripening would occur independently of the presence of ethylene, which is very low in the tissues of the berry (Davies and Böttcher, 2009). However, these tissues have a functional ethylene synthesis route, which is triggered just before *veraison*, the moment when a transient increase in internal ethylene content is evidenced (Chervin *et al.*, 2004). This production of ethylene appears to be critical to inducing some changes during the development of the berry, such as an increase in diameter, sucrose and anthocyanin accumulation and a decrease in acidity (Chervin *et al.*, 2004, Chervin *et al.*, 2008). Abscisic acid (ABA) is likely to be the key hormone produced during this period in non-climacteric fruits (Coombe and Hale, 1973, Davies *et al.*, 1997). ABA is likely to affect the expression of the MYBA1 transcription factor gene, the UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) gene and anthocyanin accumulation (Jeong *et al.*, 2004). An increase in endogenous ABA can be observed in berries at *veraison*, suggesting a role of this hormone in the ripening phase (Gagne *et al.*, 2006, Deytieux-Belleau *et al.*, 2007).

The treatment of grapes with 2-chloroethylphosphonic acid (CEPA), an ethylene-releasing compound, at véraison is a controversial topic (Szyjewicz *et al.*, 1984). Studies show that this treatment significantly increases the internal ethylene content, the expression of the UFGT gene and the level of anthocyanins (El-Kereamy *et al.*, 2003, Tira-Umphon *et al.*, 2007). Exogenous ABA application increases the internal ABA levels, accelerates the beginning of ripening and increases phenylalanine ammonia-lyase (PAL) activity and UFGT expression, improving the anthocyanin concentration and decreasing the leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) activity, thereby reducing the tannin content (Gagne *et al.*, 2006, Deytieux-Belleau *et al.*, 2007, Peppi *et al.*, 2008, Lacampagne *et al.*, 2009). Finally, the treatment of berries with 1-naphthaleneacetic acid (NAA), an auxinic PGR, causes a delay in the beginning of berry ripening (Böttcher *et al.*, 2010) and in the commonly observed ABA peak accumulation at *veraison*, suggesting a possible co-involvement of ABA and auxins in controlling the ripening process (Davies *et al.*, 1997, Deytieux-Belleau *et al.*, 2007). However, research in this field shows that the hormonal control of grape berry development remains controversial (Davies and Böttcher, 2009).

The application of PGRs has become an interesting industrial practice for the control of maturation and the improvement of grape and wine quality. The use of ABA as an agrochemical has become feasible recently due to lower productivity costs. Improved synthesis procedures make it easier to obtain S-ABA, an active enantiomer with potential use in viticulture, as seen in several patent applications (Venburg *et al.*, 2008, Venburg *et al.*, 2009).

The objective of this study was to determine the effects of the PGRs S-ABA, CEPA and indole-3-acetic acid (IAA) at *veraison* on the internal hormonal profile of grape skins and on the accumulation of flavonoid compounds in cv. Cabernet Sauvignon grapes and wine.

MATERIALS AND METHODS

This line of research has emerged from a concern in the wine industry. After achieving good results with a cv. Carmenère 2008 field conditions experiment, in 2009 it was decided to extend the research to include cv. Cabernet Sauvignon (a variety widely cultivated and more comparable) in field conditions and in a more controlled environment. In this manuscript, these two 2009 experiments are reported.

Plant Material and Sample Collection

The grape samples were collected from a commercial vineyard experiment (CVE) and a plants-in-70-L containers experiment (PCE), located at the Colchagua Valley (34.28ºS 71.27ºW) and the Maipo Valley (33.50ºS 70.62ºW) of Chile, respectively, during 2009. The plants of *Vitis vinifera* L. Cv. Cabernet Sauvignon were planted as a traditional north/south vertical trellis with spur pruning. All the plants had rooted on their own and were between 12 and 15 years old and the CVE planting density was 2,667 vines per hectare.

The CVE layout consisted of three completely randomized 16-plant replicates and the PCE layout consisted of three completely randomized 12-bunch replicate blocks. Thirty random 5-berry bunch fragment samples were collected from the CVE layout at three phenological stages (table 1). In the PCE layout, 60 random berry samples per replicate were collected at five phenological stages (table 1).

Experimental Treatments

In these experiments S-ABA, the naturally-occurring enantiomer is used instead of synthetic ABA, which is a racemic mixture of (+)-S-ABA and (-)-R-ABA, molecules that can be different in inducing gene expression and physiological responses (Zaharia et al., 2005). The PGR concentrations were based on manufacturer-recommended doses, 400 mg L^{-1} for the S-ABA (VBC-30051, Valent BioSciences Corporation, USA) and 480 mg L^{-1} for the CEPA (Ethrel, Bayer CropScience AG, Germany). For the IAA (Sigma-Aldrich, USA), an equimolar concentration to S-ABA was applied, corresponding to 265 mg L^{-1}. The treatment involved applying the PGR solution containing 0.1 % Tween80 as a wetting agent on the treated grapes or water containing the same Tween80 concentration on the control grapes. The application was done three days after *veraison* (DAV, with *veraison* defined as one per-cent color change in the grapes), corresponding to a 40 % color change. In the CVE, all the clusters of the entire plants were treated using an agricultural hand sprayer at a water rate of 1,000 L ha^{-1}. In the PCE, all the grapes were treated by complete cluster immersion for one minute in the

respective PGR solution. All treatments were performed at sunset to minimize the photodestruction of the ABA.

Skin Phenolic Extractions

Immediately after sampling, the fresh berries were weighed and the berry skins were hand separated. The skins were rinsed with distilled-deionized water, frozen and then lyophilized, before final grinding. One gram of ground lyophilized skins was extracted (Kennedy and Jones, 2001) in 10 mL of a 2:1 acetone/water solution for one hour in a shaker at room temperature and then centrifuged at 4,000 rpm for six minutes. After centrifugation, the supernatant extract was concentrated under reduced pressure at 35 °C in a rotary evaporator to remove the acetone and then dissolved in a 100 mL model wine solution (12 % v v^{-1} ethanol, pH 3.6 and 0.033M tartaric acid). To minimize oxidation, the solutions were sparged with nitrogen gas. From the homogenized berry pulp, we determined the total soluble solids (TSS) by direct reading in a digital refractometer (Pocket PAL-1, Atago, Japan), the pH using a pH meter (Orion 5-Star, Thermo Scientific, Singapore) and the titratable acidity using a pH meter and 0.1N NaOH.

Winemaking

Small-scale winemaking was performed in the CVE for all the biological replicates. The wine was made through a traditional red wine fermentation protocol. The grapes were picked on the commercial harvest date, April 6, 2009

(corresponding to 79 DAV). Twenty-five kilograms of grapes were harvested from each of the 12 replicates of the control and the PGR-treated plants. Fermentation was carried out in plastic 25-L containers in a controlled temperature room with Lalvin EC-1118 selected yeast. Complete spontaneous malolactic fermentation was performed and an adjustment to 30 mg L^{-1} free sulfur dioxide was made in all the wines prior to bottling.

ABA and IAA Analysis

Internal “free” ABA was determined following the method described by Antolin *et al.* (2003) and internal IAA was determined according to the method of Abbas *et al.* (2000). For the extraction of both hormones, 500 mg of the ground lyophilized skins of 100 berries were extracted with 100 mL methanol 80 % v v^{-1}, containing BHT (2.6-di-*tert*-butylphenol) as an antioxidant, with continuous stirring at 4°C overnight. After filtration, the extract was concentrated under reduced pressure at 35°C to remove the methanol and adjusted to pH 3.0 ($\pm$ 0.05). The residue was dissolved in ultrapure water and mixed with polyvinyl poly-pyrrolidone (5 %, w/v) at 4°C for 20 min. After filtration and the adjustment of the pH to 2.5 ($\pm$ 0.05), the extract was subjected to three consecutive liquid-liquid extractions with diethyl ether (v/v). The organic phases, containing free ABA and IAA, were pooled and dried under reduced pressure. The residue was dissolved in one mL diethyl ether and stored at -80°C until analysis. Prior to HPLC analysis, the extract

was dried under nitrogen, dissolved in 300 µL of methanol and filtered through a 0.45 µm membrane.

The extract was automatically injected and processed by an HPLC (Thermo Fisher Scientific, Illkirch, France) equipped with a reverse-phase column (4.6x250 mm Hypersyl® BDS C18, 5 µm). A gradient solvent system was used with methanol as solvent A and 0.1 M H_3PO_4 in ultrapure water as solvent B. The elution program had the following proportions of solvent A: 0-7 min, 60 %; 7-7.5 min, 60-65 %; 7.5-11 min, 65 %; 11-11.5 min, 65-60 %; 11.5-20 min, 60 %. The flow rate was one mL per minute and the column was kept at room temperature. The detection was performed in combination with UV spectrophotometry for the ABA (λ: 254 nm) and with fluorescence spectrometry for the IAA at 280 and 360 nm excitation and emission wavelengths, respectively. The data acquisition and processing were performed using the Chromquest 4.2 software (Thermo Fisher Scientific, Illkirch, France).

The ABA and IAA calibration curves were established using commercial ±-*cis*,*trans*-ABA (Sigma-Aldrich, Saint Quentin Fallavier, France) and IAA (Sigma-Aldrich, Saint Quentin Fallavier, France) and these curves were used to quantify the endogenous free ABA and IAA in the skin extracts. A sample of the ±-*cis*,*trans*-ABA and IAA external standard was included after every five samples in each HPLC sequence to check both the retention time and the concentration of ABA and IAA. The purity of the PGR peaks was first controlled by mass spectrometry coupled to HPLC to validate the procedure and then independently checked on

randomly chosen samples. The extraction and the quantification were repeated three times per sample.

Phenolic Compounds Analysis

The phenolic composition of the grapes and wine was determined using a UV/Vis spectrophotometer model Spectronic Genesys 2. The total anthocyanins were determined at 520 nm according to the method described by Puissant and Leon (1967), the total phenols were determined by DO280 and the total tannin was determined by precipitation with methyl cellulose (Sarneckis *et al*., 2006). The tannin determination by the 4-(dimethyl-amino)cinnamaldehyde (DMAC) assay, in accordance with the method described by Vivas *et al.* (1994), was performed to secure a tannin polymerization index (TPI), calculated as the quotient between the total tannin and the DMAC index. All the analyses were performed in duplicate.

Anthocyanins Analysis by HPLC

The chromatographic system for the HPLC-DAD analysis of the anthocyanins consisted of a 1,024 photodiode-array detector model L-2455, a high throughput analysis pump model L-2130, a column oven model L-2350 and an auto sampler model L-2200 (Hitachi LaChrom Elite, Japan). Separation was performed using a Purospher® STAR (Merck, Germany) reverse-phase C_{18} column (250 mm X 4,6 mm i.d., 5µm) at 20ºC. The detection was carried out at 520

nm. The elution gradient consisted of two solvents: Solvent A was water/formic acid 90:10 v/v and solvent B was acetonitrile, following the methodology described by Fanzone *et al.* (2010). After filtering through a 0.45 µm pore size membrane, a 150 µL aliquot of grape skin extract was injected. Delphinidine-3-glucoside, peonidine-3-glucoside and malvidin-3-glucoside (Extrasynthese, Lyon, France), were used as standards and the other anthocyanins were identified by comparison of the standard retention times.

Wine Sensory Analysis

A nine-person sensory analysis panel was formed consisting of students and staff of the Enology Laboratory of the Faculty of Agricultural Sciences, "Pontificia Universidad Católica de Chile". All the panel members were familiarized with the method of sensory analysis and aromatic standards during a 2-week training (four sessions). For the vegetal aroma, 3-isobutyl-2-methoxypyrazine and 3-isopropyl-2-methoxypyrazine standards (Centro de Aromas y Sabores, DICTUC, Chile) were used. For the astringency, tannic intensity and bitterness, the method proposed by Delteil (2000) was used. A structured 9-unit linear scale was used. The samples were individually three-digit coded and presented in random order to the panelists. The assessments were made following general requirements for sensory testing conditions. Each wine sample was tasted three times by each panelist.

Statistical Analysis

The results were compared by one-way (for completely randomized experiments) and two-way (for completely randomized block experiments) analysis of variance (ANOVA) and Tukey's HSD multiple comparison procedure, with $P<0.05$ statistical significance between treatments, using Statgraphics Plus (Statistical Graphics Corp., Princeton, NJ, USA).

RESULTS

Skin Internal “Free” ABA and IAA Status

The variation in internal “free” ABA content in the grape skins during *veraison* and ripening is shown in figure 1. Soon after the onset of ripening, at the first sampling point (5 DAV, two days post treatment for the CVE and 12 DAV, nine days post treatment for the PCE), when 50-80 % of the berries had changed color, the treatments showed significant differences. The S-ABA-treated grapes showed significantly higher levels of internal “free” ABA, as compared with all the other treatments at 5, 15 and 23 DAV in the PCE, expressed either as a concentration or on a per-berry basis. There were no significant differences in internal “free” ABA contents between the control and the S-ABA treated grapes in the CVE, skins of the S-ABA-treated grapes showed higher contents when compared with the CEPA-treated grapes and the skins of the CEPA-treated grapes showed lower contents when compared with the control skins expressed as a per-berry basis. The skins of

the IAA-treated grapes showed no significant differences when compared with the control grapes.

The variation in the internal IAA content in the grape skins during *veraison* and ripening is shown in figure 2. In the CVE, the CEPA-treated grapes showed a significant reduction in internal IAA levels as compared with all the other treatments at 12 DAV, expressed either as a concentration or on a per-berry basis. No significant differences between treatments were observed at 47 & 72 DAV in the CVE or at any of the three sampling dates in the PCE.

Berry Maturation

No significant berry weight differences between the treatments were found in the PCE or in the CVA and no significant differences in crop weight in CVE were evidenced between the treatments at harvest time (figure 3). However, the PGR applications altered the pulp maturation parameters (figure 4). The CEPA-treated grapes showed a decrease in TSS content (figure 4A and 4D). This reduction was significant for the first sampling date (12 DAV) in the CVE and for the two final sampling dates (42 and 72 DAV) in the PCE. When the grape TSS concentration in the CEPA-treated grapes in the CVE is compared with the other treatments, the concentration increased across the ripening phase, producing the treatment with the highest insignificant value at the last sampling date (72 DAV). The CEPA-treated grapes showed a significant increase in pH at 72 DAV (figure 4B) and a significant decrease in total acidity at 47 DAV in the CVE (figure 4C). Similar but

not significant tendencies for the pH and total acidity could be observed in the PCE (figure 4E and 4F).

HPLC-DAD Grape Skin Anthocyanin composition

The HPLC-DAD grape skin anthocyanin compositions for the PCE at 42 DAV and for the CVE at 47 DAV are shown in figure 5. For the PCE at 42 DAV, of the total glucoside anthocyanins, only the total malvidin and the total delphinidin showed significant differences. When the levels were compared to the control grapes, the skins from the IAA-treated grapes showed a significant decrease in the total malvidin and the skins from the S-ABA- and CEPA-treated grapes showed a significant increase in the total malvidin, with a greater increase found in the skins from the CEPA-treated grapes. In the case of total delphinidin, the skins from the S-ABA-treated grapes were significantly higher when compared to the IAA-treated grapes, but similar to the skins from the CEPA-treated grapes and they were not significantly different from the control grapes (figure 5A). When the anthocyanins were grouped into di- or trihydroxylated forms, the effects of the treatments were only significant for the trihydroxylated anthocyanins (*i.e.*, malvidin, petunidin and delphinidin) and these forms showed the same effect as the total anthocyanins, with the skins from the CEPA- and the S-ABA-treated grapes showing higher concentrations than the skins from the IAA-treated and the control grapes (figure 5B). In the CVE at 72 DAV, only a few significant differences could be found between the treatments for petunidin-3-glucoside, cyaniding-3-acetylglucoside and total petunidin presence (results not shown). In the CVE at 47 DAV, significant

differences were observed in the total malvidin, total petunidin, total peonidin, total cyaniding, subtotal dihydroxylated, subtotal trihydroxylated and total anthocyanins. The skins from the CEPA-treated grapes showed higher significant concentrations, while the skins from the S-ABA-treated grapes did show significant differences from the control grapes (figure 5C and 5D). In the CVE at 72 DAV, the skins from the CEPA-treated grapes were significantly higher in most anthocyanin forms than those skins treated with S-ABA (results not shown).

The percent composition of the different acylated forms of the anthocyanins found in the grape skins in the PCE is shown in table 2. In the PCE at 42 DAV, the treatments significantly affected the relative content of malvidin-3-(acetyl)glucoside, delphinidin-3-(acetyl)glucoside, total non-acylated glucosides, total acetyl glucosides and total coumaroyl glucosides. In the PCE at 72 DAV, the changes were significant for petunidin-3-glucoside, cyanidin-3-(acetyl)glucoside, malvidin-3-(coumaroyl)glucoside, peonidin-3-(coumaroyl)glucoside, total coumaroyl glucosides, total petunidin and total peonidin. From the analysis of the 15 individual anthocyanins at 42 DAV, no significant differences between the treatments could be observed and at 72 DAV, the skins from the S-ABA-treated grapes were significantly different from the control in 2 of 15 cases (table 2; petunidin-3-glucoside and cyanidin-3-(acetyl)glucoside). Also for this last sampling date (72 DAV), the skins from the S-ABA-treated grapes showed a significant relative enrichment of total coumaroyl glucosides and total petunidin (table 2). In the CVE at 12 DAV, only a significant decrease in petunidin-3-acetylglucoside could be observed in the CEPA-treated grapes at 12 DAV.

Wine Composition

The effect of the treatments on wine composition is summarized in figure 6. The wine analysis showed that wines from the CEPA-treated grapes were significantly higher than the control grapes in alcohol content, while the wines from the S-ABA- and IAA-treated grapes did not show a significant difference. The wines did not show any significant difference in pH, total acidity, residual sugar, volatile acidity, color intensity, color hue and TPI. The wines from the CEPA-treated grapes showed significantly higher levels in the DO280 index, total anthocyanins and total tannin, while wines from the S-ABA treated grapes only showed a significantly higher level of total anthocyanins and the wines from the IAA-treated grapes did not show any significant difference when they were compared to the control wines (figure 6). All the wines ended fermentation with less than 2.1 g L^{-1} of residual sugar and less than 0.52 g L^{-1} of volatile acidity.

The HPLC wine anthocyanin composition is shown in figure 7. Only wines made from the CEPA-treated grapes had significantly higher concentrations of total malvidin, total cyanidin, trihydroxylated and total anthocyanins when compared to the control wines. No significant effect on the wines made from the S-ABA-treated grapes could be observed (figure 7A and 7B).

Wine Sensory Analysis

The effect of treatments on wine sensory analysis is summarized in figure 8. CEPA-treated grapes produced wines with significantly higher bitterness than S-

ABA-treated wines and significantly higher tannic intensity than IAA-treated wines. No significant difference between control and PGR-treated wines could be observed. Moreover no significant differences in the red fruit aroma, vegetal aroma and astringency could be found between any treatments.

DISCUSSION

The results shown here demonstrate that grape PGR treatments at *veraison*, have significant effects on the grape ripening process, the grape TSS, the pH and titratable acidity levels, the wine alcohol concentration and the accumulation of phenolic compounds in the grapes and wine, including the DO280 index, total tannin, anthocyanin concentration and composition. No effects on crop yield could be found and some effects on the wine aromatic or mouth-feel sensory attributes could be found.

Hormonal signaling is a complex process that is involved in the different events of the berry development, including *veraison*: the expression of genes related to the metabolism of ABA, auxins, ethylene and brassinosteroids were shown to be significantly modified at the onset of ripening (Symons *et al*., 2006, Pilati *et al*., 2007). In this study, the exogenous application of S-ABA increased the level of internal “free” ABA in the grape skins. This effect was shown in previous studies by applying a racemic mixture of synthetic S- and R-ABA (Deytieux-Belleau *et al*., 2007). However, no effect of the S-ABA treatment over internal IAA was found. The decrease in internal IAA obtained with the CEPA treatment may be explained by an acceleration of the normal peroxidase activity that could be

responsible for increasing the IAA degradation, thereby rendering the tissue sensitive to ethylene and enhancing ripening (Szyjewicz *et al*., 1984). The results of this study suggest that the role of ethylene as a promoter of ripening could serve to reduce the levels of inhibitors to ripening, such as the auxins. Previously, it has been reported that pre-*veraison* benzothiazole-2-oxyacetic acid treatment caused increases in grape ethylene concentration (Coombe and Hale, 1973, Weaver and Singh, 1978). At 47 and 72 DAV in the present study, there were no significant effects on the internal ABA or IAA. This is expected as hormone peaks occurs while the color change developing and after that time, no additional changes in the hormonal profile take place (Deytieux-Belleau *et al*., 2007).

In accordance (El-Kereamy *et al*., 2003) or in contrast (Davies *et al*., 1997, Chervin *et al*., 2008) with previous reports, we did not find any significant differences in mean berry weight with the CEPA or IAA treatments. Our results show that the IAA and S-ABA treatments did not affect the grape TSS and wine alcohol content. These results agree with the hypothesis that ABA treatments may be able to hasten the initiation of sugar accumulation when applied early (before *veraison*) but cannot enhance it once ripening has already commenced (Davies and Böttcher, 2009). On the other hand, our results showed that the IAA treatment did not affect the TSS content. Böttcher *et al*. (2010) showed that pre-*veraison* treatment of cv. Shiraz berries with NAA, another auxinic hormone, significantly delayed ripening as measured by the accumulation of the TSS and anthocyanins. These discrepancies may be explained by the differences in application time (Giribaldi *et al*., 2010), the applied products and the varieties studied. A generally controversial subject is the effect of CEPA treatments on TSS content (Szyjewicz

et al., 1984). In this study, CEPA application significantly decreased TSS content of grapes in some sampling dates in both experiments. This effect was also observed by Delgado et al. (2004) with cv. Tempranillo. But as is shown in figure 3, CEPA-treated grapes in the CVE increased their TSS levels as ripening advanced. Even when CEPA-treated grapes significantly increased in grape pH and decreased in total acidity in the PCE, no significant differences could be found in these parameters in CVE grapes and wines. CEPA-treated grapes in the PCE evidenced higher pH and lower total acidity values, but simultaneously lower TSS values were found, showing an independent control of acid degradation and TSS accumulation.

An important objective of this work was to study the effect of the PGRs on the grapes and on wine quality, in particular on the accumulation of the phenolic compounds. The results show a greater accumulation of anthocyanins in skins from the CEPA- and S-ABA-treated grapes. This issue is also reported in other studies (Ban *et al*., 2003, El-Kereamy *et al*., 2003, Jeong *et al*., 2004, Gagne *et al*., 2006, Deytieux-Belleau *et al*., 2007, Peppi *et al*., 2008). This effect was significantly higher in the skins from the CEPA-treated grapes than in the skins from the S-ABA-treated grapes, contrary to the results obtained by Peppi *et al*. (2006) in cv. Flame Seedless table grapes, where ABA applied at *veraison* was superior to CEPA applied at any of the times tested. Higher accumulation of anthocyanin (and other phenolic compounds) as a consequence of the S-ABA and CEPA treatments could be explained by the effect of these PGRs on the structural and regulatory anthocyanin pathway gene expression. The ABA treatment has shown the ability to modify transcript accumulation of several structural genes, including PAL, chalcone synthase, chalcone isomerase, dihydroflavonol reductase,

flavonoid-3-hydroxylase, LAR, ANR and UFGT (Ban *et al*., 2003, Jeong *et al*., 2004, Lacampagne *et al*., 2009). Some of these genes are controlled by a single transcriptional regulator, MYBA1, also regulated by ABA (Ban *et al*., 2003, Jeong *et al*., 2004). On the other hand, treatments with exogenous ethylene are able to stimulate the gene expression for the anthocyanin biosynthesis pathway (El-Kereamy *et al*., 2003). Moreover, treatment with 1-methylcyclopropene, a specific inhibitor of ethylene receptors, inhibited UFGT transcript accumulation (Chervin *et al*., 2009). These results contribute to the hypothesis that the ethylene signal is likely a regulator of grape UFGT expression in grapes and this stimulation has shown to be independent from the MYBA1 transcription factor (Tira-Umphon *et al*., 2007). Additionally in the study of structural and regulatory gene transcript accumulation, proteomic studies have shown that ABA treatment acts through the over- or under-expression of the same pool of proteins involved in the ripening process, where anthocyanin biosynthesis enzymes are up-regulated (Giribaldi *et al*., 2010). Cell culture experiments in Cabernet Sauvignon have shown that applied CEPA as a single inducer failed to induce the PAL expression or trigger anthocyanin production (Faurie *et al*., 2009), while exogenous ABA treatment increased the cell ABA content and induced both the structural and regulatory genes involved in anthocyanin production, suggesting that ABA (and not ethylene) initiates the anthocyanin production (Gagné *et al*., 2010). This evidence suggests that S-ABA and CEPA treatments could be acting by different mechanisms but in the same direction and there may be a positive interaction between the ABA and the ethylene in the expression of the UFGT gene (Chervin *et al*., 2009). The effects of S-ABA and CEPA were particularly significant for the trihydroxylated

anthocyanins in PCE, therefore S-ABA and CEPA treatments may have more effect on the expression/activity of the flavonoid-3'5'-hydroxylases than on the flavonoid-3'-hydroxylases (Castellarin *et al*., 2006). There is evidence that some environmental factors, such as light, also affect the anthocyanin composition (Ristic *et al*., 2007). This could have an effect on the color of grapes and wine as dihydroxylated anthocyanins produce predominantly orange hues while trihydroxylated ones confer red-purple hues (Heredia *et al*., 1998). Our results did not show any significant difference in wine hues, but other reports showed that ABA treatments altered the color value and decreased the berry hue (Delgado *et al*., 2004).

IAA, which corresponds to the most abundant auxin, has been described as a plant hormone that promotes cell elongation in the first stages of berry development and could have a negative effect on maturation by delaying *veraison* and diminishing coloration by reduction of the anthocyanin concentration in some non-climacteric fruits such as strawberries and grapevines (Given *et al*., 1988, Ban *et al*., 2003, Deytieux-Belleau *et al*., 2007, Böttcher *et al*., 2010). In accordance with these studies, applied IAA in this study showed a negative effect on some grape skin anthocyanin glycoside content.

Information about the effects of PGR applications on wine composition and sensory characteristics is poor. We observed that CEPA treatment caused significant increments in wine total phenols, while S-ABA treatments caused only minor non-significant increments in the wine. These results confirm the superior effect of CEPA over S-ABA in the conditions of the present study for improving the accumulation of phenolic compounds in wine. The results show a greater

accumulation of anthocyanins in wines from the CEPA- and S-ABA-treated grapes. This effect was higher with the CEPA treatment than with the S-ABA treatment, as Venburg *et al.* (2009) showed. The total tannin was increased by the CEPA treatment.

Wine sensory analysis showed an absence of significant differences between the control and the PGR-treated wines. This result agrees with the results obtained by Böttcher *et al.* (2010), who did not find significant differences in sensory properties between small-scale wine lots made from control and NAA-treated fruit. Wines from the CEPA-treated grapes had higher levels of bitterness and tannic intensity (significant with respects to the S-ABA and IAA treatments, respectively). This result could relate to the high concentration of tannin found in these wines. No significant differences in TPI could be found in the grapes or the wine, therefore no relation with the differences in sensory attributes could be explained with this tannin quality index.

Our results have shown an important effect of the ethylene-releasing compound CEPA, which is greater than the effect of S-ABA, showing the relevance of ethylene levels at *veraison* on the grape ripening process and the grape and wine quality. Even if changes in the ethylene gas levels during grape berry ripening are small compared to the climacteric fruits, the physiological response to ethylene may be modulated more by changes in the sensibility of the perception of this hormone than in its levels (Davies and Böttcher, 2009).

In conclusion, the exogenous PGR application at *veraison* modifies the hormonal balance in grape skins, affecting the development of the grape ripening phase and modifying several grape and wine quality parameters. A better

understanding of each step in the molecular pathway (gene expression and enzyme activity) is required for elucidation of the mechanism for these effects.

ABBREVIATIONS USED

ABA, abscisic acid; ANOVA, analysis of variance; ANR, anthocyanidin reductasa; CEPA, 2-chloroethylphosphonic acid; CVE, commercial vineyard experiment; DAV, days after *veraison*; DMAC, 4-(dimethyl-amino)cinnamaldehyde; IAA, indole-3-acetic acid; LAR, leucoanthocyanidin reductase; NAA, 1-naphthaleneacetic acid; PAL, phenylalanine ammonia-lyase; PCE, plants in containers experiment; PGR, plant growth regulators; TSS, total soluble solids; S-ABA, (+)-S-abscisic acid; TPI, tannin polymerization index; UFGT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase.

ACKNOWLEDGMENTS

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González *et al.*; Table 1

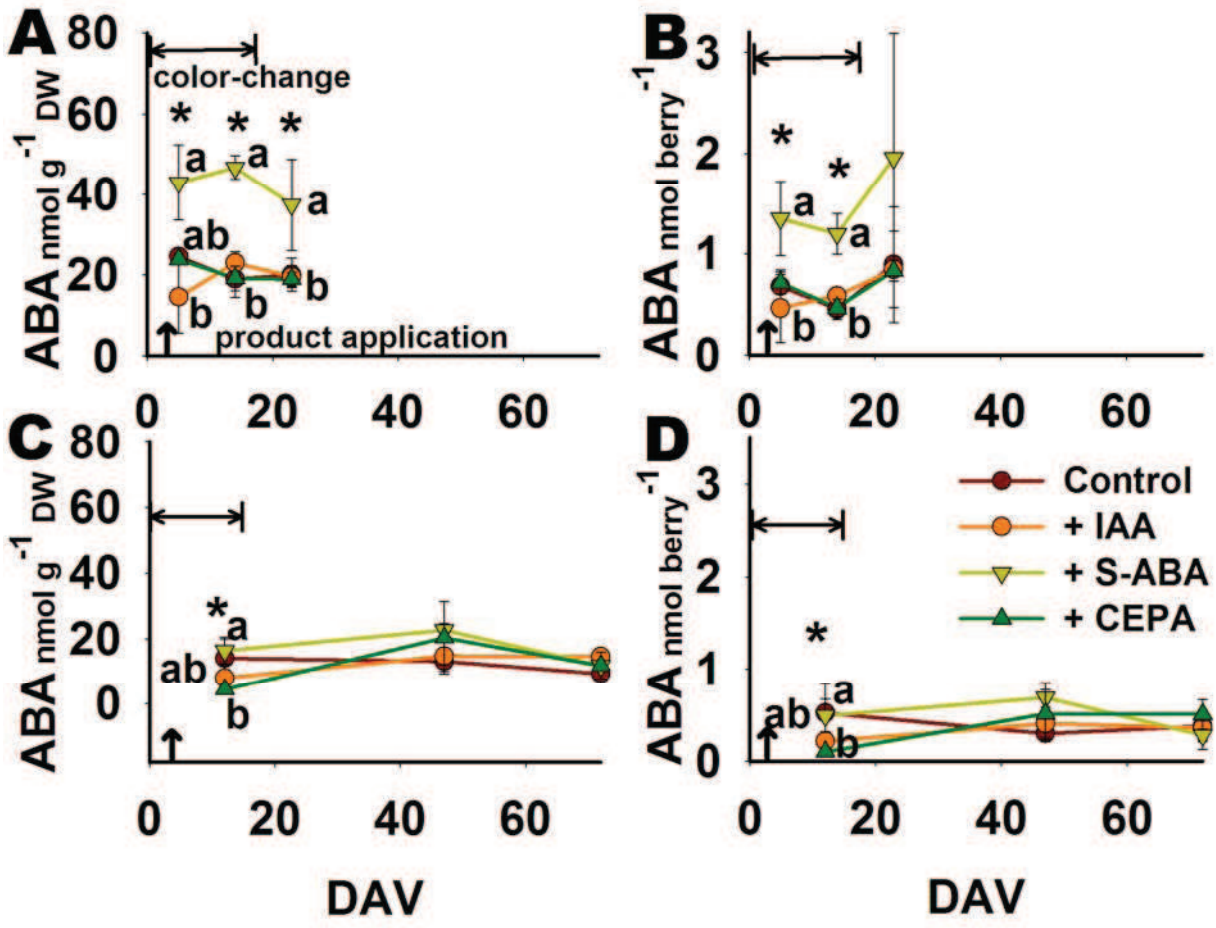
	Sample	Stage[a]	Date	DAV	TSS (°Brix)
Container Plants	1	35: Berries begin to color and enlarge	19/01	5	12,7
	2	35: Berries begin to color and enlarge	28/01	14	16,2
	3	36: Berries with intermediate sugar values	06/02	23	20,1
	4	37: Berries not quite ripe	25/02	42	21,5
	5	38: Berries harvest-ripe	27/03	72	23,9
Commercial Vineyard	1	35: Berries begin to color and enlarge	29/01	12	16,7
	2	37: Berries not quite ripe	05/03	47	22,6
	3	38: Berries harvest-ripe	30/03	72	23,9

a Grapevine growth stages as defined by the Modified E-L system (Coombe, 1995).

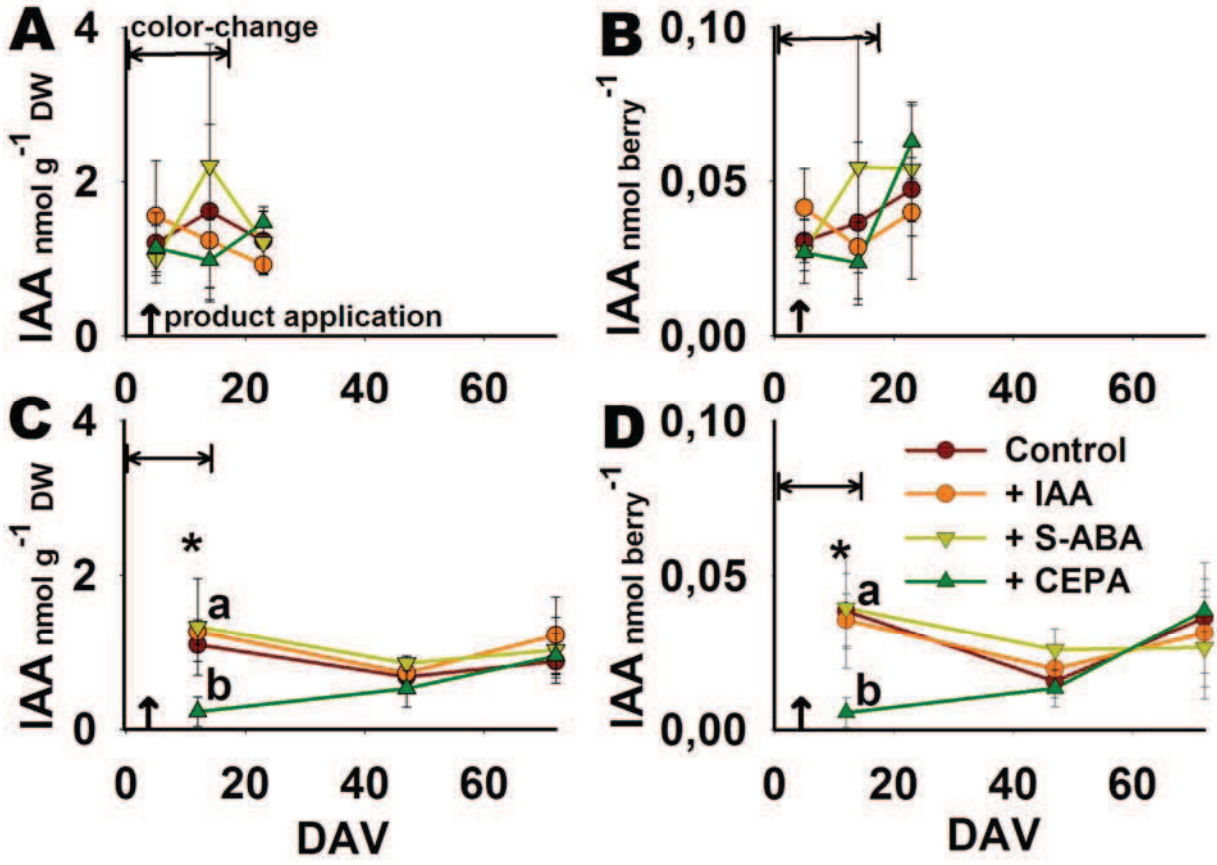
González *et al.*; Table 2

		NON-ACYLATED GLUCOSIDE					ACETYL GLUCOSIDE					COUMAROYL GLUCOSIDE					TOTAL GLUCOSIDE				
	DAV	CONTROL	IAA	S-ABA	CEPA	SIGNIFICANCE	CONTROL	IAA	S-ABA	CEPA	SIGNIFICANCE	CONTROL	IAA	S-ABA	CEPA	SIGNIFICANCE	CONTROL	IAA	S-ABA	CEPA	SIGNIFICANCE
Malvidin	42	51,1	51,8	50,2	53,9	ns	17,2	15,6	15,6	17,0	**	3,5	3,5	3,3	4,3	ns	71,9	70,9	69,1	75,2	ns
	72	54,9	50,3	51,7	52,5	ns	16,2	18,2	16,9	17,2	ns	**$5,4^{ab}$**	**$5,1^{ab}$**	**$4,3^{b}$**	**$5,7^{a}$**	*	76,5	73,6	72,9	75,4	ns
Petunidin	42	7,8	7,4	8,9	4,4	ns	0,8	1,1	1,0	0,7	ns	0,4	0,4	0,2	0,4	ns	9,0	8,8	10,1	5,4	ns
	72	**$5,4^{b}$**	**$6,4^{ab}$**	**$7,3^{a}$**	**$6,4^{ab}$**	*	0,8	0,9	1,1	0,5	ns	0,5	0,4	0,4	0,4	ns	**$6,7^{b}$**	**$7,7^{ab}$**	**$8,8^{a}$**	**$7,2^{b}$**	*
Delphinidin	42	4,0	3,1	5,3	4,5	ns	1,1	1,1	1,5	1,3	**	2,7	3,3	2,3	2,1	ns	7,8	7,5	9,1	7,9	ns
	72	2,1	3,7	3,9	3,6	ns	1,2	1,1	1,3	1,0	ns	3,0	2,3	2,7	2,3	ns	6,4	7,1	8,0	6,9	ns
Peonidin	42	6,6	7,3	7,0	6,7	ns	1,9	1,8	1,8	2,1	ns	0,8	0,8	0,8	0,9	ns	9,2	9,9	9,5	9,8	ns
	72	6,2	6,9	6,0	6,2	ns	1,4	2,1	1,8	1,8	ns	**$0,8^{ab}$**	**$0,9^{a}$**	**$0,6^{b}$**	**$0,9^{ab}$**	*	**$8,3^{b}$**	**$9,9^{a}$**	**$8,4^{ab}$**	**$8,9^{ab}$**	*
Cyanidin	42	1,3	1,9	1,5	1,1	ns	0,3	0,5	0,3	0,2	ns	0,5	0,6	0,4	0,4	ns	2,1	2,9	2,2	1,7	ns
	72	1,8	1,4	1,6	1,4	ns	**$0,0^{b}$**	**$0,1^{ab}$**	**$0,1^{a}$**	**$0,1^{ab}$**	*	0,3	0,2	0,2	0,2	ns	2,1	1,7	1,9	1,6	ns
Total	42	70,7	71,4	72,8	70,6	**	21,4	20,1	20,2	21,3	**	**$7,9^{ab}$**	**$8,5^{a}$**	**$7,0^{b}$**	**$8,1^{a}$**	*	100,0	100,0	100,0	100,0	
	72	70,5	68,8	70,5	70,0	ns	19,6	22,3	21,2	20,6	ns	**$9,9^{a}$**	**$8,8^{ab}$**	**$8,3^{b}$**	**$9,4^{ab}$**	*	100,0	100,0	100,0	100,0	

González *et al.*; Figure 1



González *et al.*; Figure 2



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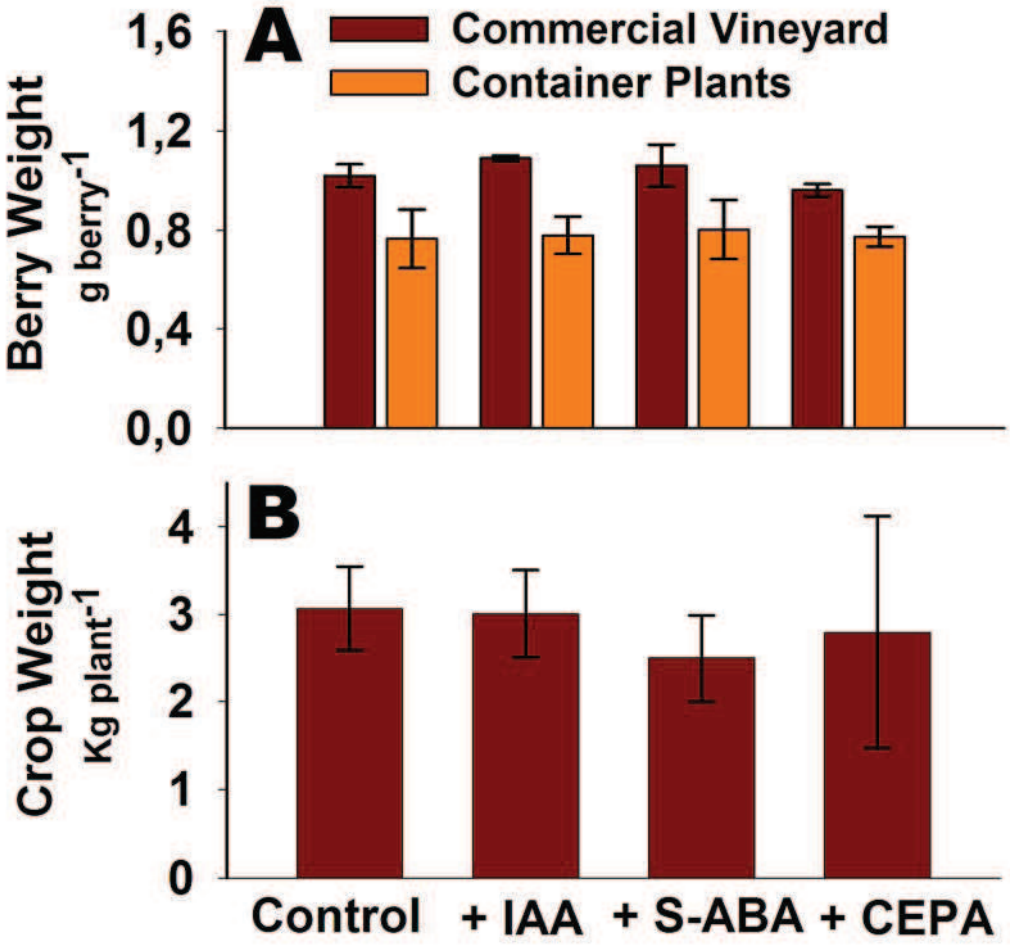

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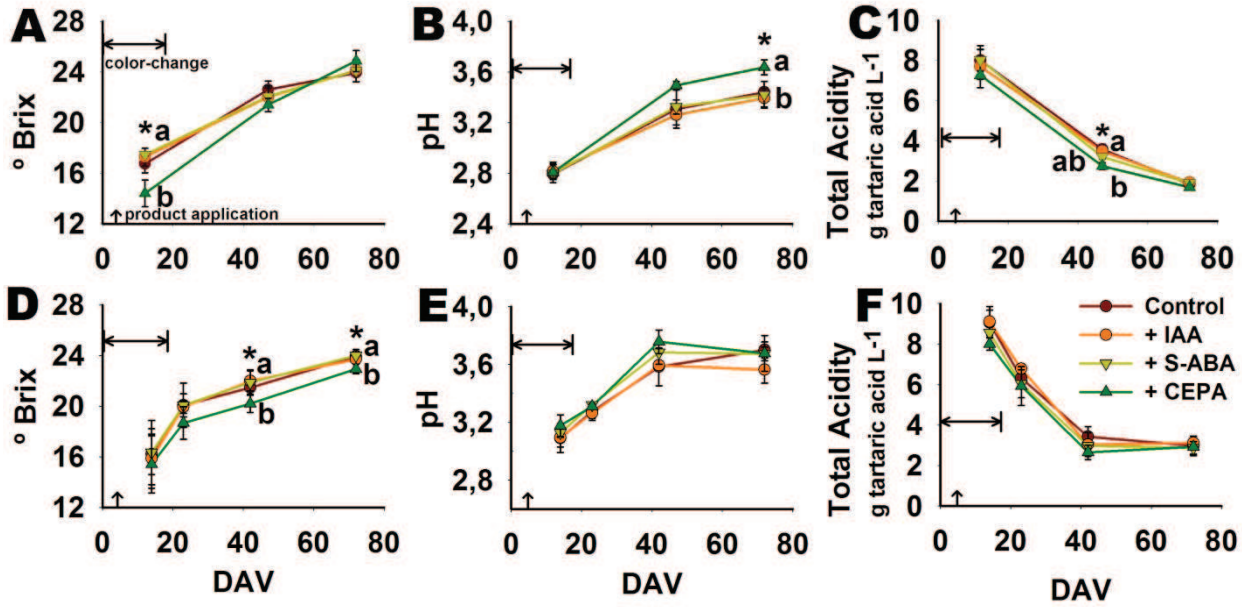

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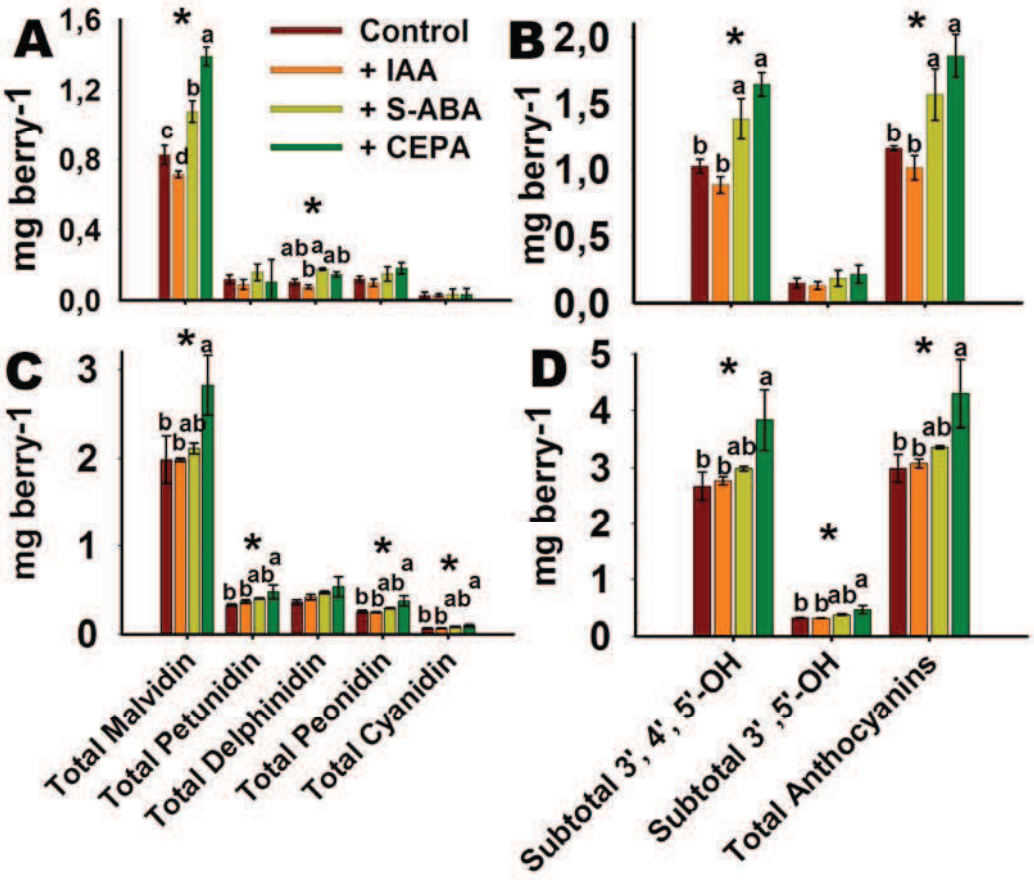

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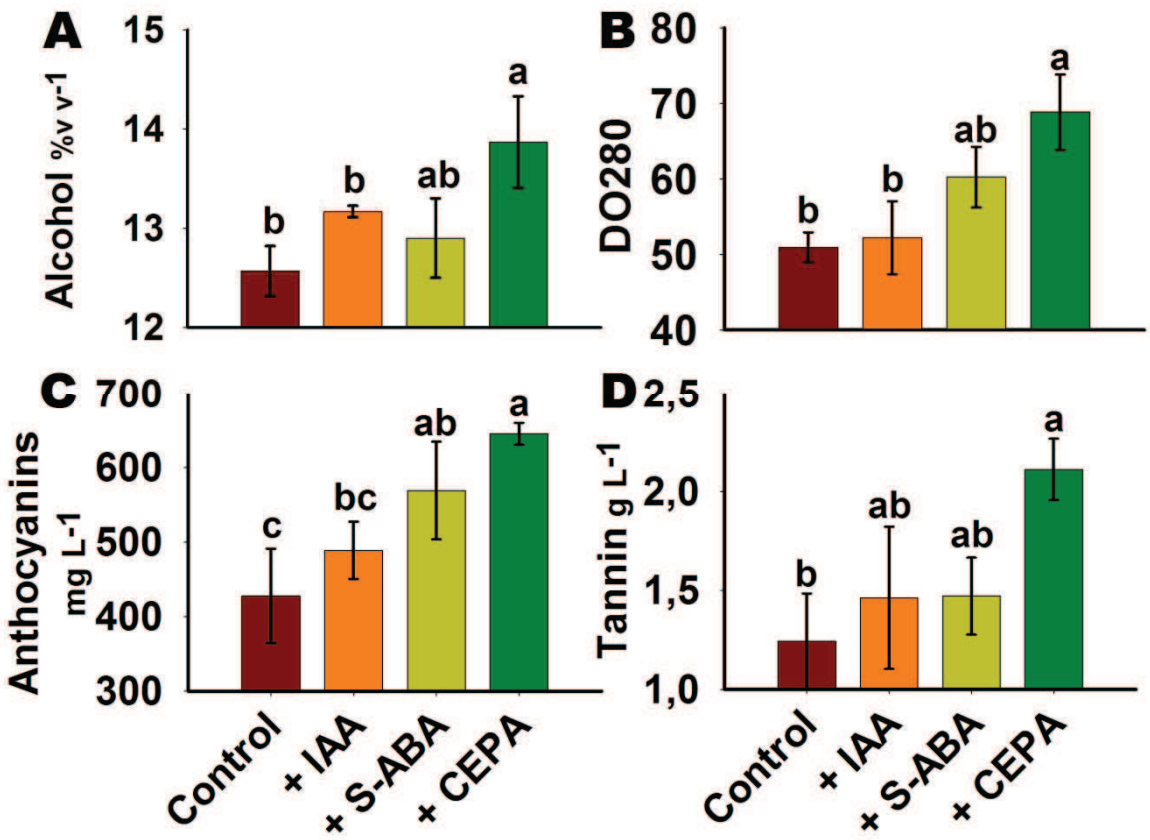
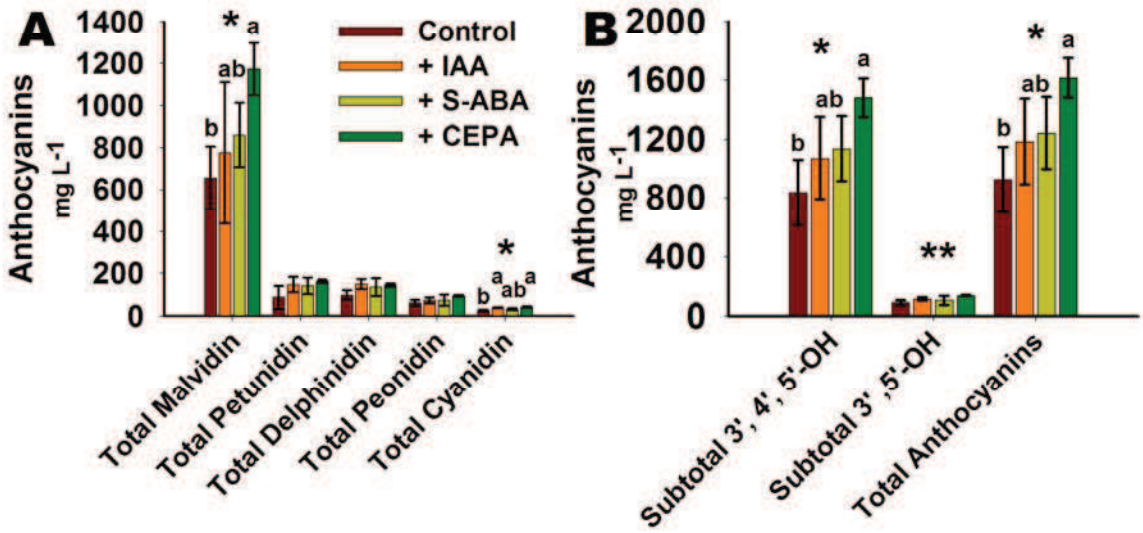


González *et al.*; Figure 7



González *et al.*; Figure 8

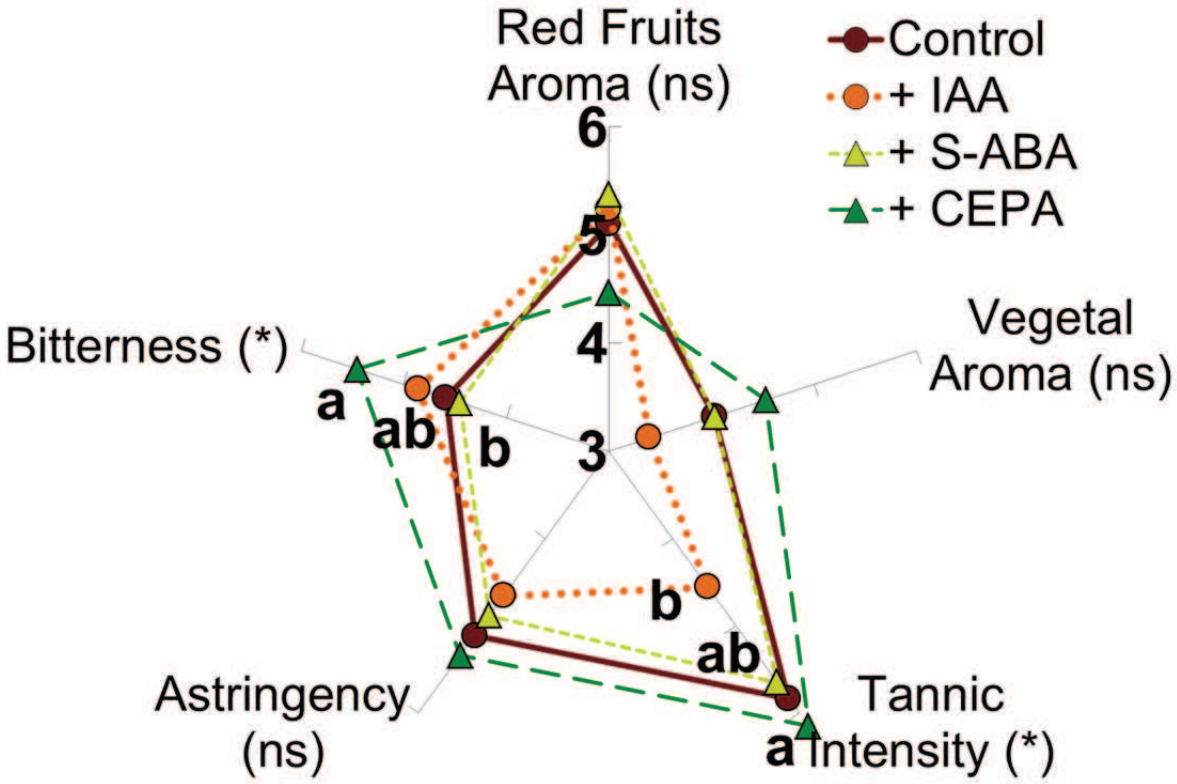


TABLE AND FIGURE LEGENDS

Table 1. Grapevine growth stage, sampling date, days after veraison and total soluble solids of the grape samples.

Table 2. Percent composition of different acylated forms of anthocyanins in the berry skins of Cabernet Sauvignon container plants at 42 and 72 DAV (corresponding to 21.5 and 23.9 ºBrix respectively) during the 2009 season for treated and control grapes. The effect of the application of growth regulators on the proportion of each anthocyanin is shown; ns indicates no significant difference, while an asterisk indicates a significant difference between the treatments at the same date at $p<0.05$ (ANOVA) and a double asterisk indicates a significant difference between treatments at the same date at $p<0.1$ (ANOVA). Superscripts indicate differences between the treatments (Tukey's HSD multiple comparison procedure). An arcsine-root transformation was performed.

Figure 1. Skin internal “free” ABA content in nanomoles per gram dry weight (A, C) and nanomoles per berry (B, D) in a cv. Cabernet Sauvignon containers (A, B) and commercial vineyard (C, D), of treated and control plants. DAV: days after veraison. The data represent the mean of three independent replicates ± standard deviation (error bars). The asterisk and letters indicate, for the same date, a significant difference between treatments ($p<0.05$; ANOVA and Tukey's HSD multiple comparison procedure).

Figure 2. Skin internal IAA content in nanomoles per gram dry weight (A, C) and nanomoles per berry (B, D) in a cv. Cabernet Sauvignon containers (A, B) and commercial vineyard (C, D) of treated and control plants. DAV: days after veraison. The data represent the mean of three independent replicates ± standard deviation (error bars). The asterisk and letters indicate a significant difference between the treatments ($p<0.05$; ANOVA and Tukey's HSD multiple comparison procedure).

Figure 3. Total Soluble Solids (A, D), pH (B, E) and titratable acidity (C, F) of a commercial vineyard (A, B, C) and a container plants experiment (D, E, F) for berry pulp samples of treated and control grapes. DAV: days after veraison. The data represent the mean of three independent replicates ± standard deviation (error bars) and an asterisk indicates a significant difference between the treatments at the same date ($p<0.05$; ANOVA).

Figure 4. Total Soluble Solids (A, D), pH (B, E) and titratable acidity (C, F) of a commercial vineyard (A, B, C) and a container plants experiment (D, E, F) for berry pulp samples of treated and control grapes. The data represent the mean of three independent replicates ± standard deviation (error bars) and an asterisk indicates a significant difference between the treatments at the same date ($p<0.05$; ANOVA).

Figure 5. Grape skin anthocyanin composition, including malvidin, petunidin, delphinidin, peonidin and cyanidin (A, C) and trihydroxylated, dihydroxylated and total anthocyanins (B, D) of PGR-treated and control grapes from cv. Cabernet Sauvignon container plants at 42 DAV (A, B) and a commercial vineyard at 47 DAV

(C, D). The data represent the mean of three independent replicates ± standard deviation (error bars) and an asterisk indicates a significant difference between treatments at the same date ($p<0.05$; ANOVA).

Figure 6. Wine composition, including alcohol content (A), DO280 index (B), total anthocyanins (C) and total tannin (D) in wines made from PGR-treated and control grapes from a cv. Cabernet Sauvignon commercial vineyard. The data represent the mean of three independent replicates ± standard deviation (error bars) and an asterisk indicates a significant difference between the treatments at the same date ($p<0.05$; ANOVA).

Figure 7. Wine anthocyanin composition, including malvidin, petunidin, delphinidin, peonidin and cyanidin (A) andtrihydroxylated, dihydroxylated and total anthocyanins (B) from PGR-treated and control grapes from a cv. Cabernet Sauvignon commercial vineyard. The data represent the mean of three independent replicates ± standard deviation (error bars) and an asterisk indicates a significant difference between the treatments at the same date ($p<0.05$; ANOVA).

Figure 8. Wine sensory analysis, including red fruits aroma, vegetal aroma, tannic intensity, astringency and bitterness of wines made from PGR-treated and control grapes from a cv. Cabernet Sauvignon commercial vineyard. Vertical bars indicate the standard deviation (three biological replicates). Asterix indicates significant differences and “ns” indicates non-significant differences between treatments as calculated by ANOVA ($P<0.05$). Different letters indicate significant differences

between treatments as calculated by Tukey's HSD multiple comparison procedures ($P < 0.05$).

Chapter 4

S-Abscisic Acid and 2-Chloroethylphosphonic Acid Treatments Modify Grape Skin Flavonoid Biosynthetic and Regulatory Gene Expression and Grapes and Wine Quality in *Vitis vinifera* L. cv. Carmenère

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ABSTRACT

Red wine quality is highly determined by its phenolic composition and aromas. In some vineyards, environmental conditions could represent a challenge for the achievement of an adequate wine quality. In these cases, plant growth regulators (PGRs) could help to improve grapes and wine quality. In the present study, PGR treatments in developing grapes were established for determining their effects on flavonoid biosynthetic and regulatory genes expression and grapes and wine phenolic and general composition. This experiment was performed under field conditions in a cv. Carmenère vineyard during 2008 and 2010. (+)-S-abscisic acid and 2-chloroethylphosphonic acid treatments were applied at two phenological stages: ten days after anthesis and *veraison*. Applied PGRs had significant effects on grapes and wine phenolic composition measured by HPLC-DAD and spectrophotometry, but these effects were highly variable between seasons and in particular in 2010 season, poor treatment effects were found. In 2010, flavonoid biosynthetic and regulatory genes expression, assessed by QRT-PCR, showed up-regulation of CHS2, LAR2, OMT, MYB5a and MYB5b and surprisingly down-regulation of LDOX, UFGT and MYBA1 in grapes treated at *veraison*, showing an up regulation of tannin biosynthesis and a down regulation of anthocyanin biosynthesis. Effects on wine chemical composition and sensory attributes showed a high seasonal dependence. While in 2008 both PGRs produced significant diminutions on wine alcohol degree (reductions of 0.4 and 0.5 % v v^{-1}), no effect on this parameter was found in 2010. In 2008, wine chemical composition was clearly affected by treatments with up to 22.6% increase in color intensity and up to 33%

increase in total tannin concentration. Wine sensory attributes were also positively affected, with a reduction in bitterness and vegetative aroma.

KEYWORDS: polyphenol; anthocyanin; tannin, RT-PCR, plant growth regulators, CHS2, UFGT, LAR2

S-Abscisic Acid and 2-Chloroethylphosphonic Acid Treatments Modify Grape Skin Flavonoid Biosynthetic and Regulatory Gene Expression and Grapes and Wine Quality in *Vitis vinifera* L. cv. Carmenère

INTRODUCTION

The Plant growth regulator (PGR) 2-chloroethylphosphonic acid (CEPA) is an ethylene releasing compound and it has been demonstrated that CEPA treatments significantly increase endogenous ethylene content (El-Kereamy *et al.*, 2003). The treatment of grapes with CEPA, has been extensively studied (Szyjewicz *et al.*, 1984). CEPA treatment at *veraison* increases grape color and the content of anthocyanin and seems to increase primarily the end product anthocyanins peonidin and malvidin (El-Kereamy *et al.*, 2003, Human and Bindon, 2008, Amiri *et al.*, 2009, Szyjewicz *et al.*, 1984, Powers *et al.*, 1980, Tira-Umphon *et al.*, 2007, Roubelakis-Angelakis and Kliewer, 1986). Early CEPA treatment seems to reduce total soluble solids (TSS) and pH (Szyjewicz and Kliewer, 1983).

On the other hand, exogenous Abscisic Acid (ABA) treatment increases the internal ABA levels (Deytieux-Belleau *et al*., 2007), accelerate the beginning of ripening and decrease the firmness of the berry (Peppi and Fidelibus, 2008,

Deytieux-Belleau *et al.*, 2007, Gagne *et al.*, 2006, Peppi *et al.*, 2008, Lacampagne *et al.*, 2009). ABA treatments induce higher grape skin coloration and a greater accumulation of anthocyanins (Peppi *et al.*, 2008, Jeong *et al.*, 2004, Omran, 2011, Ban *et al.*, 2003, Gagne *et al.*, 2006, Deytieux-Belleau *et al.*, 2007, Villalobos, 2011, Hiratsuka *et al.*, 2001). Early ABA treatments have reduced grape tannin content (Lacampagne *et al.*, 2009) while applied days before *veraison* they have increased tannin and (-)-epicatechin contents and have reduced (+)-catechin content (Villalobos, 2011).

Cultivar Carmenère is widely cultivated in Chile and is defined by the "Wines of Chile" organization like an emblematic, unique and differentiating element for the Chilean wine industry (WinesofChile, 2010). Even when cv. Carmenère has no difficulties to achieve an adequate anthocyanin and color accumulation, it has characteristic herbaceous notes (Domínguez and Agosín, 2010), that can reach undesirable levels. These notes seem to be the effect of high 3-isobutyl-2-methoxypyrazine (IBMP) and 3-isopropyl-2-methoxypyrazine (IPMP) concentrations, much higher than in other varietal wines (Belancic and Agosin, 2007). It has been reported that IBMP and IPMP levels descend with ripeness level (Allen and Lacey, 1999), so the control of ripening with PGRs application could represent a way to control herbaceous notes of cv. Carmenère.

The application of growth regulators has become an interesting industrial practice for the control of maturation and the improvement of grapes and wine quality. The use of ABA as an agrochemical has become feasible recently due to lower production costs. Improved synthesis procedures allow the obtention of (+)-

S-abscisic acid (S-ABA), an active enantiomer with potential use in viticulture, as seen on several patent applications (Venburg *et al.*, 2008, Venburg *et al.*, 2009). In Chile, there are only a few experiences with growth regulators applied to wine grape varieties, and almost no studies on their impact on wine quality are avalaible.

The objective of this study was to determine the effect of PGRs promoters of ripening S-ABA and CEPA on flavonoid biosynthetic and regulatory gene expression of grape skins and on the accumulation of flavonoid compounds in cv. Carmenère grapes and wine.

MATERIALS AND METHODS

Plant Material and Sample Collection

The grape samples were collected from a commercial vineyard experiment, located at the Cachapoal Valley of Chile (34.28ºS, 71.27ºW) during 2007/2008 and 2009/2010 seasons. The plants of *Vitis vinifera* L. cv. Carmenère were conducted as a traditional north-west/south-east vertical trellis with cane pruning. Vines were rooted on their own, 12 years old and planting density was 2,667 vines per hectare.

The experiment layout consisted of three completely randomized 16-plant replicates. Thirty random 5-berry bunch fragment samples were collected per each replicate at three and seven phenological stages in 2007/2008 and 2009/2010 respectively (table 1).

Experimental Treatments

In these experiments S-ABA, the naturally-occurring enantiomer was used, instead of synthetic ABA, a racemic mixture of (+)-S-ABA and (-)-R-ABA, molecules that can be different in inducing gene expression and physiological responses (Zaharia *et al.*, 2005). The PGR concentrations were based on manufacturer-recommended doses, 400 mg L^{-1} for the S-ABA (VBC-30051, Valent BioSciences Corporation, USA) and 480 mg L^{-1} for the CEPA (Ethrel, Bayer CropScience AG, Germany). Half doses were applied in 2008 and early applications (10 days after anthesis, DAA) were done in 2010. The treatment involved applying the PGR solution containing 0.1 % Tween80 as a wetting agent on the treated grapes or water containing the same Tween80 concentration on the control grapes. The application was done six days before *veraison* in 2008 and one day after *veraison* (DAV, with *veraison* defined as one per-cent color change in the grapes) corresponding to 61 DAA and 2-3 % color-change in 2010. All clusters of entire plants were treated using an agricultural hand sprayer at a water rate of 1,000 L ha-1. All treatments were performed at sunset to minimize photodestruction of ABA.

Skin Phenolic Extractions

Immediately after sampling, the fresh berries were weighed and the berry skins were hand separated. The skins were rinsed with distilled-deionized water, frozen and then lyophilized, before final grinding. One gram of ground lyophilized skins was extracted (Kennedy and Jones, 2001) in 10 mL of a 2:1 acetone/water solution for one hour in a shaker at room temperature and then centrifuged at 4,000 rpm for six minutes. After centrifugation, the supernatant extract was concentrated under reduced pressure at 35 °C in a rotary evaporator to remove acetone and then dissolved in a 100 mL model wine solution (12 % v v^{-1} ethanol, pH 3.6 and 0.033M tartaric acid). To minimize oxidation, the solutions were sparged with nitrogen gas. From the homogenized berry pulp, we determined the TSS by direct reading in a digital refractometer (Pocket PAL-1, Atago, Japan), the pH using a pH meter (Orion 5-Star, Thermo Scientific, Singapore) and the titratable acidity (TA) using a pH meter and 0.1N NaOH.

Winemaking

Small-scale winemaking was performed only for the high doses treatments in 2008 and for all the treatments in 2010, for all biological replicates. The wine was made through a traditional red wine fermentation protocol. The grapes were picked on the commercial harvest date, April 18 for 2008 (corresponding to 85 DAV) and April 28 for 2009 (corresponding to 90 DAV and 150 DAA). Twenty-five kilograms of

grapes were harvested from each of the replicates of the control and the PGR-treated plants. Fermentation was carried out in plastic 25-L containers in a controlled temperature room with Lalvin EC-1118 yeast. Complete spontaneous malolactic fermentation was performed and an adjustment to 30 mg L-1 free sulfur dioxide was made in all the wines prior to bottling.

RNA extraction and cDNA synthesis

RNA extraction, cDNA synthesis and quantitative comparison of gene expression were done on the 2010 experiment in samples 1 to 5 (10 to 81 DAA; table 1). Grape skins from 25-to-50-frozen-berries were carefully removed for analysis using razor blades and the tissue was grounded with liquid nitrogen. A tissue pool mix was done of every three biological replicates. Total RNA was isolated from berry skins according to the procedure of Reid *et al*. (2006), using a CTAB-Spermidine extraction buffer. RNA integrity was verified using denaturing formaldehyde agarose gel electrophoresis with ethidium bromide. A DNAse treatment was performed using RQ1 RNase-Free DNase (Promega, Wisconsin, USA). For cDNA synthesis, one µg of total RNA was reverse transcribed with random hexamer primers in an 18 µl reaction mixture using the SuperScript™ II reverse transcriptase (Invitrogene, California, USA) according to the manufacturer's instructions. The cDNA product purity was checked by PCR amplification and agarose gel electrophoresis, using gene-specific primers, to verify the absence of genomic DNA.

Quantitative comparison of gene expression throughout berry skin development

Relative transcript quantification of isolated genes was performed by real-time RT-PCR, using the Sensimix® SYBR® Kit (Bioline) and the Mx3000P detection system (Stratagene, California, USA) as described in the manufacturer's manual. Amplification of a fragment of the ACTIN1 gene (72 bp) was used as an internal control for the calibration of relative expression (Serrano, unpublished results). PCR conditions, standard quantification curves for each gene (table 2), primer efficiency values and relative gene expression calculations were conducted according to Matus *et al.* (2008). Standard quantification curves with serial dilutions of PCR products were constructed for each gene to calculate amplification efficiency according to:

$$\mathrm{E} = \left[10^{\left(-\frac{1}{\mathrm{slope}}\right)}\right] - 1$$

This value was then used to obtain an accurate ratio between the expression of the gene of interest (GOI) and the housekeeping gene, using:

$$\frac{(1 + E_{GOI})^{-\Delta Ct}}{(1 + E_{Ubiquitin})^{-\Delta \mathrm{Ct}}} = \frac{(1 + E_{GOI})^{-(\mathrm{Ct\ GOI\ -\ Ct\ GOI\ calibrated})}}{(1 + E_{Ubiquitin})^{-(\mathrm{Ct\ Ubi\ -\ Ct\ Ubi\ calibrated})}}$$

Gene expression levels were normalized to the expression of the first sampling date control sample, in order to obtain a calibrated DCt for each gene. In all cases,

R^2 values of standard curves were above 95%. All experiments were performed with three technical replicates. Reaction specificities were tested with melt gradient dissociation curves, electrophoresis gels, and cloning and sequencing of each PCR product.

Phenolic Compounds Analysis

The phenolic composition of the grapes and wine was determined using a UV/Vis spectrophotometer model Spectronic Genesys 2 (Milton Roy, Rochester, NY). The total anthocyanins were determined at 520 nm according to the method described by Puissant and Leon (1967), the total phenols were determined by DO280 and the total tannin was determined by precipitation with methyl cellulose (Sarneckis *et al.*, 2006). The tannin determination with the 4-(dimethyl-amino)cinnamaldehyde (DMAC) assay, in accordance with the method described by Vivas *et al*. (1994), was performed in the 2008 experiment to estimate a tannin polymerization index (TPI), calculated as the quotient between the total tannin and the DMAC index. All the analyses were performed in duplicate.

Anthocyanins Analysis by HPLC

The chromatographic system for the HPLC-DAD analysis of the anthocyanins consisted of a LaChrom Elite® HPLC system with a 1,024 photodiode-array detector (Hitachi LaChrom Elite, Japan). Separation was performed using a Purospher® STAR (Merck, Germany) reverse-phase C18 column (250 mm X 4.6

mm i.d., 5µm) at 20ºC. The detection was carried out at 520 nm. The elution gradient consisted of two solvents: Solvent A was water/formic acid 90:10 v v-1 and solvent B was acetonitrile, following the methodology described by Fanzone *et al.* (2010). After filtering through a 0.45 µm pore size membrane, a 150 µL aliquot of grape skin extract was injected. Delphinidine-3-glucoside, peonidine-3-glucoside and malvidin-3-glucoside (Extrasynthese, Lyon, France), were used as standards and the other anthocyanins were identified by comparison of the standard retention times.

Wine Sensory Analysis

A nine person sensory analysis panel was formed consisting of students and staff of the Enology Laboratory of The Faculty of Agronomy and Forestry Engineering, “Pontificia Universidad Católica de Chile”. All the panel members were familiarized with the method of sensory analysis and aromatic standards during a 2-week training (four sessions). For the vegetal aroma, 3-isobutyl-2-methoxypyrazine and 3-isopropyl-2-methoxypyrazine standards (Centro de Aromas y Sabores, DICTUC, Chile) were used. For the astringency, tannic intensity and bitterness, the method proposed by Delteil (2000) was used. A structured 9-unit linear scale was used. The samples were individually three-digit coded and presented in random order to the panelists. The assessments were made following general requirements for sensory testing conditions. Each wine sample was tasted three times by each panelist.

Statistical Analysis

The results were compared by one-way (for completely randomized experiments) and two-way (for completely randomized block experiments) analysis of variance (ANOVA) and Tukey's HSD multiple comparison procedure, with $P<0.05$ statistical significance between treatments, using Statgraphics Plus (Statistical Graphics Corp., Princeton, NJ, USA).

RESULTS

Growing seasons

Cachapoal valley growing seasons 2008 and 2010 were considerably different. Season 2008 was characterized by high water deficit as a result of winter-spring precipitations deficit (328 mm; 50% deficit) and high summer temperatures leading to pre-harvest berry shriveling. In this season two rains during berry development took place (March 17; April 11) and high grape yields were obtained. Season 2010 had normal winter-spring precipitations (500 mm) and low summer temperatures leading to good water conditions and low grape yields. The complete absence of precipitations during berry development lead to late harvest dates (Asociación Nacional de Ingenieros Agrónomos Enólogos de Chile, 2008 and 2010).

Effect of Growth Regulators Treatments on Berry Maturation

In the 10-DAA 2010 experiment, a significant higher berry weight in S-ABA-treated grapes was found at the last sampling date (129 DAA; figure 1C). In the *veraison* 2010 and 2009 experiments, no berry weight significant differences were found. At harvest time, no crop weight significant differences were evidenced between treatments in either experiment (figure 2) with the exception of CEPA applied 10 DAA in 2010, where a complete necrosis of bunches (figure 3) lead to cero production. However, growth regulator applications altered pulp maturation parameters (figure 4).

In 2008 PGR treatments lead to lower TSS and TA and higher pH values when compared with control grapes at the second sampling date (33 DAV). Some of these effects were significant: the higher dose of S-ABA leads to lower TSS and higher pH and both S-ABA doses lead to higher TA. No significant differences were found at the first and third sampling dates (4 and 67 DAV respectively). In 2010, *veraison* applications lead to small differences in TSS that became significant only when S-ABA and CEPA were compared and both PGRs lead to higher pH values, significant in the case of CEPA, when compared with control grapes at the last sampling date (129 DAA). No significant differences were found with *veraison* applications at all other sampling dates and with 10 DAA PGR applications.

Effect of Growth Regulators Treatments on Skin Phenolic Composition

The phenolic composition of grape skins was significantly modified by PGR treatments in the 2008 experiment (figure 5). S-ABA and CEPA treated grapes, showed higher anthocyanin concentrations that lead at the third sampling date, to increases of 26.9 and 70.8% respectively. This effect was significantly higher in skins from CEPA-treated grapes. Skins of half-dose-S-ABA and half-dose-CEPA-treated grapes showed significant transient increases of anthocyanin content at the second and first sampling dates (33 and 4 DAV respectively), but showed no significant differences in the third grape sample (67 DAV). The effects of PGRs on skin total phenols and tannins are less clear. Skins of S-ABA- and CEPA-treated grapes showed significant transient increases of total phenols and tannin concentration at the first and second sampling dates respectively (4 and 33 DAV). Skins of half-dose-S-ABA treated grapes showed only a significant increase of total phenols at the first sampling date, while the skins of half-dose-CEPA-treated grapes showed this significant effect at the first and second sampling dates (4 and 33 DAV). No significant effect of half-dose-PGR applications on tannin concentration was found and no significant effect of PGRs on any measured parameter was found at the third sampling date (67 DAV).

Effect of Growth Regulators Treatments on Skin Anthocyanin Concentrations

Grape skin 3-*O*-glycosylated anthocyanin concentrations of 2010 PGRs application treatments at *veraison* and 10 DAA are shown in figures 7 and 8 respectively.

Skins from *veraison*-S-ABA-treated grapes show significantly higher concentrations of peonidin at 109 DAA and of total anthocyanins, petunidin, peonidin and malvidin at 129 DAA, while skins from *veraison*-CEPA-treated grapes show significant higher concentrations of total anthocyanins, cyanidin, petunidin and peonidin at 129 DAA. No significant differences were found between treatments at 66 and 73 DAA (figure 7). Skins from 10-DAA-S-ABA-treated grapes showed significant higher concentrations of cyanidin at 73 DAA. No other significant differences were found between treatments: no significant differences in total anthocyanidin at any sampling date and no significant differences between treatments at 66, 109 and 129 DAA (figure 8).

Effect of PGR Treatments on PC biosynthesis gene expression in Grape Skin

Variation in PC biosynthetic pathway structural genes expression in grape skins during development is shown in figures 9 and 10. Skins of S-ABA and CEPA-treated grapes at *veraison* showed significant overexpression of chalcone synthase 2 (CHS2), leucoanthocyanidin reductase 2 (LAR2) and O-methyltransferase (OMT) genes and significant underexpression of leucoanthocyanidin dioxygenase (LDOX) and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) genes (figures 9A, 9C, 9E, 10A, 10C and 10E). These effects were observed in general across the three sampling dates (66-81 DAA) with some exceptions: S-ABA on LAR2 (66 DAA) and on LDOX (66 DAA) and CEPA on OMT (73 DAA). The most important effects of PGRs treatments applied at *veraison* are displayed in CHS2 and OMT gene expressions, where PGR-treated grapes were overexpressed in 120 to 590%

and 50 to 320% respectively. The effect on anthocyanidin reductasa (ANR) gene expression is more complex and there was only effect of S-ABA (73, 81 DAA). Skins of S-ABA-treated grapes at 10 DAA showed significant transient overexpression of CHS2, LAR2, and OMT genes and significant transient underexpression of LDOX and UFGT genes (figures 9B, 9D, 9F, 10B, 10D and 10F). Specifically S-ABA treatment significantly overexpressed CHS2 (73, 81 DAA), LAR2 (23, 81 DAA), ANR (81 DAA) and OMT (23, 81 DAA). While underexpressed LDOX (23, 73 DAA) and UFGT (73, 81 DAA). The effect of CEPA treatment at 23 DAA was significant only on CHS2 gene.

Effect of PGR Treatments on MYB TFs genes expression in Grape Skin

Variation in MYBA1, MYB5a and MYB5b genes expression in grape skins during development is shown in figure 11. Skins of S-ABA and CEPA-treated grapes at *veraison* showed significant overexpression of MYB5a and MYB5b genes and significant underexpression of MYBA1 gene (figures 11A, 11C, 11E). Some exceptions of these general effects were: PGRs on MYBA1 (66 DAA), S-ABA on MYB5a (73, 81 DAA), CEPA on MYB5a (81 DAA) and PGRs on MYB5b (66 DAA). Reduction of MYBA1 gene expression was between 46 to 59% and increments of MYB5a and MYB5b gene expression was between 190 to 710% and 250% to 1190%. Skins of S-ABA-treated grapes at 10 DAA showed significant overexpression of MYB5b gene expression at 81 DAA (increment of 180%). No additional effects of S-ABA or CEPA applied at 10 DAA on MYBA1, MYB5a or MYB5b were found (figures 11B, 11D, 11F).

Effect of PGR Treatments on HT1 gene expression in Grape Skin

Variation in HT1 gene expression in grape skins during development is shown in figure 12. Skins of S-ABA and CEPA-treated grapes at *veraison* showed significant underexpression of HT1 gene at 73 DAA (reduction of 45 to 50%) and skins of S-ABA-treated grapes at 10 DAA showed significant underexpression of HT1 gene at 73 DAA (reduction of 46%). Additionally, results show a significant overexpression of HT1 gene at 23 DAA when were compared to the expression of skins from CEPA-treated grapes, but no significant was found when the expression in skins of S-ABA or CEPA-treated grapes were compared with the control.

Effect of Growth Regulator Treatments on Wine Composition

The effect of treatments on wine composition is summarized in figure 13. Wine analysis showed that S-ABA and CEPA-treated grapes produced wines with significantly lower alcohol contents (- 0.4 and – 0.5 % v v^{-1}; - 2.7 and – 3.6% respectively) and higher anthocyanin contents (+ 11.9 and + 19.8% respectively) in the 2008 experiment. Additionally, CEPA-treated grapes produced wines with higher color intensity (+ 22.6%) and total phenols content (+ 14.9%) and S-ABA-treated grapes produced wines with higher tannin content (+ 33.3%) when compared with control wines in the 2008 experiment. The 2010 experiment did not show any significant effect for the analytical parameters of wine. The wines of both experiments did not show any significant difference for pH, total acidity and color hue (figure 13).

TPI was significantly modified by CEPA treatment in the 2008 experiment, showing a reduction of 17.4% (figure 14). This effect was not found in grapes, where a transient significant increase of TPI in PGR-treated grapes was found at 33 DAV and no effect of treatments were found at the third sampling date (67 DAV).

Effect of Growth Regulator Treatments on Wine Sensory Analysis

The effect of treatments on wine sensory analysis is summarized in figure 15. The sensory panel found significantly lower vegetative aromas and bitterness in S-ABA-treated wines when they were compared with control wines in the 2008 experiment. CEPA treatment didn't produce any significant difference in wine sensory attributes in both experiments. No significant differences of PGR treatments applied at *veraison* and at 10 DAA were found in any sensory attribute in the 2010 experiment.

DISCUSSION

The treatment effects show a high seasonal variability. While in 2008 treatments affected grape and wine chemical composition, in 2010 less differences were found in grapes and no differences were found in the wine. These differences can be explained by the variation in the climate conditions of each season. At berry development time, season 2008 presented higher temperatures, higher water deficit and higher yields than 2010 season. These three factors can impact the grape hormonal balance (Castellarin *et al.*, 2007b, Yamane *et al.*, 2006, Deluc *et*

al., 2009), the flavonoid compound accumulation and the quality of grapes and wine (Kennedy *et al.*, 2000, Bergqvist *et al.*, 2001, Spayd *et al.*, 2002, Mori *et al.*, 2007, Castellarin *et al.*, 2007a, Di Profio *et al.*, 2011). Other factors that could be impacting PGR treatment performance are developmental stage at the application time and growing conditions (Jeong *et al.*, 2004, Szyjewicz *et al.*, 1984, Davies and Böttcher, 2009). Growing conditions and in particular water status can influence hormonal balance of the berry. In 2008 application was earlier (6 days before *veraison*) than in 2010 experiment (1 DAV). Our results showed that the earlier 2008 treatment reduced wine alcohol content while in 2010 no differences were found for this parameter. These results differ from the hypothesis that ABA treatments may have the ability to hasten the initiation of sugar accumulation when applied early (before *veraison*), but cannot enhance it once ripening has already commenced (Davies and Böttcher, 2009).

Mean berry weight was affected only by 10-DAA-S-ABA treatment and in accordance (El-Kereamy *et al.*, 2003) or in contrast (Chervin *et al.*, 2008) with previous reports, we did not find any significant differences in mean berry weight with *veraison*-PGR treatments. A general controversial subject is the effect of CEPA treatments on TSS content (Szyjewicz *et al.*, 1984). In our experiments, CEPA application had no effect on TSS content. Like other studies (Szyjewicz *et al.*, 1984, Delgado *et al.*, 2004), S-ABA treatment significantly increased grape pH and decreased total acidity in the middle of the ripening phase, but ending without any difference at harvest time.

Results show a greater anthocyanin accumulation in skins from CEPA and S-ABA-treated grapes in 2008, lower effects with 2008 half-dose-treatments and

2010 *veraison*-treatments and almost no effect in 10-DAA-treated grapes. The positive effect of CEPA and ABA on anthocyanin accumulation is also reported in other studies (Jeong *et al.*, 2004, Gagne *et al.*, 2006, Deytieux-Belleau *et al.*, 2007, El-Kereamy *et al.*, 2003, Peppi *et al.*, 2008, Hiratsuka *et al.*, 2001, Anderson *et al.*, 2008). This effect was significantly higher in CEPA than in S-ABA 2008 treatments, contrary to the results obtained by Peppi (2006) in cv. Flame Seedless table grapes, where ABA applied at *veraison* was superior to CEPA applied at any of the times tested. These contradictory results could be consequence of the different varieties used in both experiments.

Higher flavonoid accumulation as a consequence of S-ABA and CEPA treatments can be hypothetically explained by the effect of these PGRs on the structural and regulatory flavonoid biosynthesis genes expression. Our results show that *veraison*-PGR-treated grapes up-regulates CHS2, LAR2, ANR and OMT genes. In previous studies, ABA treatments have shown the ability to modify transcript accumulation of several structural genes: PAL, CHS, CHI, DFR, F3H, LAR, ANR and UFGT (Lacampagne *et al.*, 2009, Jeong *et al.*, 2004, Ban *et al.*, 2003) and treatments with exogenous ethylene have shown to be able to stimulate anthocyanin biosynthesis pathway gene expression (El-Kereamy *et al.*, 2003).

Even if CHS2 was up-regulated in the skins of *veraison*-PGR-treated grapes, meaning that the initiation of flavonoid biosynthesis is induced, our results surprisingly show that LDOX, UFGT and MYBA1 genes are down-regulated in the skins of PGR-treated grapes (treated at 10 DAA or at *veraison*) showing a repression in the final steps of anthocyanin synthesis and its regulatory gene. This reveals a relative positive control for flavonoid accumulation and a relative negative

control of anthocyanin control. These results don't agree with previous reports and with the moderated increment in grape anthocyanin found at the last sampling date (129 DAA). While ABA has been reported to directly controls the MYBA1 regulatory gene (Jeong *et al.*, 2004, Ban *et al.*, 2003), 1-methylcyclopropene, a specific inhibitor of ethylene receptors, inhibited UFGT transcript accumulation (Chervin *et al.*), showing the positive relation of ethylene and UFGT gene. This effect can be explained by the fact that even if the carbon flux is limited in the final steps of anthocyanin synthesis, the overexpression of CHS2 should induce an increase of the carbon flux in the first steps of flavonoid synthesis. Additionally, other regulations are acting on the final metabolite concentration: post-transcriptional regulation, translation, and enzymatic activity. Cell culture experiments in Cabernet Sauvignon have shown that applied CEPA as a single inducer, failed to induce PAL expression or trigger anthocyanin production (Faurie *et al.*, 2009), while exogenous ABA treatment increased cell ABA content and induced both structural and regulatory genes involved in anthocyanin production, suggesting that ABA (and not ethylene) initiates the anthocyanin production (Gagné *et al.*, 2011). This evidence suggests that S-ABA and CEPA treatments can be acting by different mechanism but in the same direction, and even more there may be a positive interaction between ABA and ethylene for the expression of the UFGT gene (Chervin *et al.*, 2009).

The OMT gene was up-regulated in skins from treated grapes, especially in grapes treated at *veraison*. As is expected by this effect on OMT gene expression, anthocyanin composition was enriched in more methoxylated derivatives such as malvidin and peonidin (increment from 38 to 54%) and less in the intermediary

derivatives such as delphinidin, cyanidin and petunidin (increments from 19 to 33%). Even if no differences were found in wine hue, an increase in the methoxylation degree could shift coloration towards more purple colors (Heredia *et al.*, 1998).

It has been suggested that the control of ABA on berry ripening could be a process that begins early in berry development. Early ABA treatments have shown to control the ripening stage and flavonoid biosynthesis (Gagne *et al.*, 2006, Lacampagne *et al.*, 2009). On the other hand, CEPA is considered to be a promoter or inhibitor of ripening depending on the phenological stage of the berries at treatment time (Hale *et al.*, 1970). Early treatments seem to delay ripening and treatments just before véraison appeared to promote ripening (Coombe and Hale, 1973). In the 2010 experiment, 10-DAA treatments had several effects that were not produced with *veraison*-treatments. 10-DAA-S-ABA treatment produced higher berry weight at harvest time, almost no effects on grape skin anthocyanin concentration, up-regulation of LAR2 at the first stage of berry development, up-regulation of CHS2, LAR2, ANR, OMT and MYB5b and down regulation of HT1 near *veraison* time and no effects on wine chemical composition and sensory attributes. The 10-DAA-CEPA-treatment originated a complete bunch necrosis in cv. Carmenère (figure 3). Even if CEPA has been largely reported as a tool for cluster thinning leading to partial to near complete crop loss (Szyjewicz *et al.*, 1984), treatments in cv. Cabernet Sauvignon have shown absence of this necrosis, suggesting a varietal effect (González *et al*., unpublished results). Cultivar Carmenère has shown to be a highly sensitive cultivar to berry set problems and to

early bunch stem necrosis (Pszczolkóski *et al.*, 2005), a hormone-related physiological disorder (Jackson, 1991).

TPI has shown to be a reliable technique to measure tannin polymerization, an aspect related to the wine astringency sensation (McRae and Kennedy, 2011). The more the proanthocyanidins are polymerized, the less nucleophilic sites are available to the DMAC, that fixes specifically to carbons 6 and 8 of the benzenic A ring of proanthocyanidins and have little interference with other PC like phenolic acids and anthocyanins. This index is reliable on grape extract and young wines and less consistent on proanthocyanidins that has been exposed to oxidative condensations (*i.e*: the maturation and aging) with ethanal bridges intermediaries, determining heterogeneous branched structures (Vivas *et al.*, 1994). S-ABA and CEPA-treated grapes, revealed significant but transient higher TPI at 33 DAV and no significant differences at harvest time. This absence of effect is confirmed in wines made from S-ABA-treated grapes, but wines made from CEPA-treated grapes showed a significantly lower TPI suggesting an effect of treatments on skin tannin extractability (during red wine fermentative maceration) or on seed tannin content (Gonzalez-Manzano *et al.*, 2006). The acetone-water extraction solvent used for grape skin extraction is a much more effective extraction system than that observed in wine production, and therefore, under winemaking conditions, a subfraction of the grape PA is extracted. The subfraction properties (lower amount and mDP) are consistent with a nonequilibrated diffusion-dependent process (Pastor del Rio and Kennedy, 2006).

Information about the effects of growth regulator applications on wine composition and sensory characteristics is in general scarce. In our experiments

we found significant differences in wine chemical composition and sensory analysis in 2008 and no difference at all in the 2010 experiment. In 2008 we observed that CEPA treatments produced significant increments in wine color intensity and anthocyanin concentration, while S-ABA treatments did not affect color intensity and the effect on anthocyanin was significantly lower than the effect of CEPA. Venburg (2009) also reported a superior effect of CEPA (compared with ABA), on wine anthocyanin concentration and wine color density. Wine tannin concentration was increased by the S-ABA treatment without altering TPI, while the CEPA treatment did not affect tannin concentration and showed a reduction in TPI, with a possible impact in wine mouthfeel and astringency (Vivas *et al.*, 1994) that was not confirmed by the results of tannic intensity and astringency by the sensory panel (figure 15). In the same way as wine chemical parameters, wine sensory analysis showed a complete absence of significant effects in 2010, while in 2008 a reduction of herbaceous aroma and bitterness in wines from S-ABA-treated grapes was found. These results can be related to a hastening of grape ripening and its reported implications in methoxypirazine degradation (Allen and Lacey, 1999).

To obtain high quality red wines, grape ripeness is a key issue. However, in addition to the traditional pulp ripeness (TSS, TA and pH), skin and seed ripeness (phenolic compounds status) have to be taken into account (Kontoudakis *et al.*, 2010). This usually leads to the harvest of overripe grapes, and finally to sensory unbalanced wines with high alcohol content. Therefore, any method that allows improving or advancing phenolic ripeness without increasing the sugar accumulation (or even better decreasing it) is of great interest. Our results show

that the use of promoters of ripening like CEPA and S-ABA can make a contribution in this direction.

Some practical conclusions of these experiments are that the effect of CEPA and S-ABA applications are strongly influenced by seasonal growing conditions and probably by PGR application phenological time. PGR treatments had important effects on the expression of flavonoid biosynthetic genes, with a general induction but surprisingly a repression of the last steps in anthocyanin biosynthesis. When environmental factors were favorable, the effect of CEPA was more effective than S-ABA in order to improve phenolic composition and PGR treatments had a positive impact on wine quality, reducing alcohol concentration and increasing wine color intensity. Even if early treatments were tested only in 2010, they impacted gene expression, but they almost did not impact grapes and wine quality by the time of harvest

ABBREVIATIONS USED

ABA, abscisic acid; ANOVA, analysis of variance; ANR, anthocyanidin reductasa; CEPA, 2-chloroethylphosphonic acid; CHS, chalcone synthase; DAA, days after anthesis; DAV, days after *veraison*; DMAC, 4-(dimethyl-amino)cinnamaldehyde; LAR, leucoanthocyanidin reductase; LDOX, leucoanthocyanidin dioxygenase; OMT, O-methyltransferase; PAL, phenylalanine ammonia-lyase; PGR, plant growth regulators; TA, titratable acidity; TSS, total soluble solids; S-ABA, (+)-S-abscisic acid; TPI, tannin polymerization index; UFGT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase.

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Table 1

Season	Sample	Stage[a]	Date	DAA	TSS (ºBrix)
2007-2008	1	35: Berries begin to color and enlarge	29/01	4	9,5
	2	37: Berries harvest-ripe	26/02	33	25,0
	3	38: Berries over-ripe	31/03	67	26,0
2009-2010	1	29: Berries pepper-corn size (4 mm diam.)	09/12	10	
	2	31: Berries pea size (7 mm diam.)	22/12	23	
	3	35: Berries begin to color and enlarge	03/02	66	
	4	36: Berries with intermediate Brix values	10/02	73	13,8
	5	37: Berries not quite ripe	18/02	81	17,7
	6	38: Berries harvest-ripe	18/03	109	22,0
	7	39: Berries over-ripe	07/04	129	23,8

a Grapevine growth stages as defined by the Modified E-L system (Coombe, 1995).

Table 2

Gene ID (Genebank Accession)	Primer		Sequence	Amplicon length (bp)	Primer efficiency (%)	Reference
CHS2 (AB066275)	Forw	5′	GAAGATGGGAATGGCTGCTG	131	87	Jeong *et al.* (2004)
	Rev	5′	AAGGCACAGGGACACAAAAAG			
LDOX (X75966)	Forw	5′	AGGGAAGGGAAAACAAGTAG	109	88	Jeong *et al.* (2004)
	Rev	5′	ACTCTTTGGGGATTGACTGG			
UFGT (AF000372)	Forw	5′	GGGATGGTAATGGCTGTGG	152	89	Jeong *et al.* (2004)
	Rev	5′	ACATGGGTGGAGAGTGAGTT			
OMT (BQ796057)	Forw	5′	GAGAGCAGGCAGAGTCCATC	159	98	Castellarin *et al.* (2007b)
	Rev	5′	CACCATAAGCAAACCCTAAACC			
ANR (DQ129684)	Forw	5′	CTTGATGGGACAGGTCTGGT	102		Lacampagne *et al.* (2009)
	Rev	5′	TGTCTTGGAGGCAGGATAGC			
LAR2 (DQ129686)	Forw	5′	TAAACGAGCTGGCATCACTG	88		Lacampagne *et al.* (2009)
	Rev	5′	GCAGCGGCTAGTAGGTCATC			
MYBA1 (AB097923)	Forw	5′	TAGTCACCACTTCAAAAAGG	65	89	Jeong *et al.* (2004)
	Rev	5′	GAATGTGTTTGGGGTTTATC			
HT1 (AJ001061)	Forw	5′	TCAATGGCGCTGCTAAAGCT	91	93	Mayus (2008)
	Rev	5′	AGCGGCACAGACTGATTGG			
MYB5a (AY555190)	Forw	5′	GTGCAGCAGCCATCTAATGTG	101	75	Matus (2008)
	Rev	5′	GCAGCAGGTTCCCAGACAGT			
MYB5b (AY899404)	Forw	5′	GGTGTTCTTTAATTTGGCTTCA	143	80	Deluc *et al.* (2008)
	Rev	5′	CACAACAACACAACCACATACA			
ACTIN1 (Q94KC1)	Forw	5′	TCCTTGCCTTGCGTCATCTAT	72	103	Serrano, unpublished results
	Rev	5′	CACCAATCACTCTCCTGCTACAA			

Figure 1

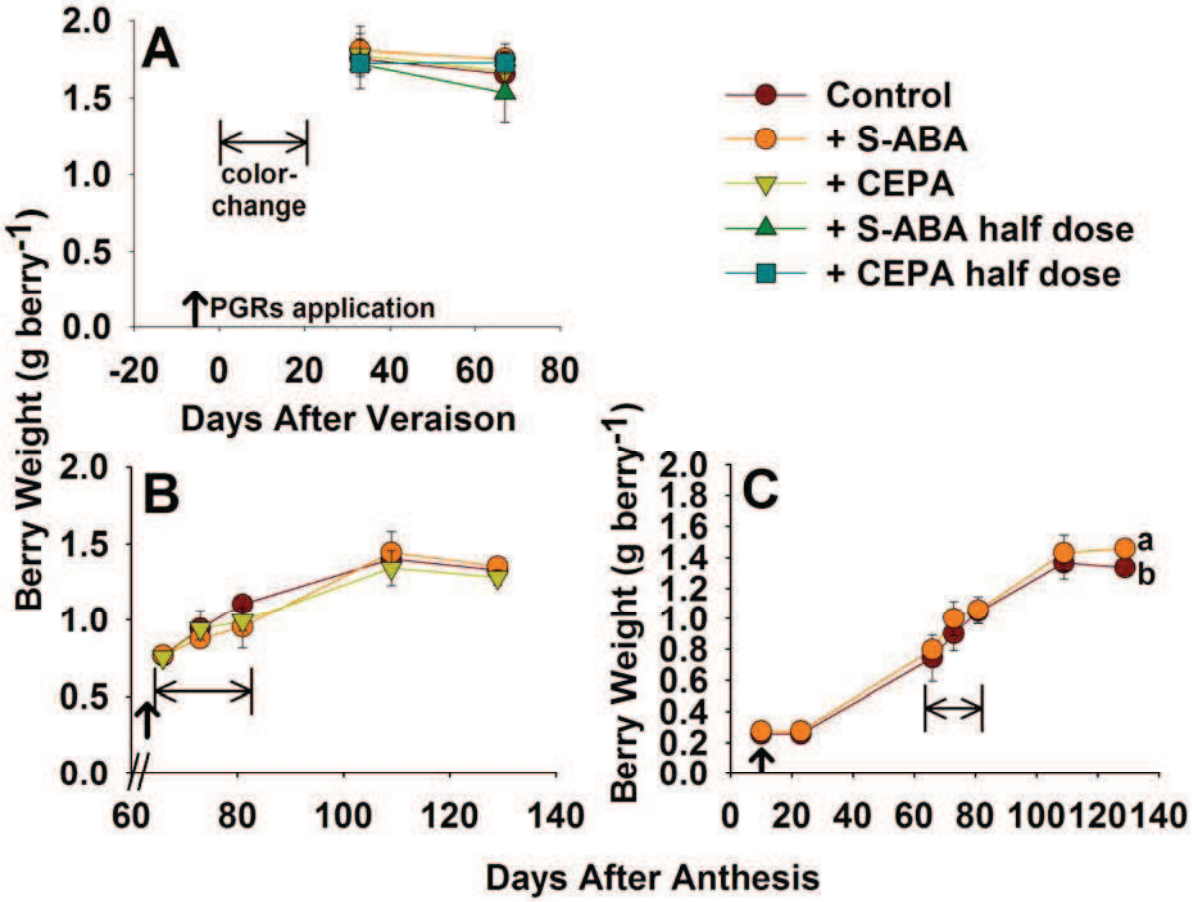
A
B
C
Control
+ S-ABA
+ CEPA
+ S-ABA half dose
+ CEPA half dose
color-
change
PGRs application
Berry Weight (g berry-1)
Days After Veraison
Days After Anthesis
a
b

Figure 2

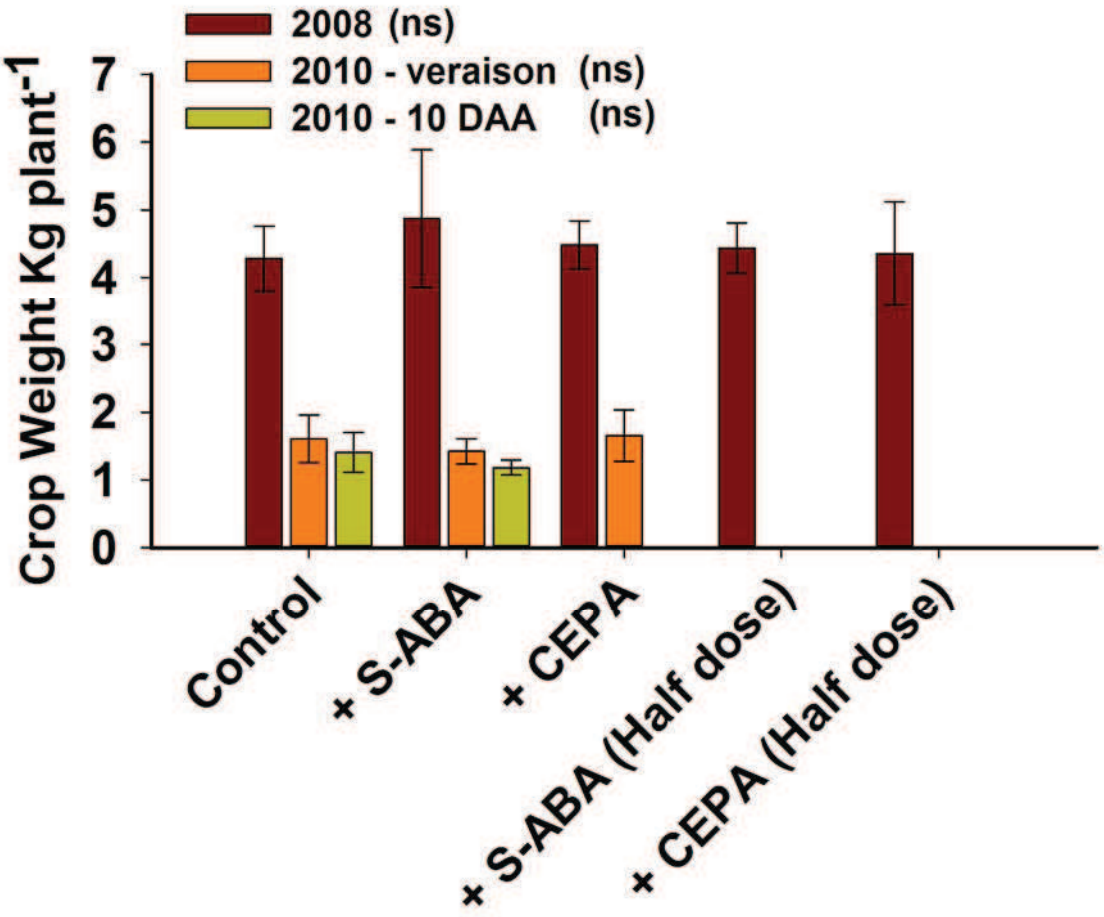
2008 (ns)
2010 - veraison (ns)
2010 - 10 DAA (ns)
Crop Weight Kg plant-1
0
1
2
3
4
5
6
7
Control
+ S-ABA
+ CEPA
+ S-ABA (Half dose)
+ CEPA (Half dose)

Figure 3

Figure 4

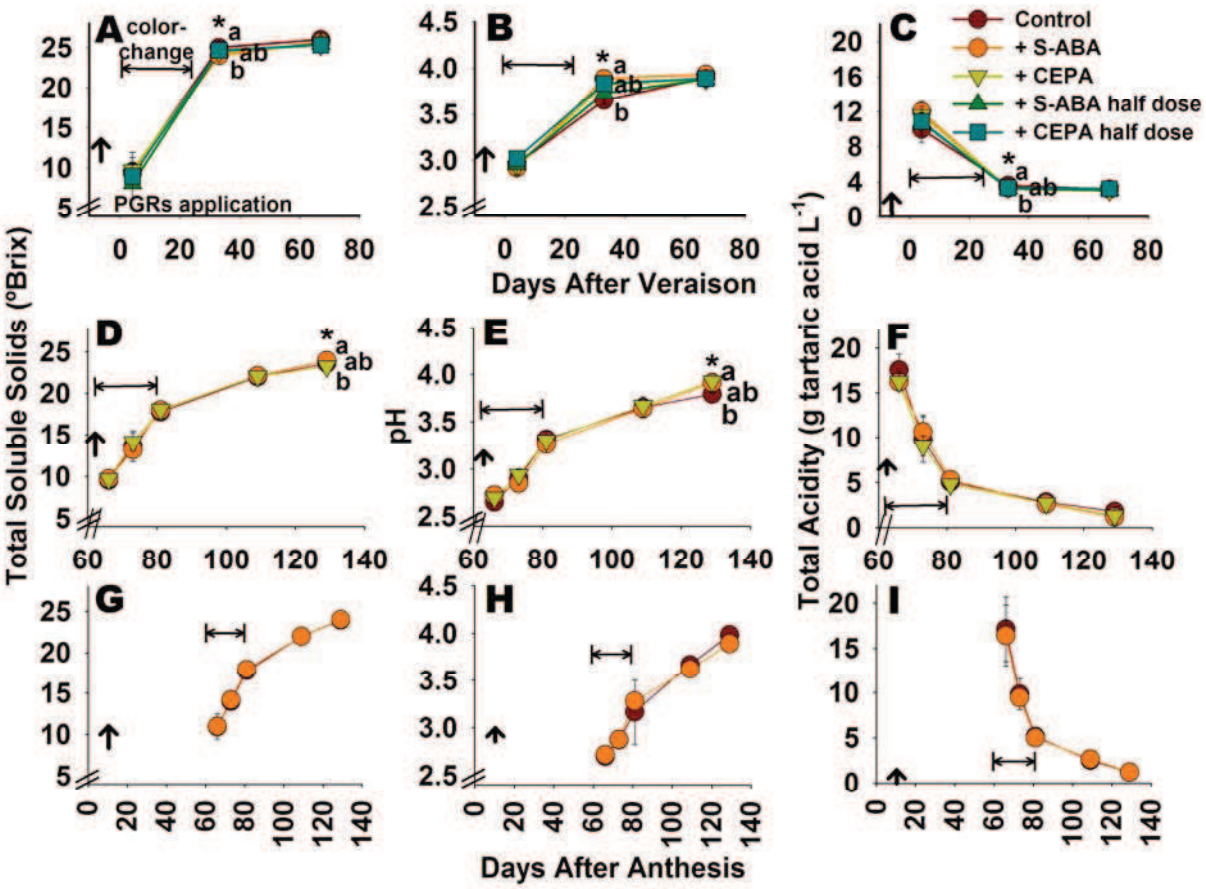
A
color-change
PGRs application
B
C
Control
+ S-ABA
+ CEPA
+ S-ABA half dose
+ CEPA half dose
D
E
F
G
H
I
Total Soluble Solids (ºBrix)
pH
Total Acidity (g tartaric acid L-1)
Days After Veraison
Days After Anthesis

Figure 5

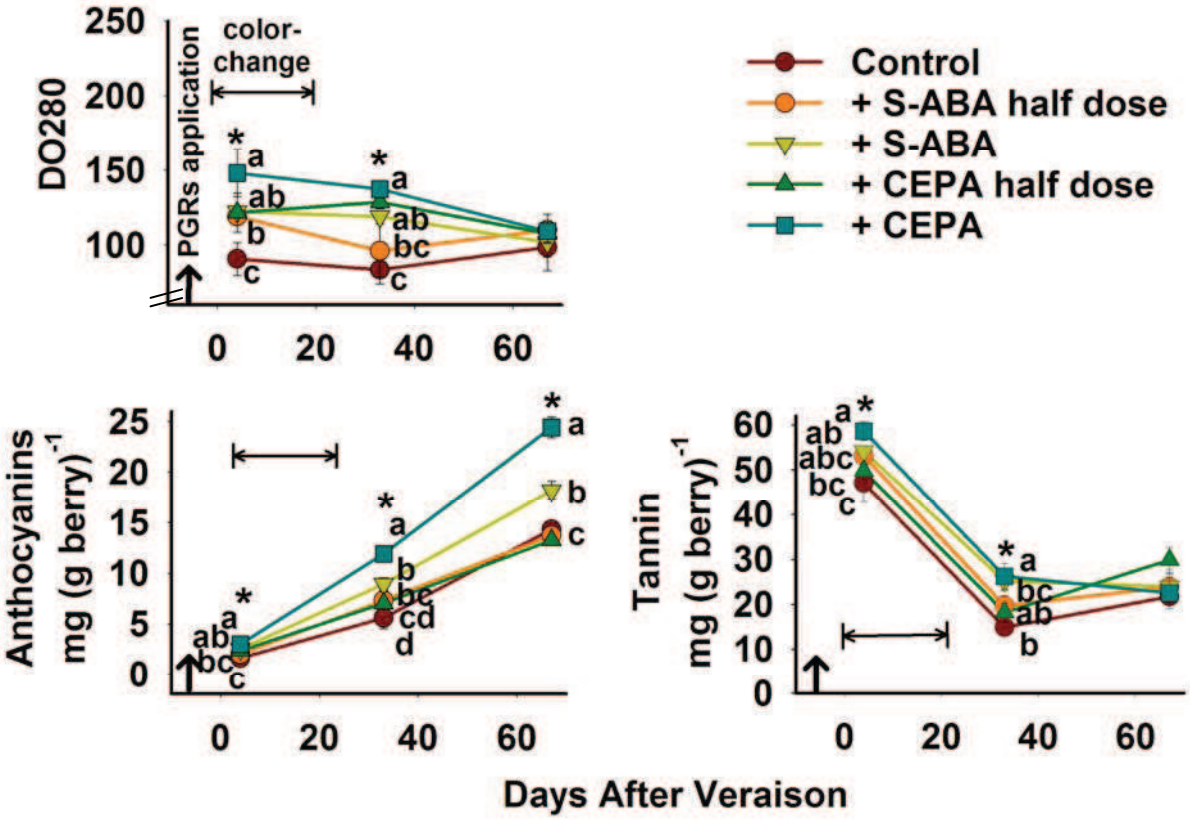
DO280
250
200
150
100
color-
change
PGRs application
Control
+ S-ABA half dose
+ S-ABA
+ CEPA half dose
+ CEPA
Anthocyanins
mg (g berry)-1
25
20
15
10
5
0
Tannin
mg (g berry)-1
60
50
40
30
20
10
0
0
20
40
60
Days After Veraison

Figure 6

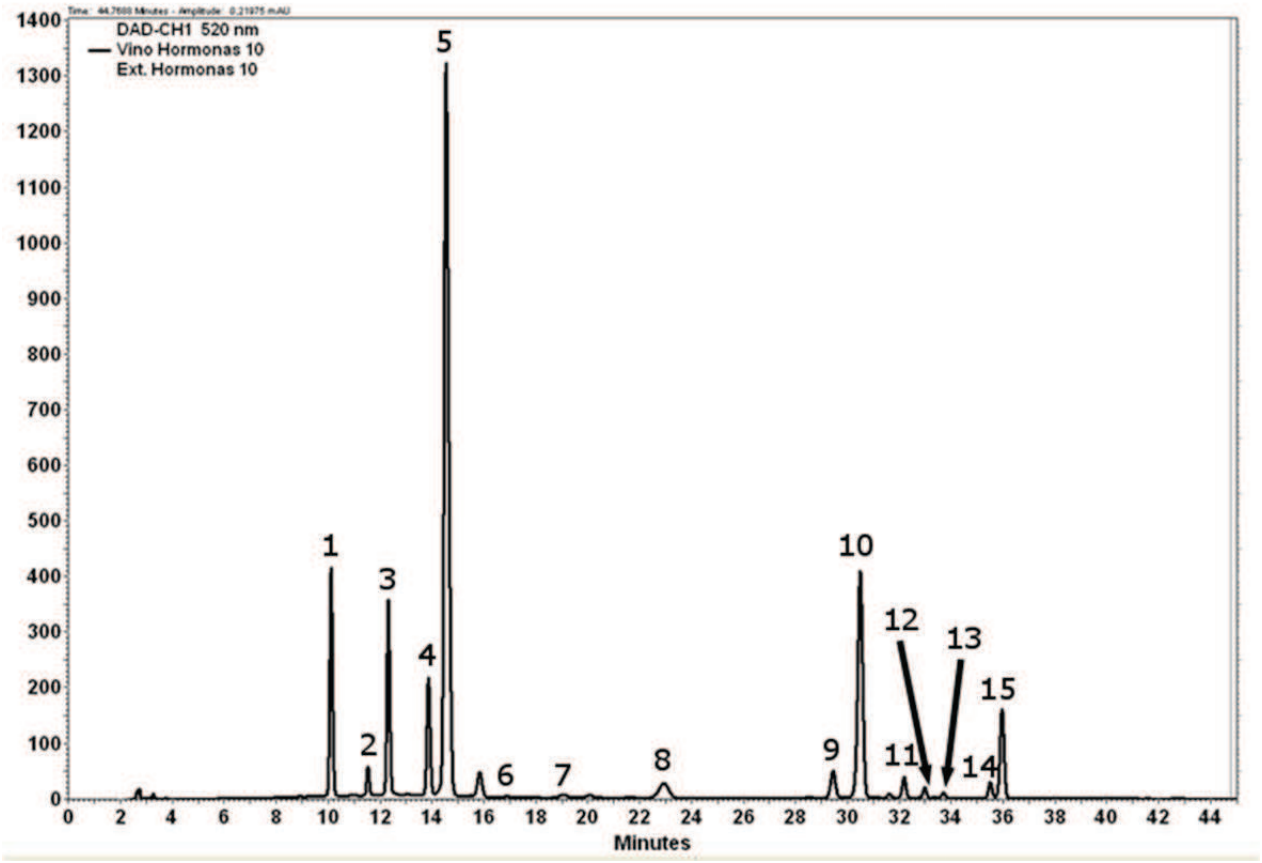
DAD-CH1 520 nm
Vino Hormonas 10
Ext. Hormonas 10
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Minutes
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 7

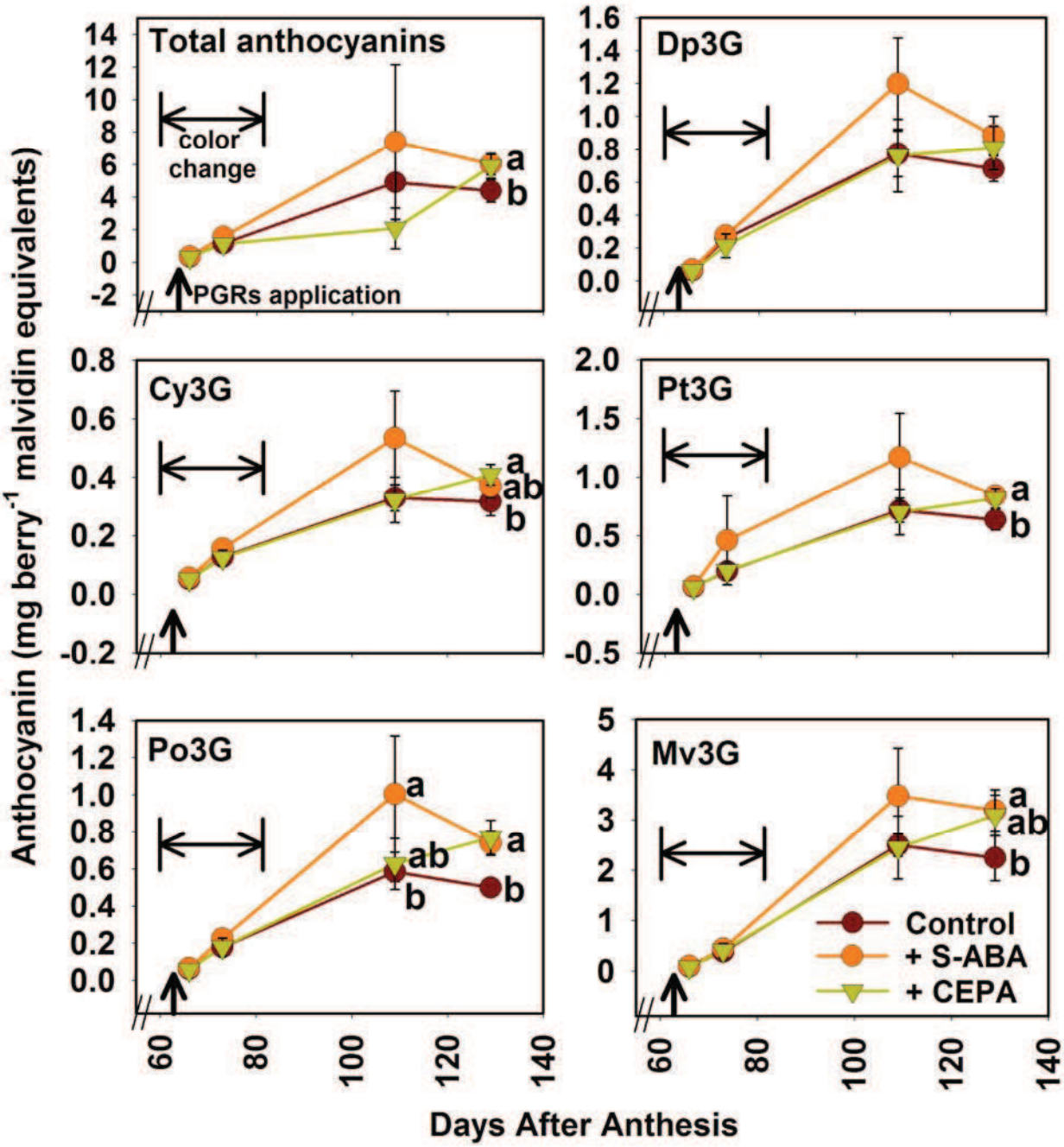
Total anthocyanins
color change
PGRs application
Dp3G
Cy3G
Pt3G
Po3G
Mv3G
Control
+ S-ABA
+ CEPA
Anthocyanin (mg berry-1 malvidin equivalents)
Days After Anthesis

Figure 8

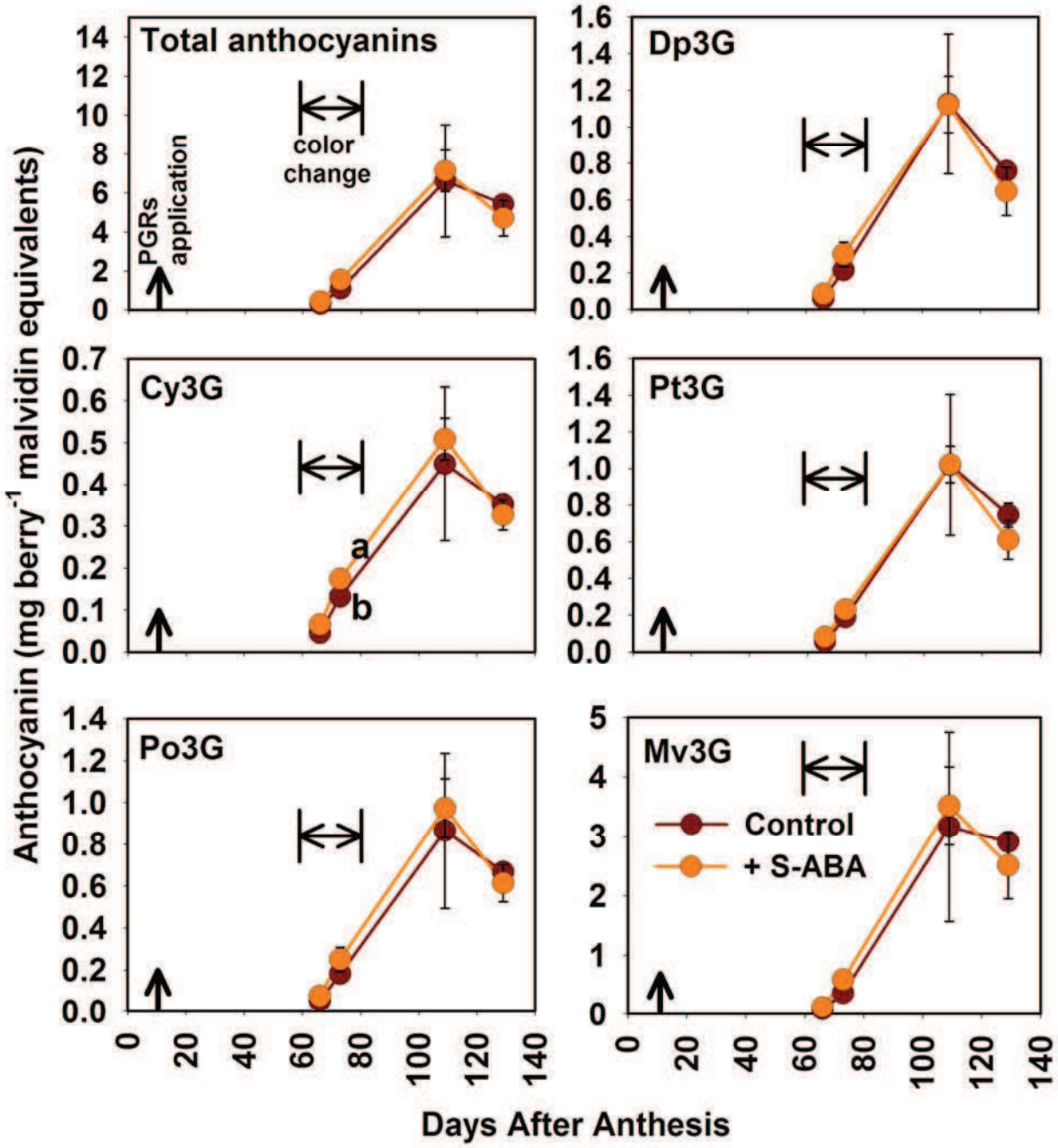
Total anthocyanins
color change
PGRs application
Dp3G
Cy3G
a
b
Pt3G
Po3G
Mv3G
Control
+ S-ABA
Anthocyanin (mg berry-1 malvidin equivalents)
Days After Anthesis

Figure 9

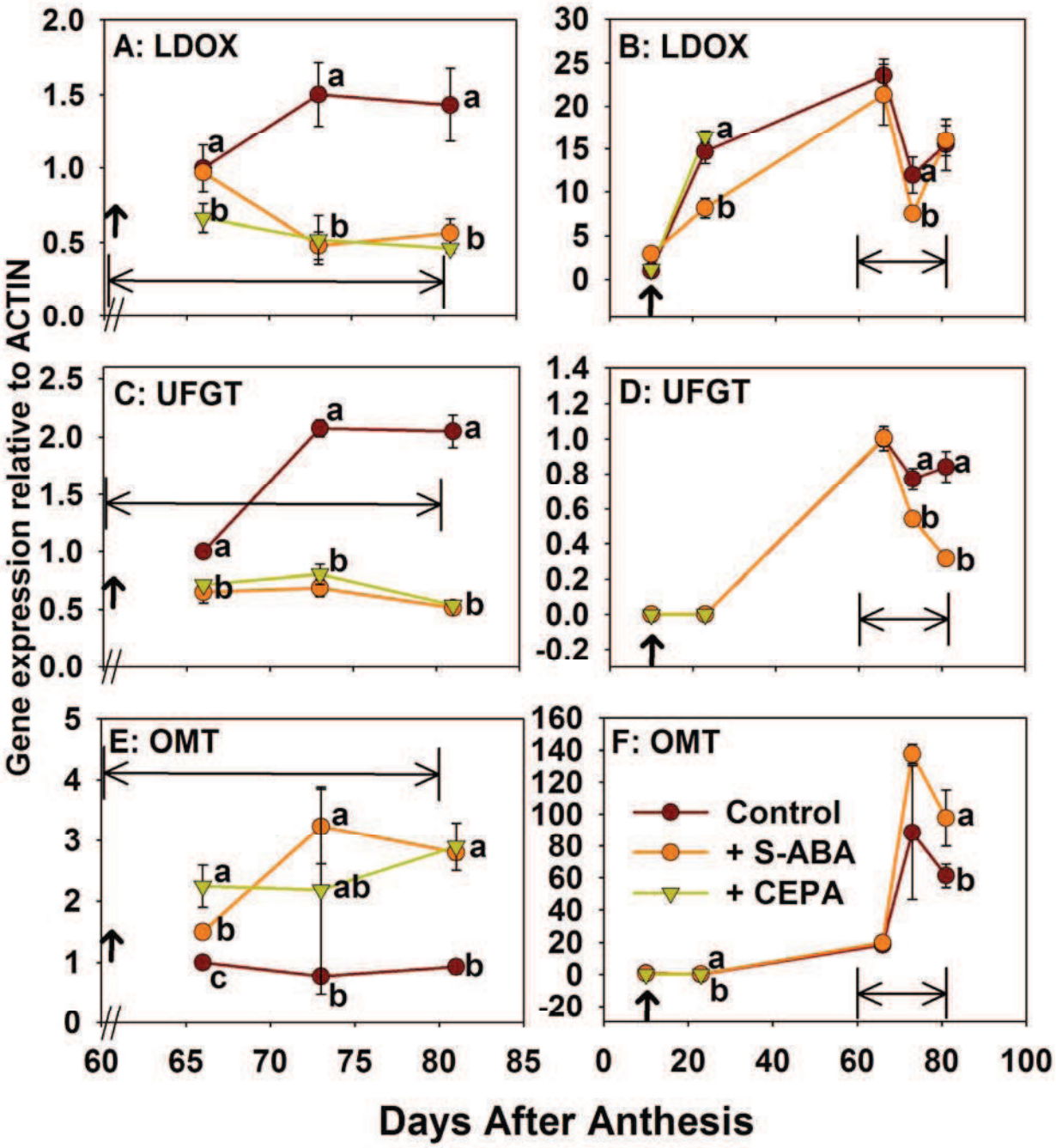
A: LDOX
B: LDOX
C: UFGT
D: UFGT
E: OMT
F: OMT
Control
+ S-ABA
+ CEPA
Gene expression relative to ACTIN
Days After Anthesis

Figure 10

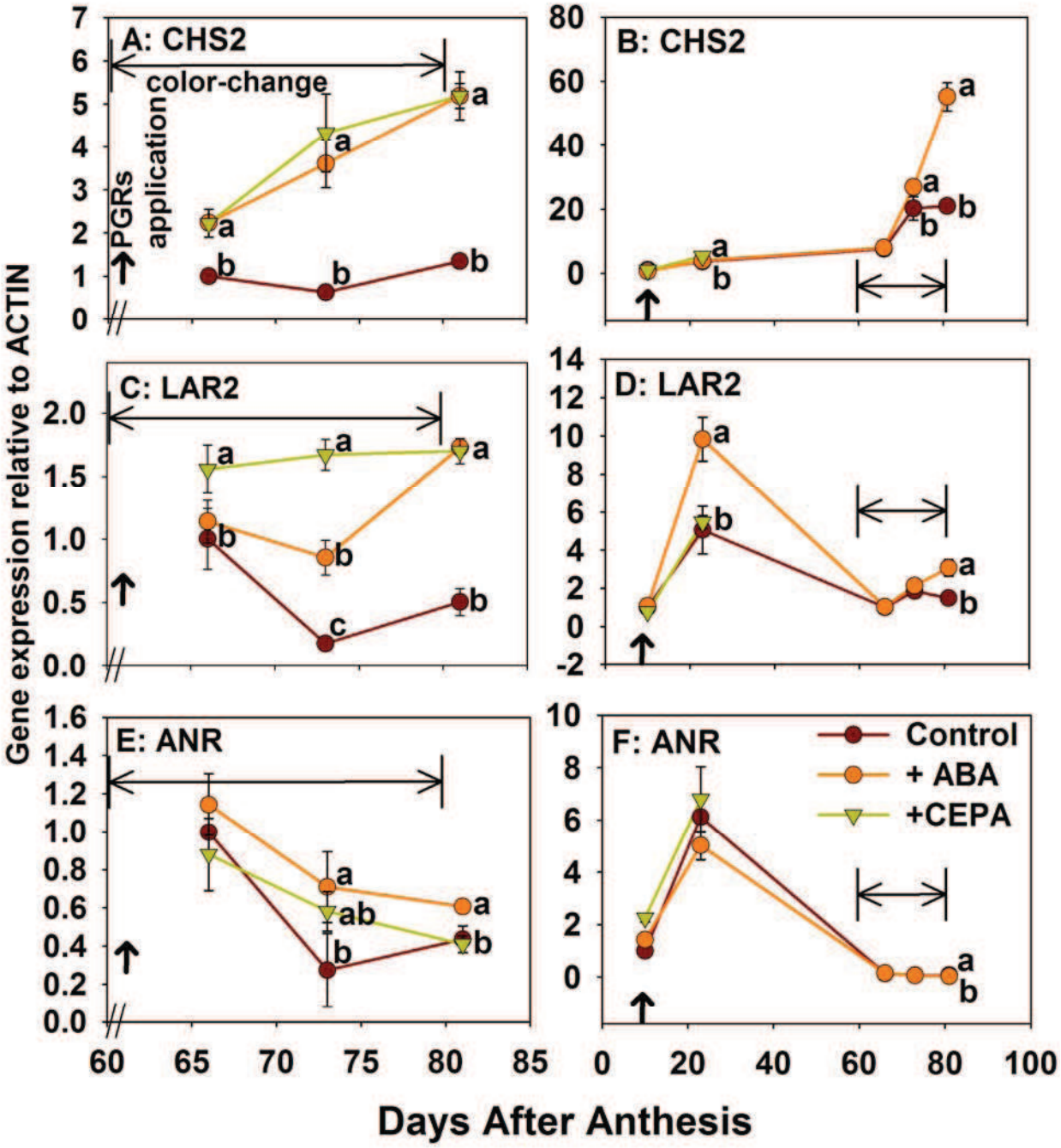
A: CHS2
color-change
PGRs application
B: CHS2
C: LAR2
D: LAR2
E: ANR
F: ANR
Control
+ ABA
+CEPA
Gene expression relative to ACTIN
Days After Anthesis

Figure 11

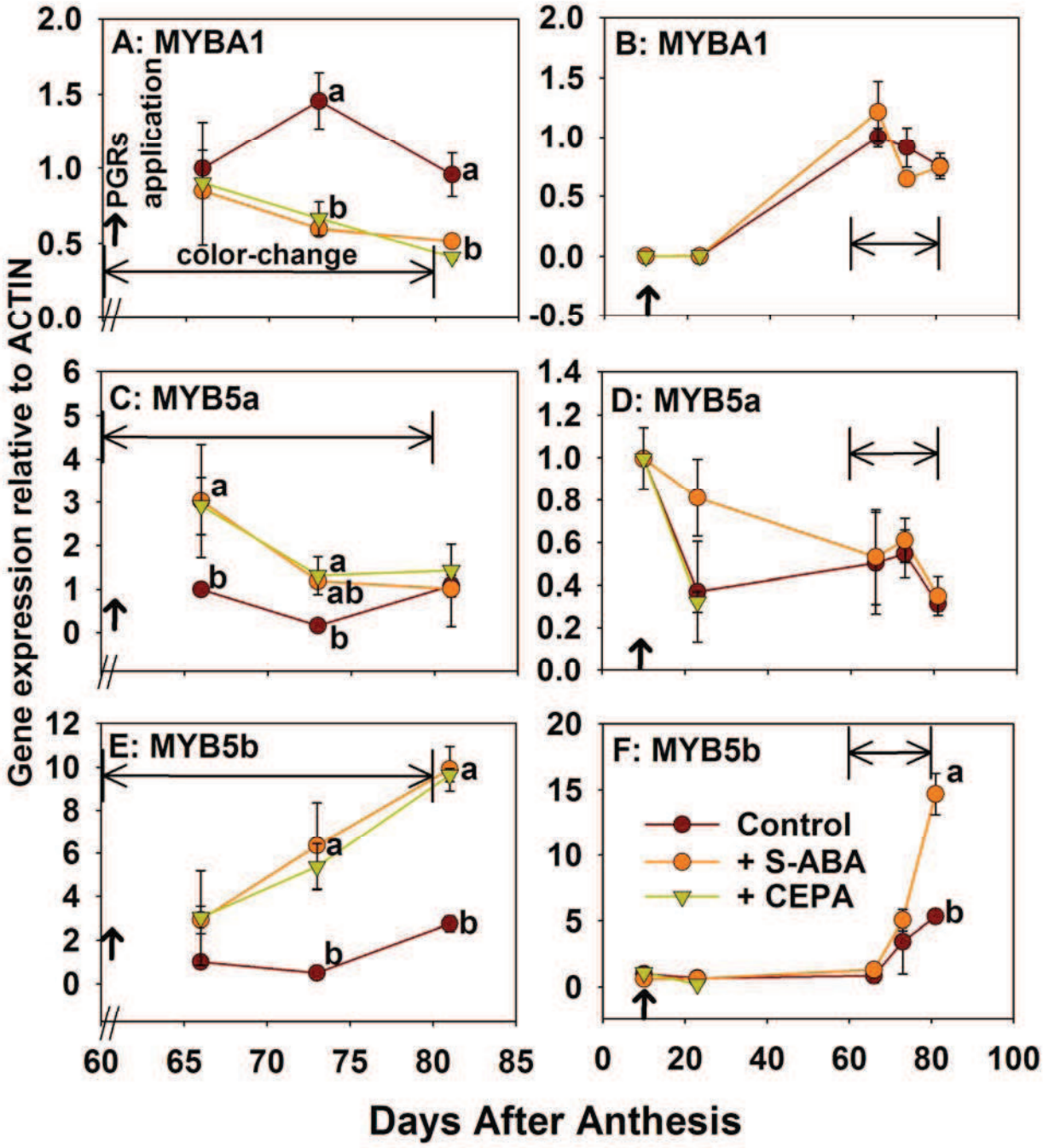
A: MYBA1
B: MYBA1
C: MYB5a
D: MYB5a
E: MYB5b
F: MYB5b
PGRs application
color-change
Control
+ S-ABA
+ CEPA
Gene expression relative to ACTIN
Days After Anthesis

Figure 12

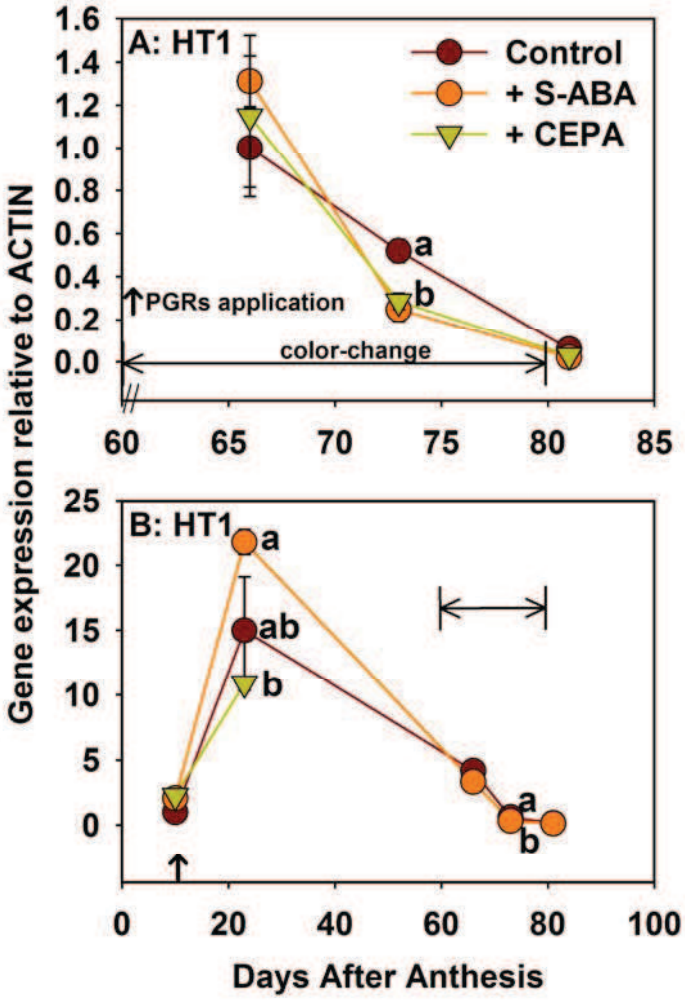
A: HT1
Control
+ S-ABA
+ CEPA
a
b
PGRs application
color-change
B: HT1
a
ab
b
a
b
Gene expression relative to ACTIN
Days After Anthesis

Figure 13

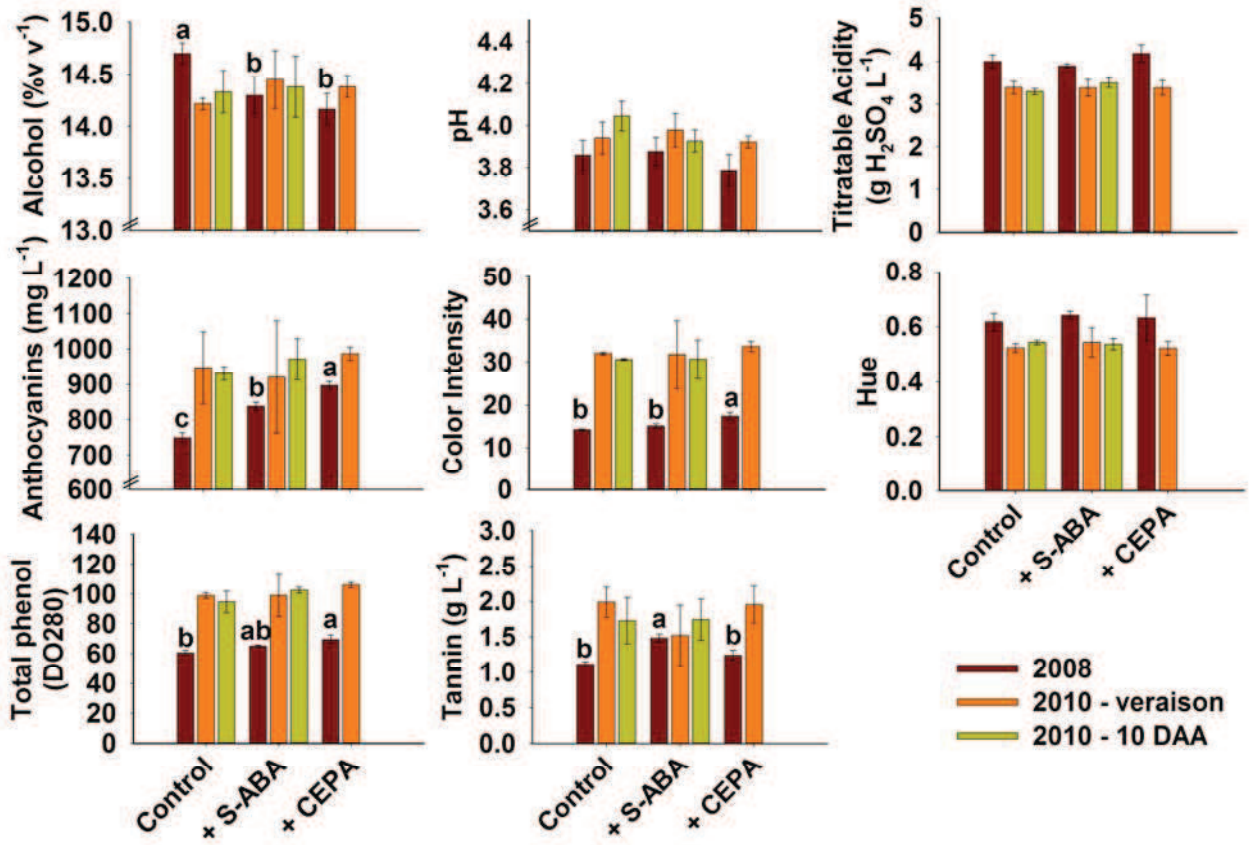
Alcohol (%v v-1)
15.0
14.5
14.0
13.5
13.0
a
b
b
pH
4.4
4.2
4.0
3.8
3.6
Titratable Acidity
(g H2SO4 L-1)
5
4
3
2
1
0
Anthocyanins (mg L-1)
1200
1100
1000
900
800
700
600
c
b
a
Color Intensity
50
40
30
20
10
0
b
b
a
Hue
0.8
0.6
0.4
0.2
0.0
Total phenol
(DO280)
140
120
100
80
60
40
20
0
b
ab
a
Tannin (g L-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
b
a
b
Control
+ S-ABA
+ CEPA
2008
2010 - veraison
2010 - 10 DAA

Figure 14

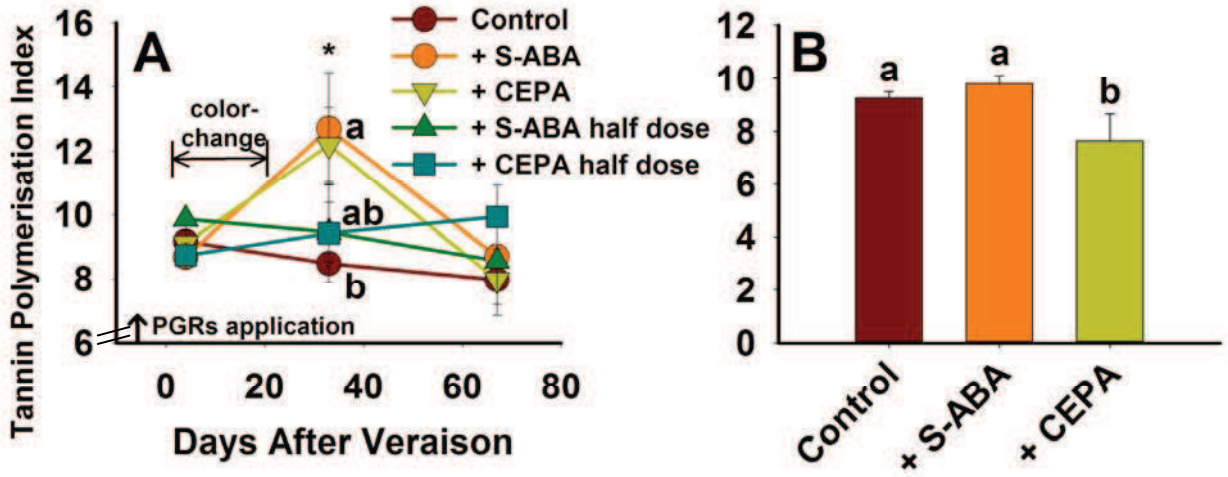
A
Tannin Polymerisation Index
16
14
12
10
8
6
color-
change
*
a
ab
b
PGRs application
0
20
40
60
80
Days After Veraison
Control
+ S-ABA
+ CEPA
+ S-ABA half dose
+ CEPA half dose
B
12
10
8
6
4
2
0
a
a
b
Control
+ S-ABA
+ CEPA

Figure 15

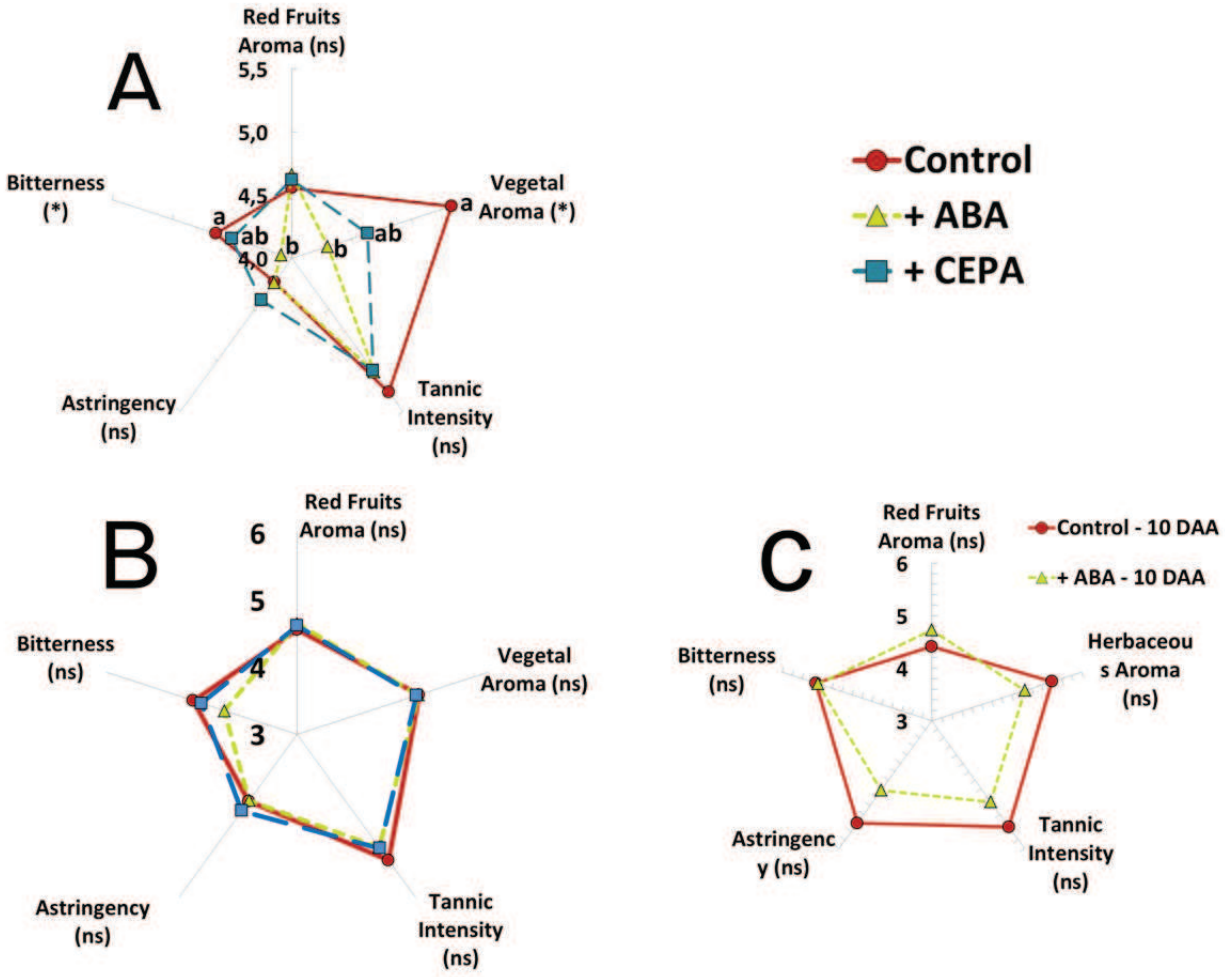
A
Red Fruits Aroma (ns)
5,5
5,0
4,5
4,0
Vegetal Aroma (*)
Tannic Intensity (ns)
Astringency (ns)
Bitterness (*)
a
ab
b
b
ab
a
Control
+ ABA
+ CEPA
B
Red Fruits Aroma (ns)
6
5
4
3
Vegetal Aroma (ns)
Tannic Intensity (ns)
Astringency (ns)
Bitterness (ns)
C
Red Fruits Aroma (ns)
6
5
4
3
Herbaceous Aroma (ns)
Tannic Intensity (ns)
Astringency (ns)
Bitterness (ns)
Control - 10 DAA
+ ABA - 10 DAA

TABLE AND FIGURE LEGENDS

Table 1. Grapevine growth stage, sampling date, days after *veraison* and total soluble solids of the grape samples of cv. Carmenère during seasons 2008 and 2010.

Table 2. Primer used for quantification of transcripts by means of real time quantitative PCR.

Figure 1. Evolution of berry weight during development in 2008 grapes treated at *veraison* (A), 2010 grapes treated at *veraison* (B) and 2010 grapes treated at 10 days after anthesis (C) experiments, of PGR-treated and control grapes. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures ($P < 0.05$).

Figure 2. Crop weight per plant in cv. Carmenère 2008 treated at *veraison*, 2010 treated at *veraison* and 2010 treated 10 days after anthesis, and their respective control plants. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures ($P < 0.05$).

Figure 3. Pictures of necrotic bunches of cv. Carmenère 2010 commercial vineyard experiment at 23 days after anthesis. Grapes treated ten days after anthesis with 2-chloroethylphosphonic acid.

.

Figure 4. Total soluble solids (A, D, G), pH (B, E, H) and titratable acidity (C, F, I) of berry pulp samples of 2008 grapes treated at *veraison* (A, B, C), 2010 grapes treated at *veraison* (D, E, F) and 2010 grapes treated at 10 days after anthesis (G, H, I) in cv. Carmenère commercial vineyard experiments of treated and their respective control grapes. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures ($P < 0.05$).

Figure 5. Total phenols (A), anthocyanin content (B) and tannin content (C) of cv. Carmenère commercial vineyard grape skins, of treated and their respective control plants during three sampling dates in 2008. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures ($P < 0.05$).

Figure 6. Image of an HPLC chromatogram for the detection of anthocyanins in a berry skins extract sample, detected at 520nm with a photodiode-array detector. Pic number details are: delphinidin-3-glucoside (1), cyanidin-3-glucoside (2), petunidin-3-glucoside (3), peonidin-3-glucoside (4), malvidin-3-glucoside (5),

delphinidin-3-(6-acetyl)glucoside (6), cyanidin-3-(6-acetyl)glucoside (7), petunidin-3-(6-acetyl)glucoside (8), peonidin-3-(6-acetyl)glucoside (9), malvidin-3-(6-acetyl)glucoside (10), delphinidin-3-(6-*p*-coumaroyl)glucoside (11), cyanidin-3-(6-*p*-coumaroyl)glucoside (12), petunidin-3-(6-*p*-coumaroyl)glucoside (13), peonidin-3-(6-*p*-coumaroyl)glucoside (14) and malvidin-3-(6-*p*-coumaroyl)glucoside (15).

Figure 7. Concentration of total and 3-*O*-glycosylated anthocyanin compounds: delphinidin (Dp3G), cyanidin (Cy3G), petunidin (Pt3G), peonidin (Po3G) and malvidin (Mv3G), from the different *veraison* PGR-treated and control cv. Carmenère berry skins in a commercial vineyard experiment. Anthocyanin concentrations are calculated in malvidin equivalents. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures ($P < 0.05$).

Figure 8. Concentration of total and 3-*O*-glycosylated anthocyanin compounds: delphinidin (Dp3G), cyanidin (Cy3G), petunidin (Pt3G), peonidin (Po3G) and malvidin (Mv3G), from the different 10-days-after-anthesis PGR-treated and control berry skins cv. Carmenère in a commercial vineyard experiment. Anthocyanin concentrations are calculated in malvidin equivalents. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures ($P < 0.05$).

Figure 9. Changes in the expression of flavonoid biosynthetic genes: LDOX, leucoanthocyanidin oxidase (A, B), UFGT, UDP-glucose flavonoid 3-*O*-glucosyltransferase (C, D) and OMT, O-methyl-transferase (E, F), under different PGR treatments applied at *veraison* (A, C, E) and ten days after anthesis (B, D, F), in a commercial vineyard experiment. Transcript levels are expressed in relation to the VvACTIN gene. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures ($P < 0.05$).

Figure 10. Changes in the expression of flavonoid biosynthetic genes: CHS2, chalcone synthase (A, B), LAR2, leucoanthocyanidin reductase (C, D) and ANR, anthocyanidin reductase (E, F), under different PGR treatments applied at *veraison* (A, C, E) and ten days after anthesis (B, D, F), in a commercial vineyard experiment. Transcript levels are expressed in relation to the VvACTIN gene. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures ($P < 0.05$).

Figure 11. Changes in the expression of MYB regulators genes: MYBA1 (A, B), MYB5a (C, D) and MYB5b (E, F), under different PGR treatments applied at *veraison* (A, C, E) and ten days after anthesis (B, D, F), in a commercial vineyard experiment. Transcript levels are expressed in relation to the VvACTIN gene. Vertical bars indicate the standard deviation (three biological replicates). Different

letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures (P <0.05).

Figure 12. Changes in the expression of HEXOSE TRANSPORTER1 under different PGR treatments applied at *veraison* (A) and ten days after anthesis (B) in a commercial vineyard experiment. Transcript levels are expressed in relation to the VvACTIN gene. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures (P <0.05).

Figure 13. Wine composition, including alcohol concentration (A), pH (B), titratable acidity (C), anthocyanin concentration (D), color intensity (E), hue (F), DO280 index (G) and tannin concentration (H) in wines made from 2008 *veraison*, 2010 *veraison* and 2010 ten-days-after-anthesis PGR-treated and control grapes from a cv. Carmenère commercial vineyard. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments as calculated by ANOVA and Tukey's HSD multiple comparison procedures (P <0.05).

Figure 14. Tannin polimerization index of grapes (A) and wines (B) from 2008 PGR-treated and control cv. Carmenère commercial vineyard grapes. Vertical bars indicate the standard deviation (three biological replicates). Different letters

indicate significant differences between treatments as calculated by ANOVA and Tukey's HSD multiple comparison procedures ($P < 0.05$).

Figure 15. Wine sensory analysis, including red fruits aroma, vegetal aroma, tannic intensity, astringency and bitterness of wines made from PGR-treated and control grapes: 2008 grapes treated at *veraison* (A), 2010 grapes treated at *veraison* (B) and 2010 grapes treated ten days after anthesis (C) from a cv. Carmenère commercial vineyard. Vertical bars indicate the standard deviation (three biological replicates). Asterix indicates significant differences and “ns” indicates non-significant differences between treatments as calculated by ANOVA ($P<0.05$). Different letters indicate significant differences between treatments as calculated by Tukey's HSD multiple comparison procedures ($P < 0.05$).

Chapter 5

Comparative study of the effects of PGR Treatments on Grape Skin and Wine Quality in *Vitis vinifera* L. cv. Cabernet Sauvignon in Maipo (Chile) and Bordeaux (France)

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ABSTRACT

Treatment of wine grapes with plant growth regulators is a potential tool for modifying grape and wine composition. Hormonal balance of developing grapes depends on environmental conditions and impacts phenolic composition of grape skins. In the present work we report the effect of low dose abscisic acid (ABA) and 2-chloroethylphosphonic acid (CEPA) treatments, applied ten days after anthesis and at veraison, in two contrasting environmental conditions as are Bordeaux (France) and Maipo (Chile). The effects of treatments were dependent on environmental conditions. Early-CEPA treatment increased TSS levels (+ 1.6ºBrix) and total 3-*O*-glycosylated anthocyanin compounds (up to + 101%), while veraison CEPA treatment reduced TSS levels (– 2.43ºBrix) and had no impact on anthocyanins. Early-ABA treatments increased tannin content (+47%) at veraison and veraison ABA treatment reduced tannin content at ripeness (-55%). The effect of treatments on Maipo grapes and wine chemical composition were limited, but some effects on sensory parameters were found: early-ABA and veraison CEPA treatments augmented vegetal aroma, whereas early-ABA treatment reduced bitterness.

KEYWORDS: polyphenol; anthocyanin; tannin; abscisic acid; 2-chloroethylphosphonic acid

INTRODUCTION

The fruit of the grapevine (*Vitis vinifera* L.) is considered non-climacteric and therefore its ripening occurs independently of the presence of ethylene (Giovannoni, 2001). Grape ripening hormonal signaling is a complex process that is activated at veraison: the expression of genes related to the metabolism of abscisic acid (ABA), auxins, ethylene and brassinosteroids (BS) were shown to be significantly modified at the onset of ripening (Broquedis, 1983, Pilati *et al.*, 2007, Symons *et al.*, 2006).

Early ABA treatments have shown the ability to control ripening and flavonoid biosynthesis, reducing tannin content of green berries but increasing it at veraison (Gagne *et al.*, 2006, Lacampagne *et al.*, 2009). Davies and Böttcher (2009) hypothesize that ABA treatments may have the ability to hasten the initiation of sugar accumulation when applied before veraison, but cannot enhance it once ripening has already commenced. On the other hand, 2-chloroethylphosphonic acid (CEPA) is considered to be a promoter or inhibitor of ripening depending on the phenological stage of the berries at treatment time (Hale *et al.*, 1970). Early treatments seem to delay ripening, reducing total soluble solids (TSS) and pH (Szyjewicz and Kliewer, 1983) while treatments just before véraison seem to promote ripening (Coombe and Hale, 1973). There are only a few experiences on early plant growth regulator (PGR) treatments, and their effects on grapes and wine quality are somehow inconsistent.

Literature on the effects of PGR applications on wine composition and sensory characteristics is poor and somehow contradictory. Wine from CEPA-

treated grapes of cv. Pinot noir (a poorly colored wine grape variety) during two seasons were 47 to 60% superior in color after 6 to 15 months of bottle aging (Powers *et al.*, 1980). Wines from CEPA-treated grapes increase total polyphenol and anthocyanin, enhancing wine coloration and color stability (Szyjewicz *et al.*, 1984). Delgado *et al*. (2004) showed that the CEPA-treated grapes lead to increases in wine alcohol content. While some reports show no influence of CEPA on sensory characteristics (Szyjewicz *et al.*, 1984), other reports show that the taste panel judged wines from CEPA-treated grapes better than control wines (Powers *et al.*, 1980). Venburg *et al.* (2009) showed a greater accumulation of anthocyanins in wines from the CEPA-plus-ABA-treated grapes. Other reports showed that ABA treatments altered wine color value (Delgado *et al*., 2004). PGR treatments could have different effects on the expression or activity of the flavonoid 3',5'-hydroxylase (F3'5'H) and flavonoid 3'-hydroxylase (F3'H) genes (Castellarin *et al.*, 2006) and this could have an effect on the color of grapes and wine as dihydroxylated anthocyanins produce predominantly orange hues while trihydroxylated ones confer red-purple hues (Heredia *et al*., 1998). Even if Böttcher *et al.* (2010) found small changes in wine volatile compounds, they did not find significant differences in sensory properties between small-scale wine lots made from control and NAA-treated fruit.

The objective of this study was to determine the effect of low-dose ABA and CEPA treatments performed early in development and at veraison in two different environmental conditions as are Bordeaux (France) and Maipo (Chile), on the accumulation of flavonoid compounds in berry skins and wine from cv. Cabernet Sauvignon treated grapes.

MATERIALS AND METHODS

Plant Material and Sample Collection

The grape samples were collected from an experimental vineyard located at Bordeaux, France (44.78ºN, 0.56ºW) during 2009 and a commercial vineyard experiment, located at the Maipo Valley of Chile (33.63ºS, 70.65ºW) during season 2009-2010. The Bordeaux and Maipo plants of *Vitis vinifera* L. cv. Cabernet Sauvignon were conducted as a traditional east/west vertical trellis with cane pruning and a traditional north/south vertical trellis with spur pruning, respectively. Bordeaux plants were grafted onto rootstock 101-14, were 39 years old and planting density was 7,576 vines per hectare (1.2 x 1.1 m). Maipo plants had rooted on their own, were 12 years old and planting density was 4,000 vines per hectare (2.5 x 1.0 m).

The Bordeaux experiment layout consisted of three completely randomized 3-plant-replicate blocks and the Maipo experiment layout consisted of three completely randomized 6-plant-replicates. Twenty random 5-berry bunch fragment samples per replicate were collected from each experiment layout at eight phenological stages (table 1).

Experimental Treatments

The PGR treatment concentration was 2 x 10^{-4} mol L^{-1} for the synthetic ± -*cis,trans*-ABA (Sigma, Saint Quentin Fallavier, France in the Bordeaux experiment; Sigma,

Schnelldorf, Germany in the Maipo experiment) and for the CEPA (Sierra®, Bayer CropScience, Lyon, France in the Bordeaux experiment; Ethrel®, Bayer CropScience, Germany in the Maipo experiment). Two application times were studied: 10 days after anthesis (DAA, with anthesis defined as 80 per-cent flower caps off) and veraison. The treatment involved applying the PGR solution containing 0.1 % Tween80 as a wetting agent on the treated grapes or water containing the same Tween80 concentration on the control grapes. The 10-DAA application was done exactly 10 DAA in both experiments and the veraison application was done 2 days after veraison (with veraison defined as one per-cent color change in the grapes) corresponding to 42 DAA and 6% color change in Bordeaux experiment and 0 DAV, corresponding to 56 DAA and 1% color change in Maipo experiment. All and each cluster of entire plants were treated with 10 mL of the respective solution using a hand sprayer. All treatments were performed at sunset to minimize photodestruction of ABA.

Glories Method for ripeness assessment

Separated berry samples were collected in the Bordeaux experiment to perform a grape berry ripeness assessment during the two final sampling dates (75 and 101 DAA). Extraction of phenolic compounds of grapes with pH 1 and pH 3.5 buffers was performed according to the method described by Glories (2001). This method of phenolic ripeness allows calculating total phenols, extractable anthocyanidin, total tannin, anthocyanin extractability and the seed tannin contribution index.

Skin Phenolic Extractions

Immediately after sampling, berries were frozen *in situ* with liquid nitrogen. The berries were weighed and their skins were hand separated after thawing. The skins were rinsed with distilled-deionized water, frozen again and then lyophilized, before final grinding. One hundred milligrams of ground lyophilized skins were extracted in 10 mL of 50% v v^{-1} aqueous ethanol pH 2.0 solution for one hour in a shaker at room temperature and then centrifuged at 4,000 rpm for six minutes. After centrifugation, the supernatant was sparged with nitrogen gas in order to minimize oxidation and retained at 4ºC until analyzed. From the homogenized berry pulp, we determined TSS by direct reading in a digital refractometer (Pocket PAL-1, Atago, Japan), the pH using a pH meter (Orion 5-Star, Thermo Scientific, Singapore) and the titratable acidity (TA) using a pH meter and 0.1N NaOH.

Winemaking

Small-scale winemaking was performed in Maipo experiment considering all biological replicates for each treatment. The wine was made through a traditional red wine fermentation protocol. The grapes were picked on the commercial harvest date, April 23 2010 (corresponding to 81 DAV and 137 DAA). Twenty-five kilograms of grapes were harvested from each biological replicate of the control and the PGR-treated plants. Fermentation was carried out in plastic 25-L containers in a controlled temperature room with Lalvin EC-1118 selected yeast.

Complete spontaneous malolactic fermentation was performed and an adjustment to 30 mg L-1 free sulfur dioxide was made in all wines prior to bottling.

Phenolic Compounds Analysis

The phenolic composition of the grapes and wine was determined using a UV/Vis spectrophotometer model Spectronic Genesys 2 (Milton Roy, Rochester, NY). The total anthocyanins were determined at 520 nm according to the method described by Puissant and Leon (1967), the total phenols were determined by DO280 (Iland *et al.*, 2004) and the total tannin was determined by precipitation with methyl cellulose (Sarneckis *et al.*, 2006).

Anthocyanins Analysis by HPLC

The chromatographic system for the HPLC-DAD analysis of the anthocyanins consisted of a LaChrom Elite® HPLC system with a 1,024 photodiode-array detector (Hitachi LaChrom Elite, Japan). Separation was performed using a Purospher® STAR (Merck, Germany) reverse-phase C18 column (250 mm X 4.6 mm i.d., 5µm) at 20ºC. The detection was carried out at 520 nm. The elution gradient consisted of two solvents: Solvent A was water/formic acid 90:10 v v^{-1} and solvent B was acetonitrile, following the methodology described by Fanzone *et al*. (2010). After filtering through a 0.45 µm pore size membrane, a 150 µL aliquot of grape skin extract was injected. Delphinidine-3-glucoside, peonidine-3-glucoside and malvidin-3-glucoside (Extrasynthese, Lyon, France), were used as standards

and the other anthocyanins were identified by comparison of the standard retention times.

Wine Sensory Analysis

A 10-person sensory analysis panel was formed consisting of students and staff of the Enology Laboratory of The Faculty of Agronomy and Forestry Engineering, “Pontificia Universidad Católica de Chile”. All the panel members were familiarized with the method of sensory analysis and aromatic standards during a 2-week training (four sessions). For the vegetal aroma, 3-isobutyl-2-methoxypyrazine and 3-isopropyl-2-methoxypyrazine standards (Centro de Aromas y Sabores, DICTUC, Chile) were used. For the astringency, tannic intensity and bitterness, the method proposed by Delteil (2000) was used. A structured 9-unit linear scale was used. The samples were individually three-digit coded and presented in random order to the panelists. The assessments were made following general requirements for sensory testing conditions. Each wine sample was tasted three times by each panelist.

Statistical Analysis

The results were compared by one-way (for completely randomized experiments) and two-way (for completely randomized block experiments) analysis of variance (ANOVA) and Tukey's HSD multiple comparison procedure, with $P<0.05$ statistical

significance between treatments, using Statgraphics Plus (Statistical Graphics Corp., Princeton, NJ, USA).

RESULTS

Growing seasons

During the seasons when experiments took place, differences in temperatures, rainfalls and grapevine phenology were found for *Vitis vinifera* cv. Cabernet Sauvignon (figures 1 – 3). Throughout berry development, Bordeaux showed higher minimal temperatures and lower maximal temperatures (figure 1). At Maipo experiment, there was no rainfall during berry development and at Bordeaux experiment, the rainfalls had continued during all the grape development period (figure 2). These differences in environmental conditions, along with other differences not measured in this study (*i.e*: edaphic conditions, irrigation, etc.), lead to differences in phenology and physiological response of the grapevine (figure 3). At Bordeaux, the onset of ripening stage took place at 40 DAA, while at Maipo, it took place at 56 DAA. Bordeaux phenology advanced faster and grape TSS accumulation was higher when compared with Maipo for equal DAA. Average berry weight was comparatively larger in Bordeaux grapes, while TSS levels were higher in Maipo grapes. Berries of Maipo experiment showed a final decline on average berry weight suggesting the occurrence of an over-ripening phase, a phenomenon which was not observed with Bordeaux grapes.

Effects on Berry Maturation

The effect of PGR treatment on berry weight, TSS levels and evolution of color change is shown in figure 4 for the Bordeaux experiment and in figure 5 for the Maipo experiment. At Bordeaux experiment, 10-DAA ABA-treated grapes showed higher berry weight at the last sampling date (101 DAA). No other effect on berry weight was found between treatments of both experiments. No significant effect of treatments was found on crop yield in the Maipo commercial vineyard experiment (figure 6). TSS levels were affected in the Bordeaux experiment, where 10-DAA ABA-treated grapes showed significantly lower TSS levels at 75 DAA (reduction by 1.3ºBrix and 6.9%), PGR-treated grapes showed significantly higher TSS levels at 101 DAA (increment of 0.5-1.6ºBrix and 2.4-7.5%) and veraison CEPA-treated grapes showed significant lower TSS levels at 105-128 DAA (reduction by 1.8-2.4ºBrix and 8.2-12.6%). No significant differences in TSS levels were found in the Maipo experiment. At the Maipo experiment, veraison CEPA-treated grapes showed higher pH values that were significant at 86 DAA and 10-DAA PGR-treated grapes showed transiently significant higher TA concentrations at 60 DAA, when there were 6% berries with color change and a posterior diminution of this effect between 60-128 DAA. No differences between treatments were found for percentage of color change berries, at Bordeaux or at Maipo, treated at 10-DAA or at veraison (figure 4)

Effect on Skin Phenolic Composition

According to the Glories Method for grape phenolic ripeness assessment, there were no differences between treatments for total phenols, total anthocyanidin, extractable anthocyanidin and anthocyanidin extractability potential (data not shown).

The phenolic composition of berry skins is shown in figures 7 and 8. At Maipo, skins from 10-DAA CEPA-treated grapes showed significantly lower total phenol index values at 105 DAA (-35.2%), reaching at the last sampling date (128 DAA) a significantly higher index value (+50.0%). Skins from 10-DAA ABA-treated grapes showed significantly higher index values at 128 DAA (+35.7%; figure 8A). No significant total phenol index differences between treatments were found in skins from 10-DAA PGR-treated grapes at Bordeaux experiment, as well as in skins from veraison PGR-treated in both experiments (figures 7A, 7B and 8B). Skins from 10-DAA ABA-treated grapes showed transient significantly higher tannin content at 54 DAA (increment of 46.9%; figure 7C) and skins from veraison ABA-treated grapes showed significantly lower tannin content at 101 DAA (reduction of 55.3%; figure 7D). No significant tannin differences between treatments were found at the Maipo experiment (figures 8C and 8D).

Effect on Skin HPLC Anthocyanins Concentration

The evolution of total and 3-*O*-glycosylated anthocyanin compounds in the Bordeaux and the Maipo experiments are shown in figures 9, 10, 11 and 12. At

Bordeaux, skins from 10-DAA CEPA-treated grapes showed significantly higher content of anthocyanin compounds: significantly higher total anthocyanin (+ 86.0%), cyanidin 3-*O*-glucoside (+ 67.2%), petunidin 3-*O*-glucoside (+ 81.4%), peonidin 3-*O*-glucoside (+ 78.3%) and malvidin 3-*O*-glucoside (+ 89.3%) at 75 DAA, leading to non-significant higher content at 101 DAA (+ 37.6-59.9%) and higher significant delphinidin 3-*O*-glucoside at 75-101 DAA (+ 100.8% and + 69.8%, respectively). Skins from 10-DAA ABA-treated grapes didn't show any significant effect (figure 9). At Maipo, skins from 10-DAA CEPA-treated grapes showed only a transient effect, a significantly higher petunidin 3-*O*-glucoside content at 86 DAA (+ 27.7%). No other effect of 10-DAA PGR treatments were observed at Maipo experiment (figure 10).

At both Bordeaux and Maipo experiments, no significant effects of veraison PGR treatments on total and 3-*O*-glycosylated anthocyanin compounds was found (figures 11 and 12).

Wine Composition and Sensory Analysis

The chemical composition of wines made from PGR-treated grapes at Maipo experiment is shown in figure 13. Wines made from 10-DAA CEPA-treated grapes showed a significantly higher TA (increase of 0.74 g L^{-1}, 19%). No other effect of PGR treatments on wine quality (alcohol content, pH, color intensity, anthocyanin concentration, total phenol index and tannin concentration) was found.

The sensory analysis of wines made from PGR-treated grapes at Maipo experiment is shown in figure 14. Wines made from 10-DAA ABA-treated grapes

showed significantly lower bitterness values and significantly higher vegetal aroma values, while wines made from veraison CEPA-treated grapes showed significantly higher vegetal aroma values. No significant differences between treatments were found for red fruit aroma, tannic intensity and astringency values.

DISCUSSION

According to Messina (2008), the regions of Bordeaux and Maipo are part of the “Köppen Climate Classification” zone C: mid-latitude with mild winters. Both regions have temperate climate with dissimilar characteristics: Bordeaux has an oceanic climate and Maipo a mediterranean climate. These climate differences lead to differences during the studied seasons: Bordeaux presented higher minimal temperatures and lower maximal temperatures, revealing lower thermal amplitude (figure 1). These differences, together with precipitation (figure 2) and other environmental differences impact on the grapevine phenology (figure 3), hormonal balance (Yamane *et al.*, 2006) and on reproductive physiology and metabolism (May, 2004). The same applied treatments (application timing, PGR concentration, studied variety) in different environmental conditions, as are Bordeaux and Maipo, show several differences in their effects on grape composition. While treatment effects at the Bordeaux experiment are significant for flesh TSS levels and skin tannin and anthocyanin contents, at Maipo only minor transient differences were found (figures 5, 8, 10 and 12). Some environmental factors have an influence on the hormonal balance of developing grapes. As it is shown in figures 1 and 2, Maipo experiment was exposed to several condition that can affect endogenous

ABA content: lower night temperatures that can induce higher endogenous ABA levels (Yamane *et al.*, 2006), absence of summer precipitations and the consequent effect on grapevine water status during grape development can induce higher endogenous ABA levels (Castellarin *et al.*, 2007) and the fact that more daily hours of sun exposure at Maipo (data not shown) can also induce higher endogenous ABA levels (Berli *et al.*, 2010). As a consequence of these environmental differences, higher levels of endogenous ABA could be expected at the Maipo experiment, explaining a lower response to exogenous ABA treatments.

The berry weight was slightly superior in those berries treated with the PGRs at 10-DAA, this effect was only significant with ABA treatment. Despite Quiroga *et al*. (2009) had reported an increase in yield associated with ABA treatments, no effect on berry weight was found by them. The crop yield was not significantly affected by PGR treatments, but it is interesting to notice that 10-DAA CEPA-treated grapes had the lower crop yield. This non-significant effect can be explained by early CEPA treatment on grape loss, possibly because of berry and bunch toxicity (Szyjewicz and Kliewer, 1983) that induced necrosis as was observed in a cv. Carmenère experiment (González *et al*., unpublished results).

Among the limited effects of PGR treatments on berry flesh ripeness parameters, grape TSS levels were affected by PGR treatments at Bordeaux experiment: CEPA seems to increase TSS levels when applied early in berry development and to decrease TSS levels when applied near veraison. These agree with previous results in cv. Cabernet Sauvignon grapes (González *et al.*, 2012) and in cv. Carmenère wines (González *et al*., unpublished results), but are contrary with the hypothesis that early treatments delay ripening, reducing TSS

and pH (Szyjewicz and Kliewer, 1983) and treatments just before véraison seem to promote ripening (Coombe and Hale, 1973). Our results agree with the idea that CEPA is a promoter or inhibitor of ripening depending on the phenological stage of the berries at treatment time (Hale *et al.*, 1970, Davies and Böttcher, 2009), but it would promote TSS accumulation when applied early in development and it would reduce TSS accumulation when applied near veraison, or at least it would be affecting sugar metabolism in that direction without affecting other parameters as color-change evolution, that was not affected by treatments. The early CEPA treatment produced a significant reduction in TA at the beginning of veraison (60 DAA; 6% color change) and a non-significant increment of this parameter at the last sampling dates (105-128 DAA) that could be revealing an effect of CEPA on the synthesis of acids during the green phase of development and an effect on acid degradation during ripening stage that could explain the increase of TA near veraison and the reduction close to ripeness at the Maipo experiment. These effects agree with the reduction in pH and increase in TA found in cv. Chenin blanc treated 1-2 weeks after full bloom reported by Szyjewicz and Kliewer (1983).

Phenolic composition of grapes was affected by treatments: ABA treatment affected tannin content in Bordeaux experiment: a transient increase when applied early in development and a final reduction when applied around veraison (figures 7C and 7D). As Lacampagne *et al*. (2009) reported, ABA seems to affect tannin biosynthesis pathway in grape skins by decreasing leucoanthocyanidin reductase and anthocyanidin reductase activity and repressing the expression of related genes a few days after treatment. In accordance with the results of Lacampagne *et al*. (2009), ABA had a positive effect on tannin biosynthesis during veraison (figure

7C). After veraison, tannin vary little or present small decreases in a "per berry" basis (Adams and Harbertson, 1999, Jordao *et al.*, 2001, Fournand *et al.*, 2006). According to this, it can be expected from a promoter of ripening treatment as the ABA treatment (Davies and Böttcher, 2009), the founded effect of reduction of tannin content (figure 7D).

Per berry total anthocyanidin levels were considerably superior in Bordeaux grapes, presumably because of the smaller Maipo average berry size (figure 3). While in the Maipo experiment there were almost no differences in total and 3-*O*-glycosylated anthocyanin compounds, in Bordeaux experiment early CEPA treatment increased all 3-*O*-glycosylated anthocyanin compounds, but no effect of veraison treatments were observed. The effect of early CEPA treatment in Bordeaux experiment is not in agreement with the ripening inhibitory effect of early CEPA treatments (Davies and Böttcher, 2009) and as far as we know it is the first report of this effect of early CEPA treatment.

The effect of PGR treatment on wine composition was shown to be very limited. This effect agrees with the limited effects observed on grapes and could be due to the low concentrations used in this experiment and the environmental conditions of the Maipo field commercial vineyard experiment. The only significant effect was the increase in TA in those wines made from veraison CEPA-treated grapes.

The effects of 10-DAA ABA and veraison CEPA treatments increasing vegetal aroma were unexpected. The reduction in bitterness with 10-DAA ABA treatment may be related to the effect of ABA controlling tannin biosynthesis (Lacampagne *et al.*, 2009). The bitterness of the red wine seems to be induced by

sub-threshold concentrations of phenolic acid ethyl esters and flavan-3-ols (Hufnagel and Hofmann, 2008) and the effect of ABA on increasing grape the IPT (González *et al*., unpublished results) could be related to a reduction in flavan-3-ols and bitterness.

As conclusions of these experiments we can report the limited effect of low dose promoter of ripening PGR treatments on wine quality and the importance of environmental conditions over the effect of these treatments on grape composition. We found that CEPA affects TSS levels positively when applied early in berry development and the opposite effect when is applied at veraison, an interesting effect in order to reduce alcohol concentration in the wine. ABA treatment affected tannin content when applied early in development, showing early ABA regulation of the phenylpropanoid pathway and with low concentrations. However, no effects of PGRs on wine chemical composition were found in the Maipo experiment.

ABBREVIATIONS USED

ABA, abscisic acid; ANOVA, analysis of variance; CEPA, 2-chloroethylphosphonic acid; DAA, days after anthesis; PGR, plant growth regulators; TSS, total soluble solids; TA, titratable acidity.

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Table 1

	Sample	Stage[a]	Date	DAA	TSS (°Brix)	Color-change
BORDEAUX	1	29: Berries pepper-corn size	22/06	10		0
	2	29: Berries pepper-corn size	23/06	11		0
	3	29: Berries pepper-corn size	26/06	14		0
	4	35: Berries begin to color and enlarge	24/07	42		6
	5	35: Berries begin to color and enlarge	27/07	45	7,0	16
	6	36: Berries with intermediate Brix values	05/08	54	12,6	65
	7	37: Berries not quite ripe	26/08	75	19,3	99
	8	38: Berries harvest-ripe	21/09	101	21,6	100
MAIPO	1	29: Berries pepper-corn size	17/12	10		0
	2	29: Berries pepper-corn size	18/12	11		0
	3	33: Berries still hard and green	20/01	44		0
	4	35: Berries begin to color and enlarge	05/02	60	8,8	6
	5	36: Berries with intermediate Brix values	15/02	70	12,5	80
	6	36: Berries with intermediate Brix values	03/03	86	15,0	100
	7	38: Berries harvest-ripe	22/03	105	22,3	100
	8	39: Berries over-ripe	14/04	128	23,3	100

a Grapevine growth stages as defined by the Modified E-L system (Coombe, 1995).

Figure 1

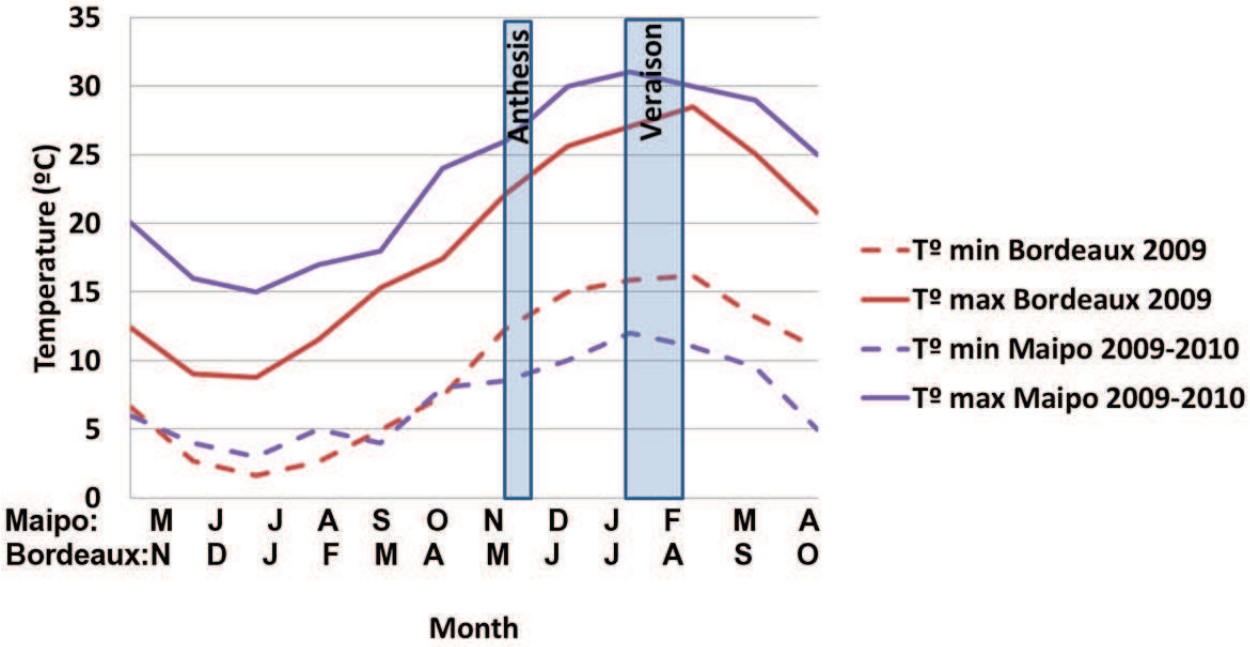
35
30
25
20
15
10
5
0
Temperature (ºC)
Anthesis
Veraison
Maipo: M J J A S O N D J F M A
Bordeaux: N D J F M A M J J A S O
Month
Tº min Bordeaux 2009
Tº max Bordeaux 2009
Tº min Maipo 2009-2010
Tº max Maipo 2009-2010

Figure 2

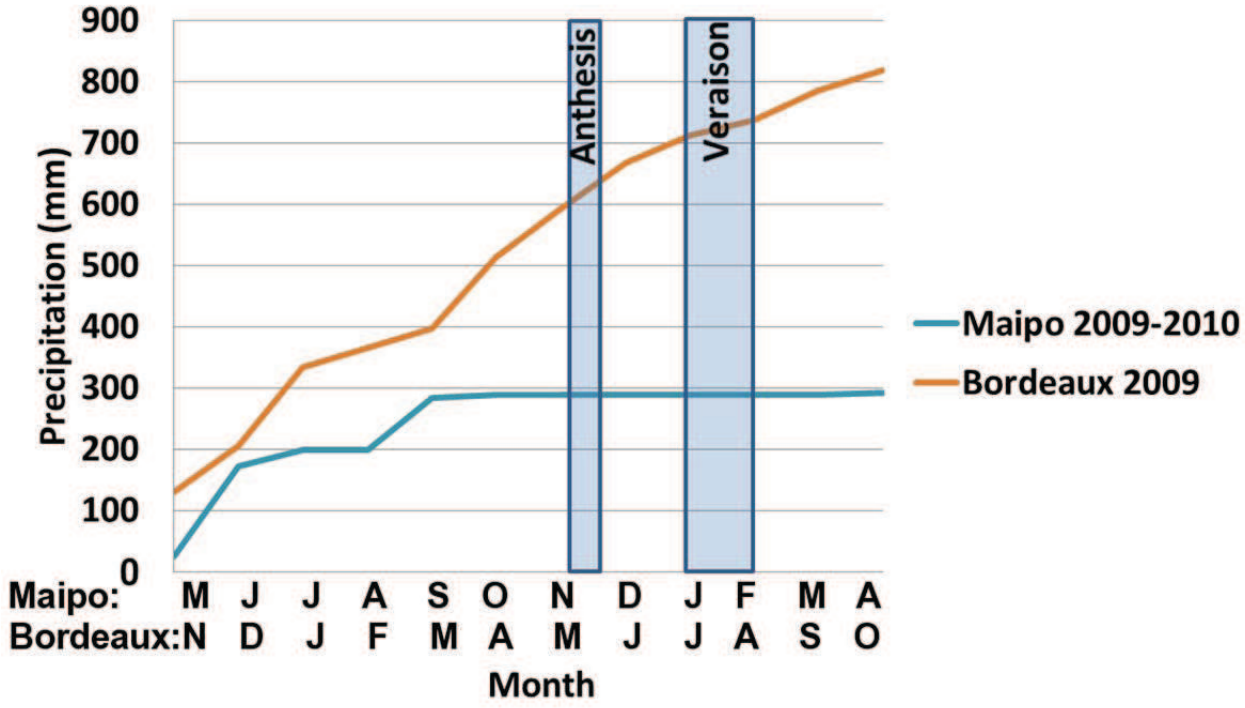
900
800
700
600
500
400
300
200
100
0
Precipitation (mm)
Anthesis
Veraison
Maipo 2009-2010
Bordeaux 2009
Maipo: M J J A S O N D J F M A
Bordeaux: N D J F M A M J J A S O
Month

Figure 3

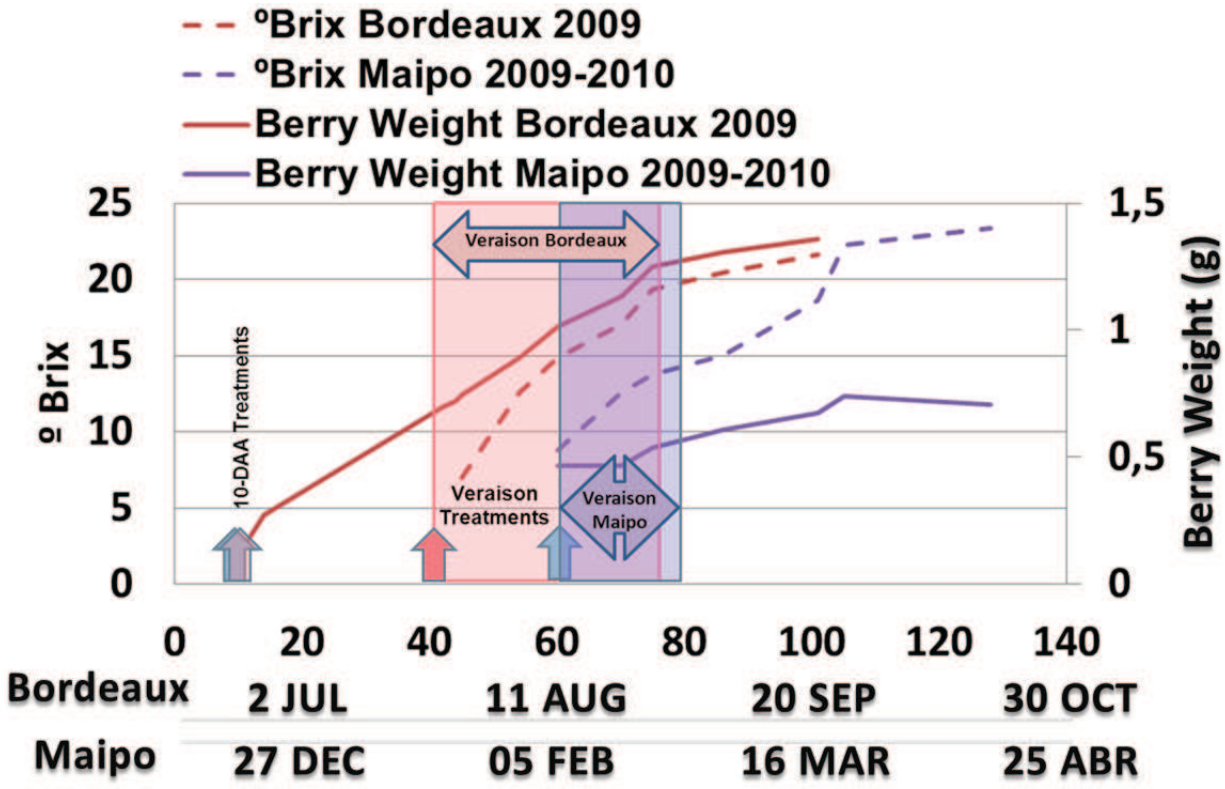
ºBrix Bordeaux 2009
ºBrix Maipo 2009-2010
Berry Weight Bordeaux 2009
Berry Weight Maipo 2009-2010
º Brix
Berry Weight (g)
25
20
15
10
5
0
1,5
1
0,5
0
10-DAA Treatments
Veraison Bordeaux
Veraison Treatments
Veraison Maipo
0
20
40
60
80
100
120
140
Bordeaux
2 JUL
11 AUG
20 SEP
30 OCT
Maipo
27 DEC
05 FEB
16 MAR
25 ABR

Figure 4

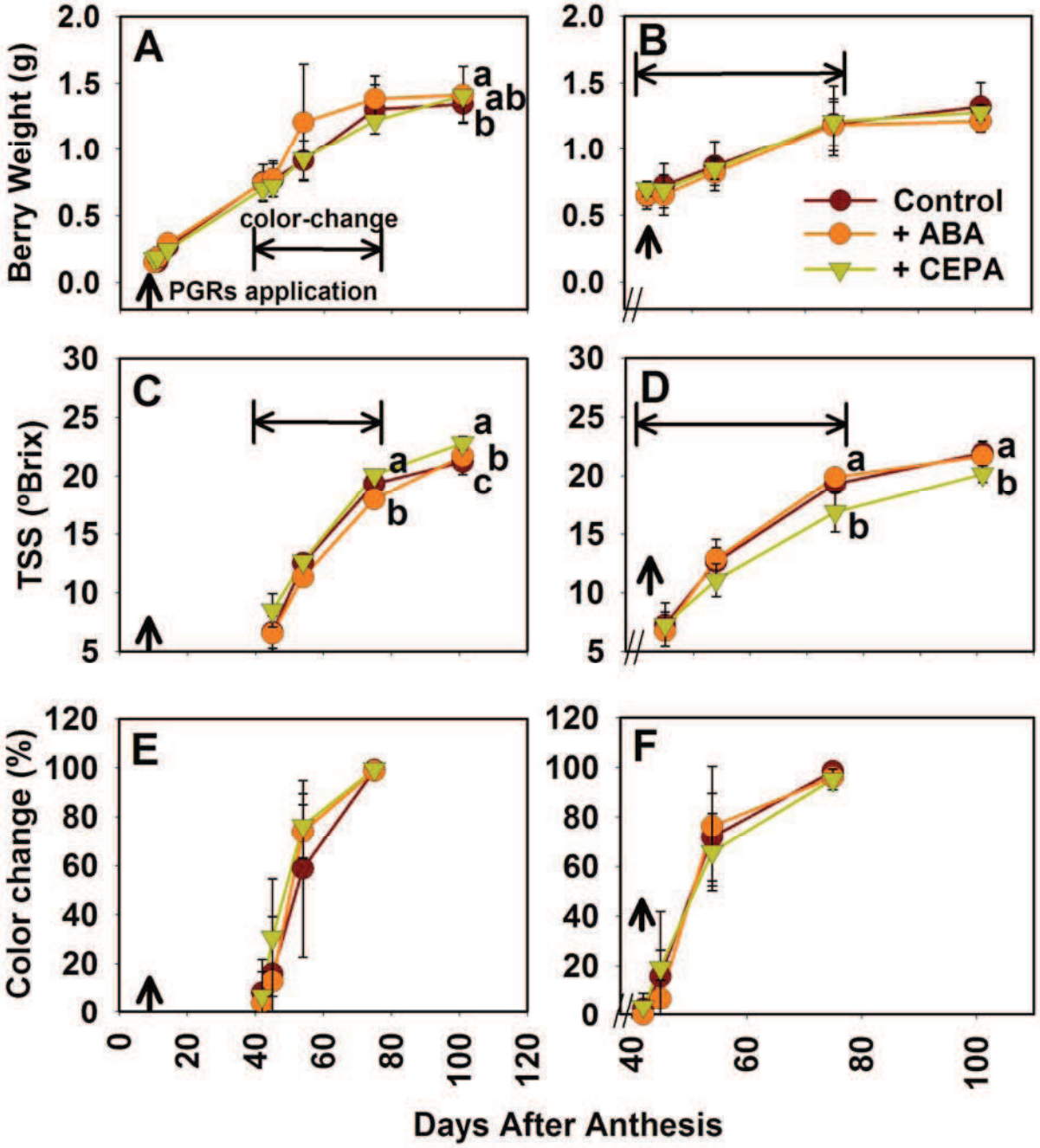
A
B
C
D
E
F
Berry Weight (g)
TSS (°Brix)
Color change (%)
Days After Anthesis
color-change
PGRs application
Control
+ ABA
+ CEPA
a
ab
b
a
b
a
b
c
a
b
a
b
2.0
1.5
1.0
0.5
0.0
30
25
20
15
10
5
120
100
80
60
40
20
0
0
20
40
60
80
100
120
40
60
80
100

Figure 5

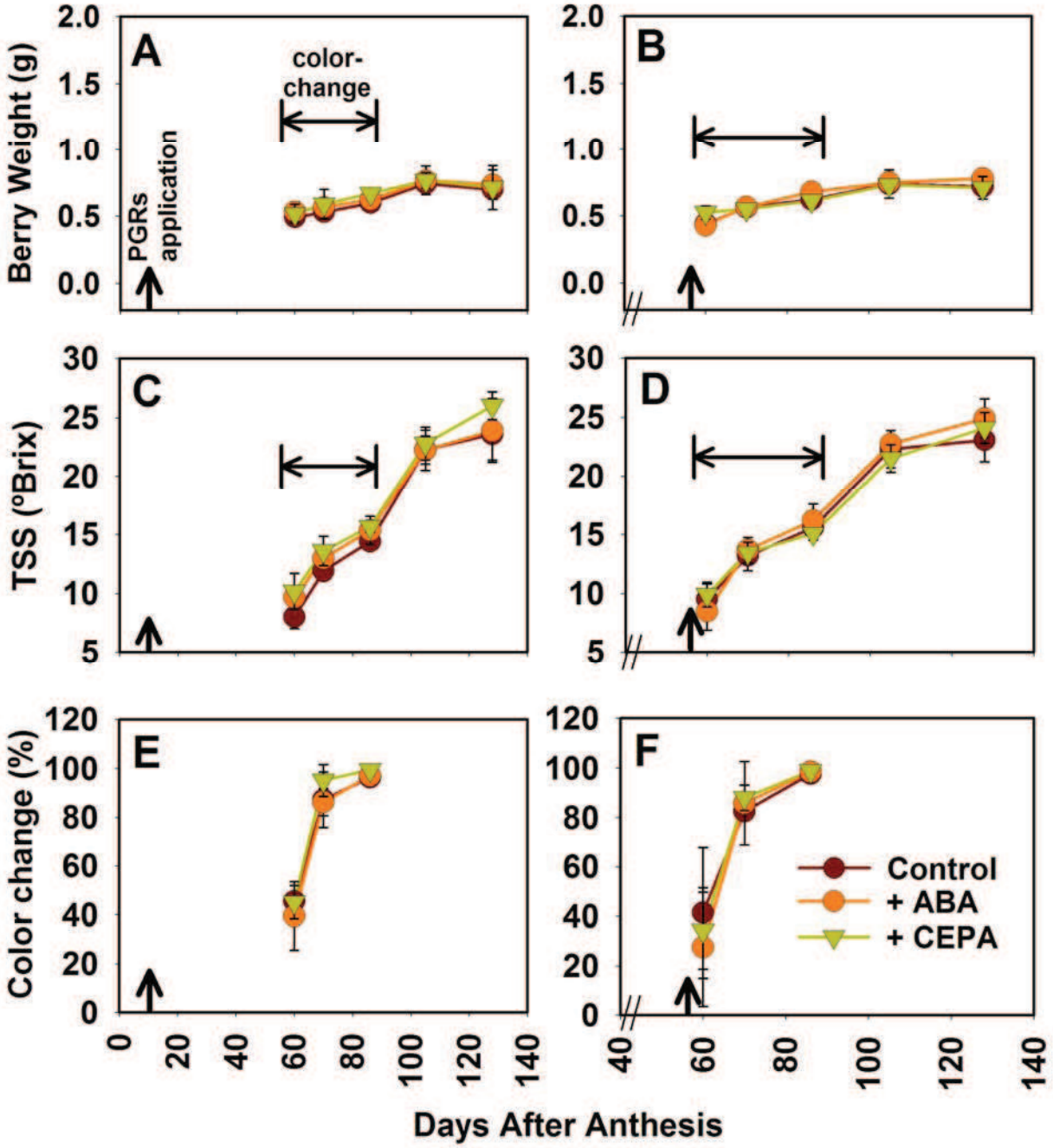
A
B
C
D
E
F
color-
change
PGRs
application
Berry Weight (g)
TSS (°Brix)
Color change (%)
Days After Anthesis
Control
+ ABA
+ CEPA

Figure 6

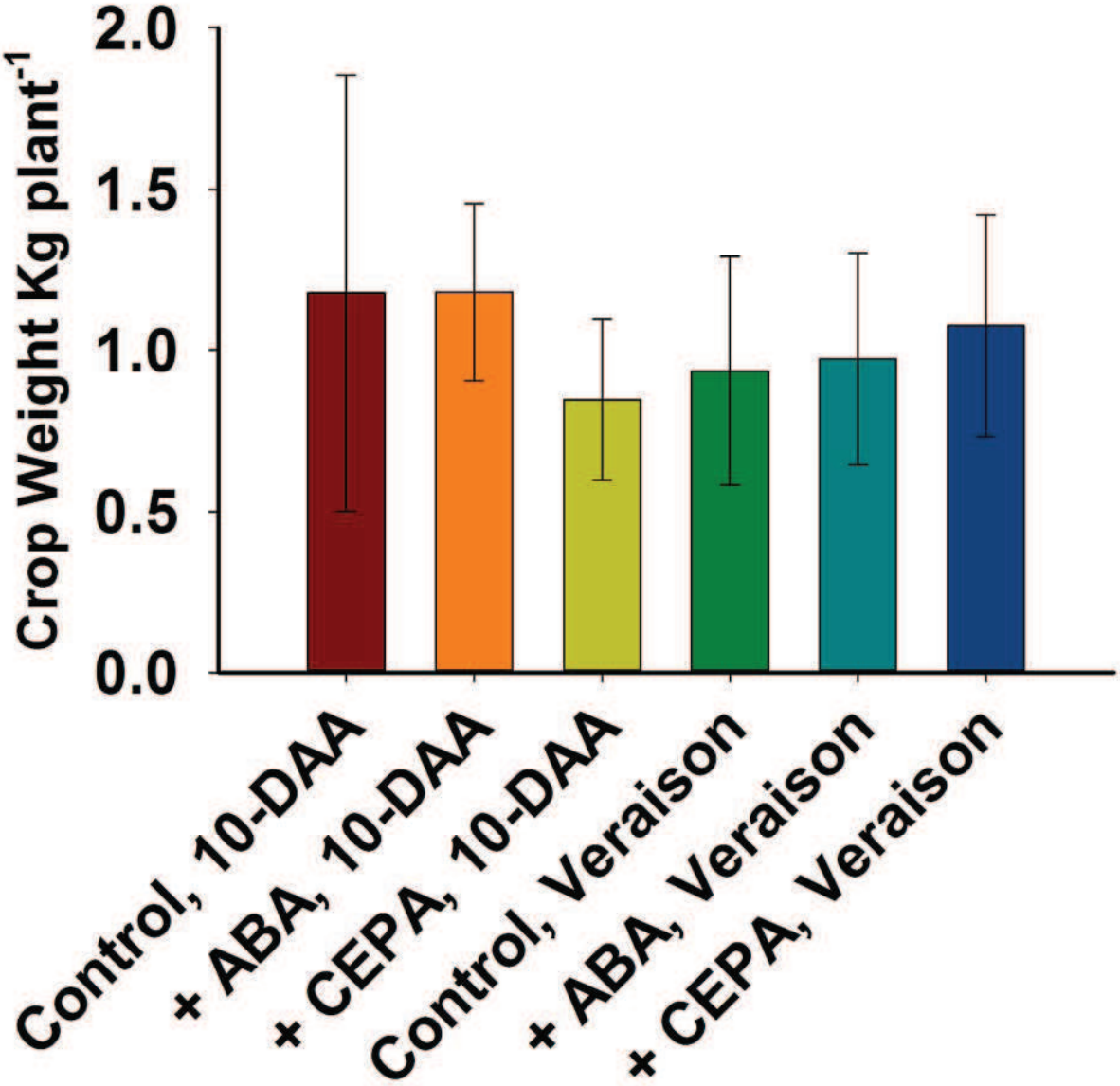
Crop Weight Kg plant-1
2.0
1.5
1.0
0.5
0.0
Control, 10-DAA
+ ABA, 10-DAA
+ CEPA, 10-DAA
Control, Veraison
+ ABA, Veraison
+ CEPA, Veraison

Figure 7

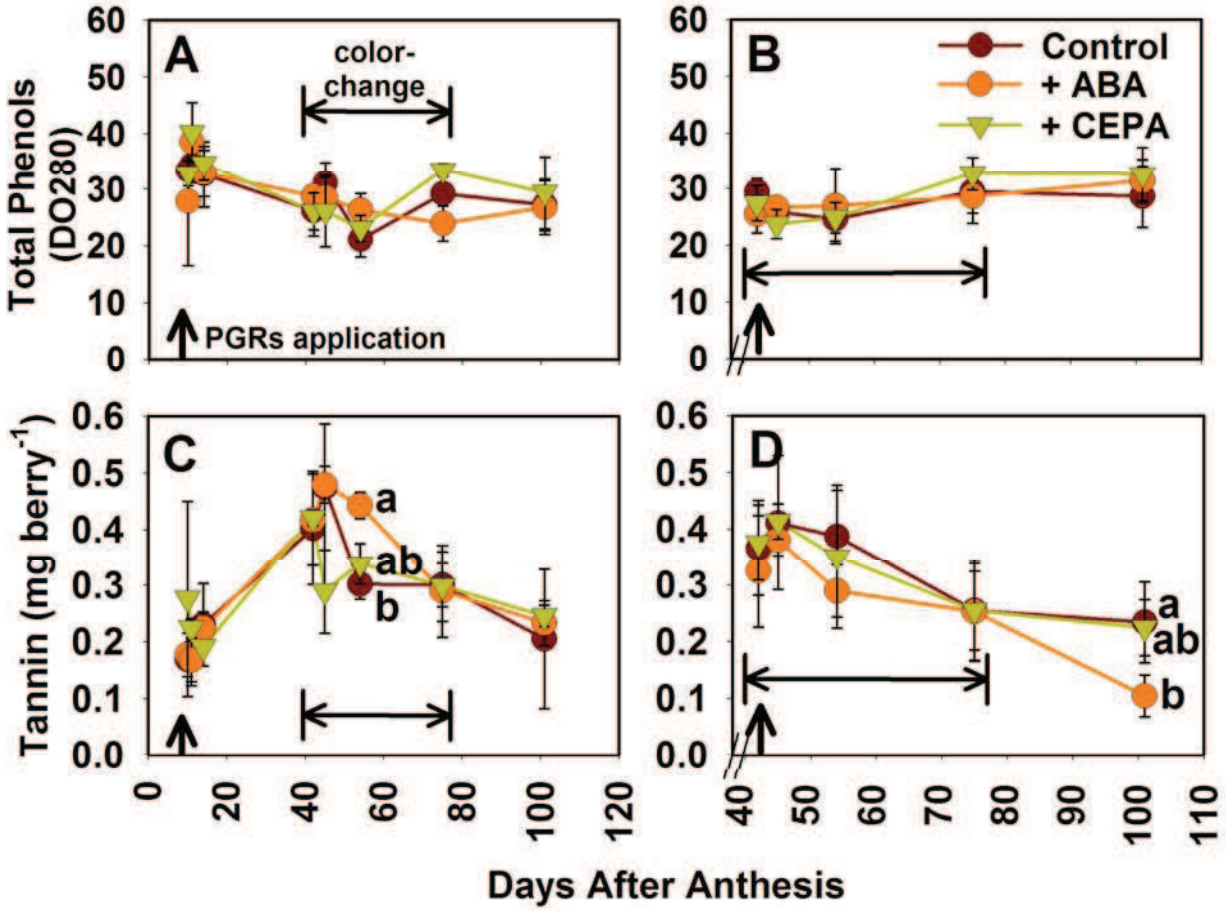
A
color-
change
PGRs application
Total Phenols
(DO280)
B
Control
+ ABA
+ CEPA
C
a
ab
b
Tannin (mg berry-1)
D
a
ab
b
Days After Anthesis

Figure 8

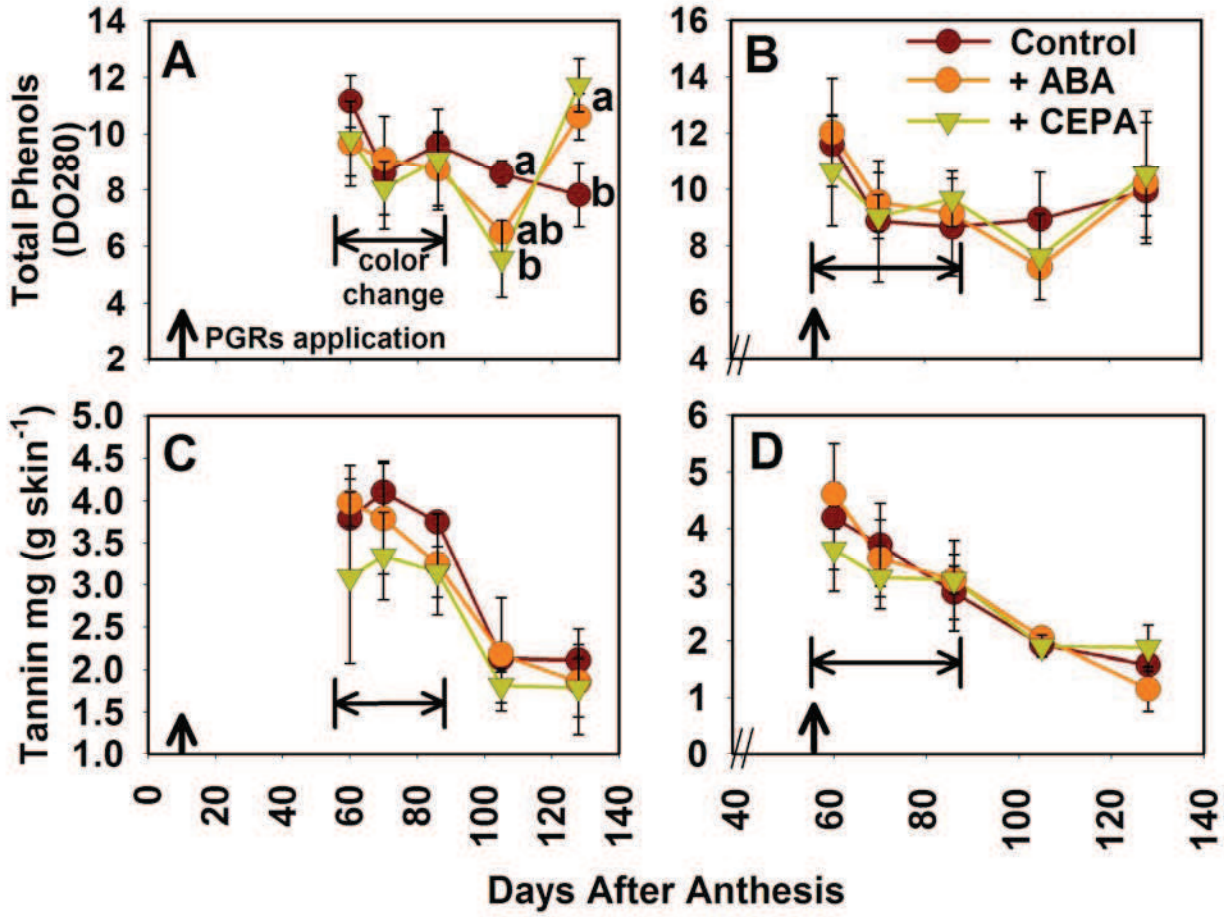
A
B
C
D
Control
+ ABA
+ CEPA
Total Phenols (DO280)
Tannin mg (g skin-1)
color change
PGRs application
a
a
b
ab
b
Days After Anthesis

Figure 9

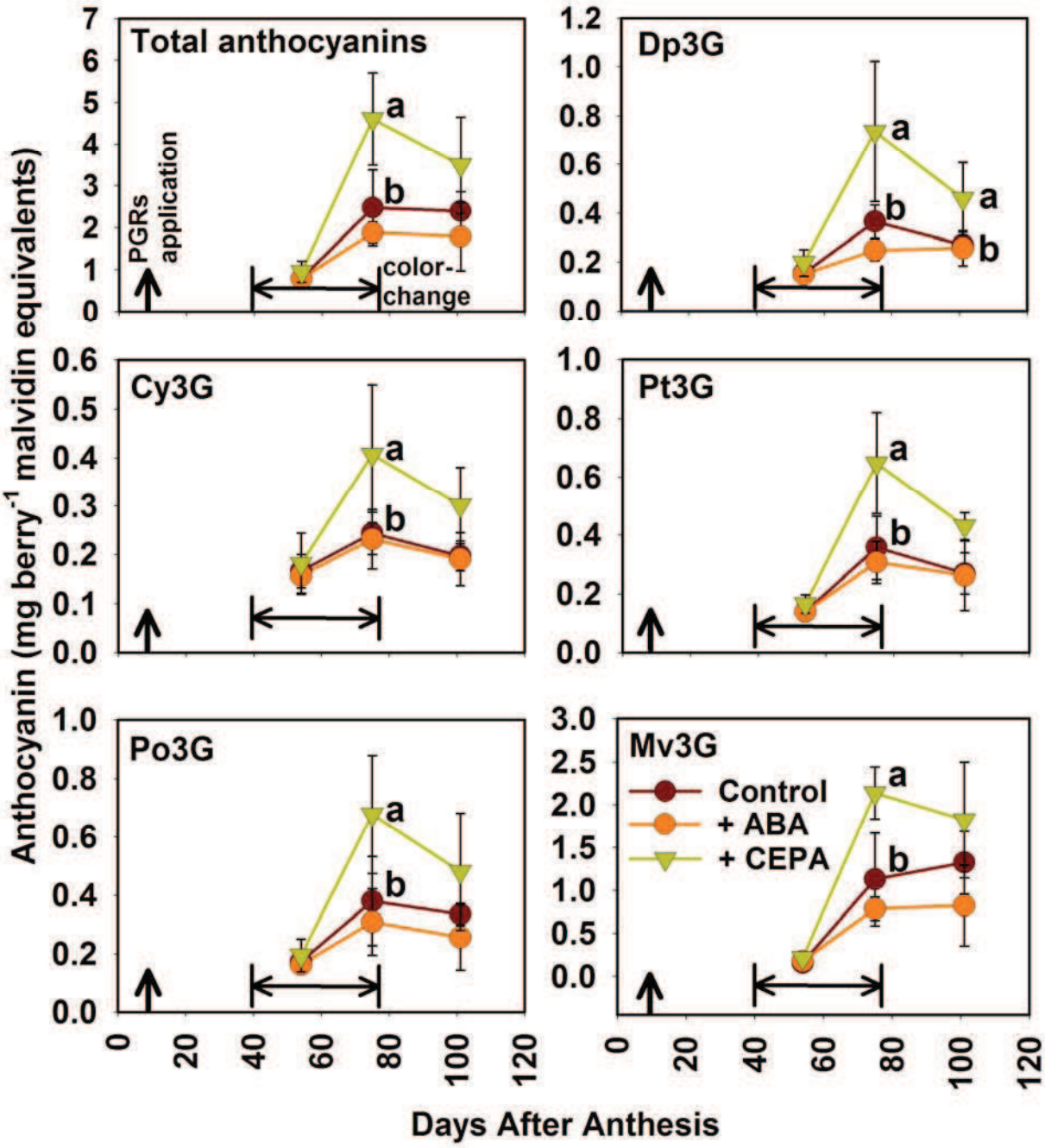
Total anthocyanins
Dp3G
Cy3G
Pt3G
Po3G
Mv3G
PGRs application
color-change
a
b
Control
+ ABA
+ CEPA
Anthocyanin (mg berry-1 malvidin equivalents)
Days After Anthesis
0
20
40
60
80
100
120

Figure 10

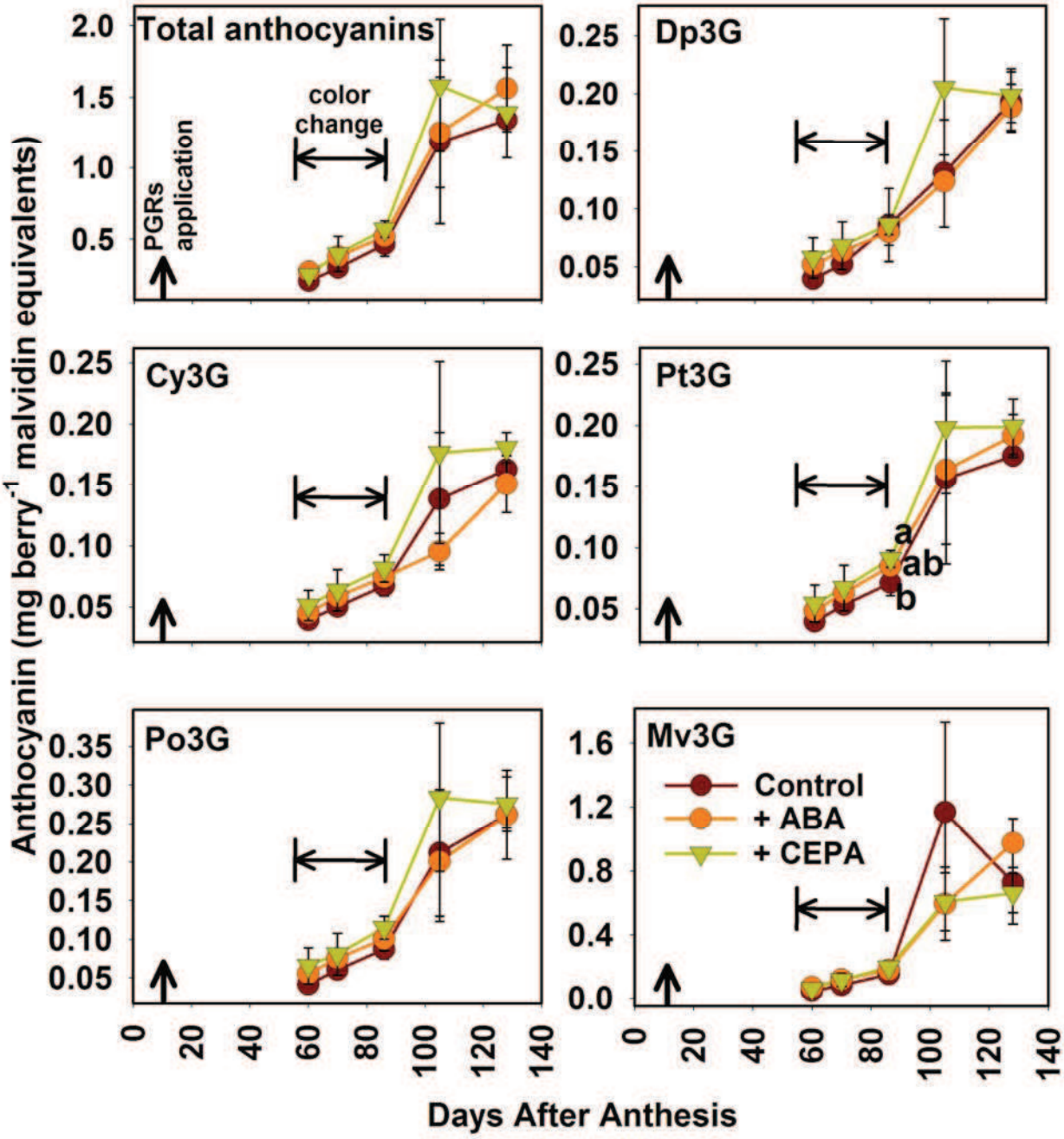
Total anthocyanins
color change
PGRs application
Dp3G
Cy3G
Pt3G
a
ab
b
Po3G
Mv3G
Control
+ ABA
+ CEPA
Anthocyanin (mg berry-1 malvidin equivalents)
Days After Anthesis

Figure 11

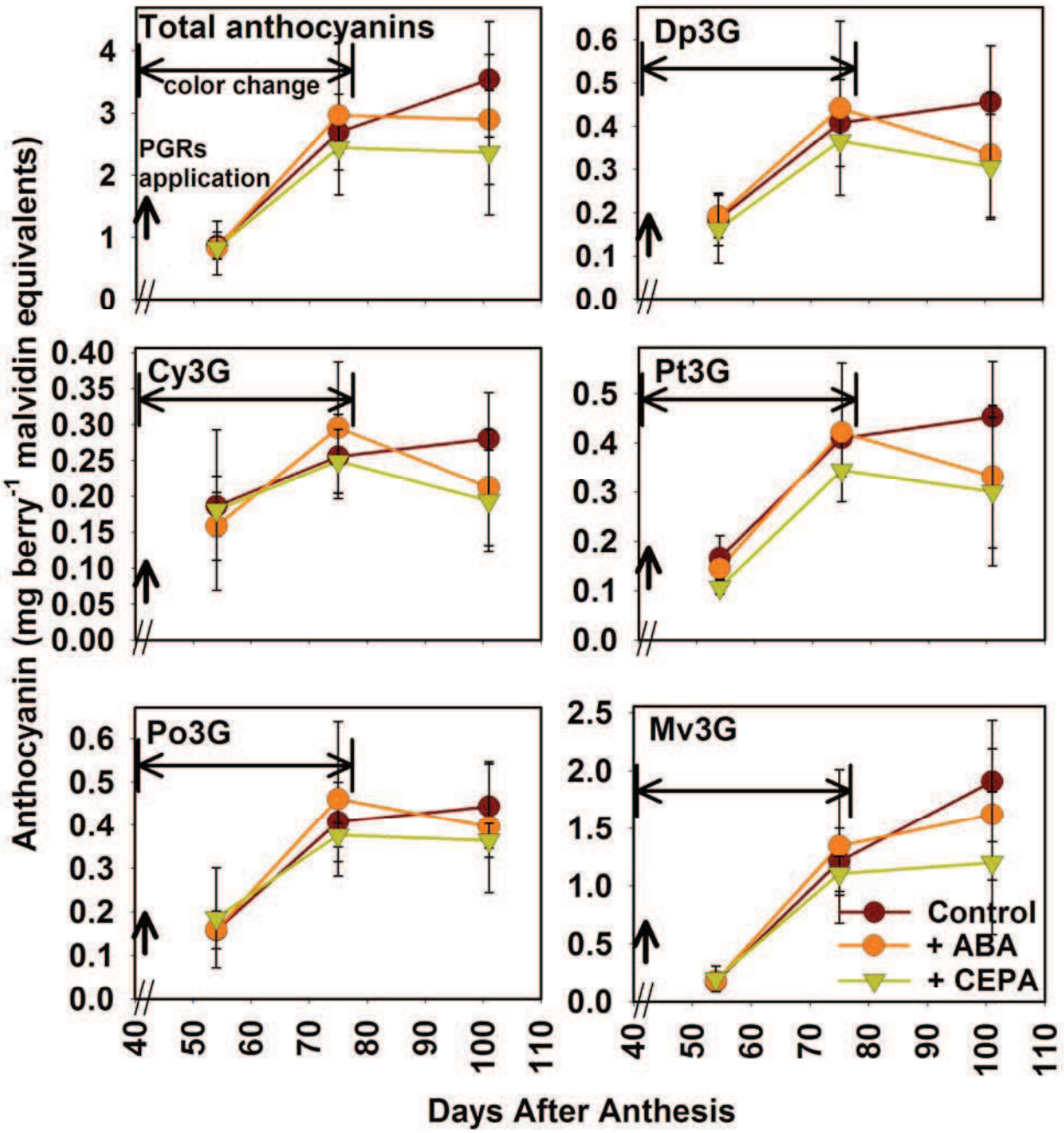
Total anthocyanins
color change
PGRs application
Dp3G
Cy3G
Pt3G
Po3G
Mv3G
Control
+ ABA
+ CEPA
Anthocyanin (mg berry-1 malvidin equivalents)
Days After Anthesis

Figure 12

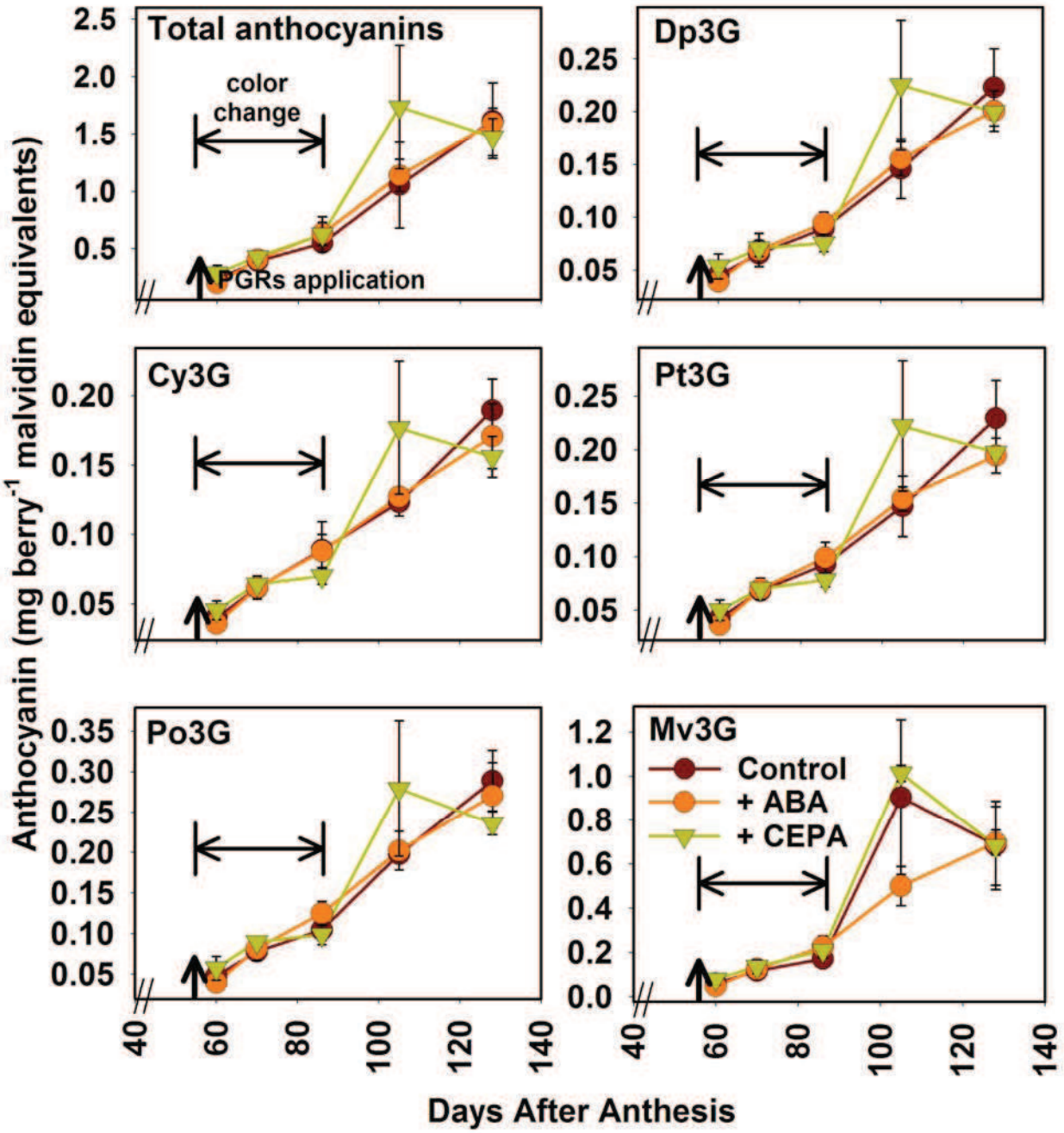
Total anthocyanins
color change
PGRs application
Dp3G
Cy3G
Pt3G
Po3G
Mv3G
Control
+ ABA
+ CEPA
Anthocyanin (mg berry-1 malvidin equivalents)
Days After Anthesis

Figure 13

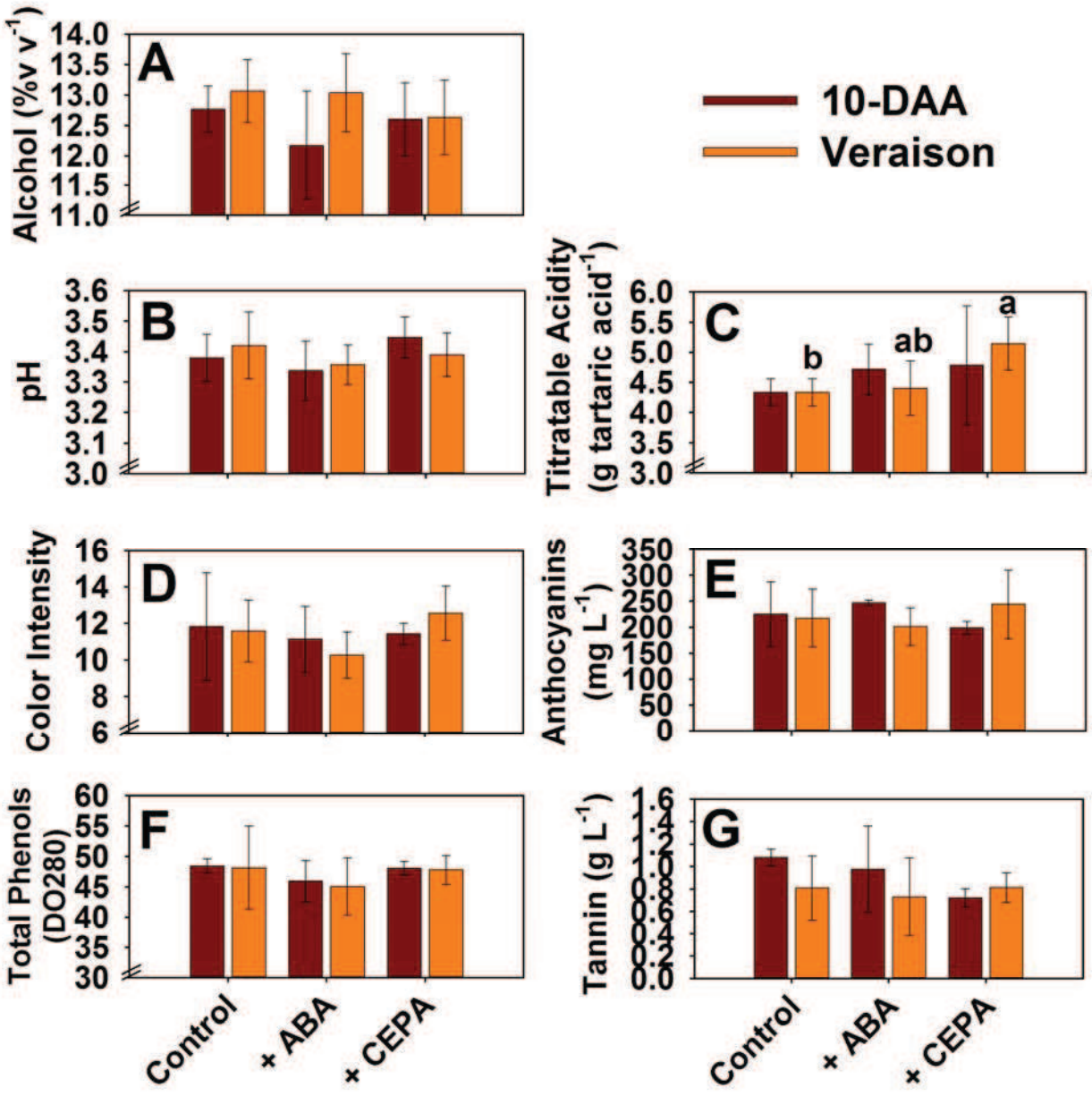
A
B
C
D
E
F
G
Alcohol (%v v-1)
pH
Titratable Acidity (g tartaric acid-1)
Color Intensity
Anthocyanins (mg L-1)
Total Phenols (DO280)
Tannin (g L-1)
10-DAA
Veraison
b
ab
a
Control
+ ABA
+ CEPA

Figure 14

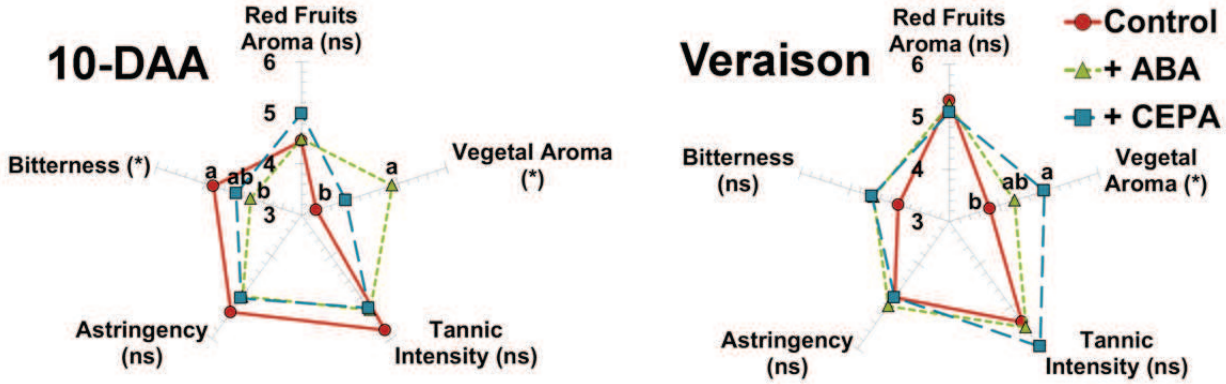
10-DAA
Red Fruits Aroma (ns)
6
5
4
3
Vegetal Aroma (*)
Tannic Intensity (ns)
Astringency (ns)
Bitterness (*)
a
ab
b
b
a
Veraison
Red Fruits Aroma (ns)
Vegetal Aroma (*)
Tannic Intensity (ns)
Astringency (ns)
Bitterness (ns)
Control
+ ABA
+ CEPA

TABLE AND FIGURE LEGENDS

Table 1. Grapevine growth stage, sampling date, days after veraison and total soluble solids of grape samples of cv. Cabernet Sauvignon at Bordeaux (France) and Maipo (Chile).

Figure 1. Annual evolution of mean monthly minimum and maximum temperatures from November 2008 to October 2009 at Bordeaux (France) and from May 2009 to April 2010 at Maipo (Chile).

Figure 2. Annual evolution of accumulated precipitations from November 2008 to October 2009 at Bordeaux (France) and from May 2009 to April 2010 at Maipo (Chile).

Figure 3. Comparative evolution of mean total soluble solids and mean berry weight during berry development from control cv. Cabernet Sauvignon grapes in experimental and commercial vineyard trials at Bordeaux (France) during 2009 and Maipo (Chile) during 2009-2010, respectively.

Figure 4. Evolution of berry weight (A, B), total soluble solids (C, D) and color change berries (E, F) of PGR-treated and control grapes treated 10 days after anthesis (A, C, E) and at veraison (B, D, F) during development in a cv. Cabernet Sauvignon experimental vineyard trial at Bordeaux (France). Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate

significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures (P <0.05).

Figure 5. Evolution of berry weight (A, B), total soluble solids (C, D) and color change berries (E, F) of PGR-treated and control grapes treated 10 days after anthesis (A, C, E) and at veraison (B, D, F) during development in a cv. Cabernet Sauvignon commercial vineyard experiment at Maipo (Chile). Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures (P <0.05).

Figure 6. Crop weight per plant in cv. Cabernet Sauvignon 2009-2010 treated 10 days after anthesis, at veraison and their respective control plants. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures (P <0.05).

Figure 7. Skin total phenols (A, B), skin tannin content (C, D) and skin anthocyanin content (E, F) of PGR-treated and control grapes treated 10 days after anthesis (A, C, E) and at veraison (B, D, F) during development in a cv. Cabernet Sauvignon experimental vineyard trial at Bordeaux (France). Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures (P <0.05).

Figure 8. Skin total phenols (A, B), skin tannin content (C, D) and skin anthocyanin content (E, F) of PGR-treated and control grapes treated 10 days after anthesis (A, C, E) and at veraison (B, D, F) during development in a cv. Cabernet Sauvignon experiment during 2009-2010 at Maipo (Chile). Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures ($P < 0.05$).

Figure 9. Concentration of total and 3-*O*-glycosylated anthocyanin compounds: delphinidin (Dp3G), cyanidin (Cy3G), petunidin (Pt3G), peonidin (Po3G) and malvidin (Mv3G), from the different 10-days-after-anthesis PGR-treated and control berry skins cv. Cabernet Sauvignon, in a experimental vineyard trial at Bordeaux (France). Anthocyanin concentrations are calculated in malvidin equivalents. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures ($P < 0.05$).

Figure 10. Concentration of total and 3-*O*-glycosylated anthocyanin compounds: delphinidin (Dp3G), cyanidin (Cy3G), petunidin (Pt3G), peonidin (Po3G) and malvidin (Mv3G), from the different 10-days-after-anthesis PGR-treated and control berry skins cv. Cabernet Sauvignon, in a commercial vineyard experiment at Maipo (Chile). Anthocyanin concentrations are calculated in malvidin equivalents. Vertical bars indicate the standard deviation (three biological replicates). Different letters

indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures (P <0.05).

Figure 11. Concentration of total and 3-*O*-glycosylated anthocyanin compounds: delphinidin (Dp3G), cyanidin (Cy3G), petunidin (Pt3G), peonidin (Po3G) and malvidin (Mv3G), from the different veraison PGR-treated and control berry skins cv. Cabernet Sauvignon, in a experimental vineyard trial at Bordeaux (France). Anthocyanin concentrations are calculated in malvidin equivalents. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures (P <0.05).

Figure 12. Concentration of total and 3-*O*-glycosylated anthocyanin compounds: delphinidin (Dp3G), cyanidin (Cy3G), petunidin (Pt3G), peonidin (Po3G) and malvidin (Mv3G), from the different veraison PGR-treated and control berry skins cv. Cabernet Sauvignon, in a commercial vineyard experiment at Maipo (Chile). Anthocyanin concentrations are calculated in malvidin equivalents. Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments for the same date, as calculated by ANOVA and Tukey's HSD multiple comparison procedures (P <0.05).

Figure 13. Wine composition, including alcohol concentration (A), pH (B), titratable acidity (C), color intensity (D), anthocyanin concentration (E), total phenols (F) and tannin concentration (G) of wines made from 10-days-after-

anthesis and veraison PGR-treated and control grapes from a cv. Cabernet Sauvignon commercial vineyard experiment at Maipo (Chile). Vertical bars indicate the standard deviation (three biological replicates). Different letters indicate significant differences between treatments as calculated by ANOVA and Tukey's HSD multiple comparison procedures ($P < 0.05$).

Figure 14. Wine sensory analysis, including red fruits aroma, vegetal aroma, tannic intensity, astringency and bitterness of wines made from PGR-treated and control grapes treated either at 10 days after anthesis (A) or at veraison (B) from a cv. Cabernet Sauvignon commercial vineyard experiment at Maipo (Chile). Vertical bars indicate the standard deviation (three biological replicates). Asterix indicates significant differences and “ns” indicates non-significant differences between treatments as calculated by ANOVA ($P<0.05$). Different letters indicate significant differences between treatments as calculated by Tukey's HSD multiple comparison procedures ($P < 0.05$).

Chapter 6

Congress Presentations

GONZÁLEZ, A.S., E. BORDEU. 2009. Aplicación exógena de reguladores de crecimiento durante el desarrollo de la piel de bayas de *Vitis vinifera* L. XII Congreso Latinoamericano de Viticultura y Enología. 11-13 of November, Montevideo, Uruguay. Poster Presentation.

GONZÁLEZ, A.S., P. OLEA, E. BORDEU, L. GENY, J.A. ALCALDE, A. ZUÑIGA. 2010. Aplicación exógena de reguladores de crecimiento durante el desarrollo de la piel de bayas de *Vitis vinifera* L. 61º Congreso Agronómico de Chile - 56 Reunión anual de la Sociedad Interamericana de Horticultura Tropical (ISTH) - 11º Congreso de la Sociedad Chilena de Fruticultura. Sociedad Agronómica de Chile. 26-29 of September, Santiago, Chile. Oral Communication.

GONZÁLEZ, A.S., P. OLEA, E. BORDEU, L. GENY, J.A. ALCALDE, A. ZUÑIGA. 2010. Effect of abscisic acid, (2-chloroethyl) phosphonic acid and indole-3-acetic acid on *Vitis vinifera* L. cvs Cabernet Sauvignon and Carmenère

grapes and wine. V Reunión de Biología Vegetal. Universidad de Chile. 1-3 of December, Olmué, Chile. Oral Communication.

GONZÁLEZ, A.S., E. BORDEU, P. ARCE-JOHNSON, J.A. ALCALDE, L. GENY. 2011. Efecto del tratamiento con reguladores de crecimiento vegetal sobre la regulación de la vía fenilpropanoide y la calidad de uvas y vinos *Vitis vinifera* L. Cv. Carmenère. XIII Congreso Latinoamericano de Viticultura y Enología. 21-23 of November, Santiago, Chile. Oral Communication.

GONZÁLEZ, A.S., P. OLEA, E. BORDEU, J.A. ALCALDE, L. GENY. 2011. Tratamientos con reguladores de crecimiento vegetal modifican la calidad de uvas y vinos *Vitis vinifera* L. Cv. Cabernet Sauvignon. XIII Congreso Latinoamericano de Viticultura y Enología. 21-23 of November, Santiago, Chile. Poster Presentation.

Chapter 7

Concluding Remarks

Plant growth regulators (PGRs) are substances that promote or inhibit plant growth and development affecting agriculture product quality. On the other hand, phenolic compounds are a big family of plant secondary metabolites with different chemical and biological activities. They are responsible for red wine color, bitter taste, mouthfeel and antioxidant, anti-inflammatory and anti-carcinogenic activities. This phenolic composition determines red wine quality.

In this thesis project, PGRs abscisic acid (ABA), 2-chloroethylphosphonic acid (CEPA) and indole-3-acetic acid (IAA) applications on cvs Cabernet Sauvignon and Carmenère wine grapes have been studied and their effects on red wine quality have been evaluated, in particular their effects on phenolic composition. Exogenous treatments with promoters of ripening ABA and CEPA at *veraison* are able to positively influence grape and wine quality, enhancing grape berry skin and wine flavonoid content. Results show CEPA as the most effective PGR for inducing higher flavonoid accumulation. These effects are highly variable depending on environmental factors, as climatic conditions of the growing season, that also affect vine balance between productivity and vegetative growth, leading to

limited or absence of effects in some conditions. The specific environmental factors affecting PGR treatment effects have not been determined in this thesis.

Nowadays in hot climate viticulture, enologists use to wait for phenolic ripeness, which normally takes place in overripe grapes with high total soluble solids (TSS) concentration leading to high wine alcohol, with consequences in wine sensory balance and health benefits of its consumption. Exogenous CEPA treatment is able to reduce grape TSS and hence wine alcohol content. Moreover, CEPA treatment also decreases grape acidity and increases grape pH, skin anthocyanin and skin phenolic composition. This is an important effect that can improve phenolic composition without the necessity of overripening grapes which leads to higher TSS. It is interesting to note the independent CEPA effect on TSS and other general ripeness parameters (*i.e.* acidity, pH, phenolic composition), suggesting a specific regulation of grape sugar uptake and metabolism, as demonstrated by the reduction of the “hexose transporter 1” gene expression.

The effects of PGR treatments on grape skin tannin content are limited or null and are not well correlated with the corresponding effects on wine tannin content, where CEPA and ABA treatment increase wine tannin concentration. These differences can be explained by the fact that wine also contains seed tannin or by a possible treatment effect on skin tannin extractability, subjects that were not resolved in this thesis.

Exogenous treatment with the inhibitor of ripening IAA at *veraison* is able to induce opposite effects to those of promoters of ripening, reducing the content of some grape skin phenolic compounds but without effects on wine composition, at least under the dose tested.

Hormonal balance of cv. Cabernet Sauvignon grape skin is modified by CEPA and ABA treatments. Exogenous S-ABA treatment increases the internal free ABA content and CEPA treatment decreases internal IAA content. Exogenous IAA treatment did not modify internal IAA or ABA levels. It can be concluded that PGR treatments modify hormonal balance of berry skin, in particular S-ABA and CEPA.

Flavonoid pathway biosynthetic and regulatory gene expression of cv. Carmenère grape skin is modified by CEPA and ABA treatments. In this thesis an up-regulation of the first step of flavonoid synthesis pathway was found, together with an up-regulation of the genes responsible for tannin synthesis and anthocyanin methoxylation. However, a surprising down-regulation of the two last metabolic steps in anthocyanin synthesis was found. These effects lead to minor or null effects on skin and wine phenolic composition, showing that even if a treatment might not have an effect on grape or wine composition, grape skin

flavonoid metabolic pathway is modified by PGR treatments. Other subsequent regulations must be taking place, possibly modulated by environmental factors.

The effect of treatments on wine sensory attributes was determined by a sensory panel. S-ABA reduces or increases wine vegetal aroma depending if application time is early in berry development or at *veraison*, while low doses of *veraison* CEPA treatment increases wine vegetal aroma. These effects are dependent on grapevine growing conditions. There is no effect of IAA treatment on this kind of aromas. Another effect of treatments on sensory attributes was a reduction in bitterness caused by ABA, an issue that may be associated to the effect of ABA in controlling tannin biosynthesis and increasing tannin polymerization level.

Treatments shortly after fruit set are not always able to promote greater effects than at *veraison*. CEPA presents contrasting effects depending on application time: CEPA increase TSS levels when applied early in berry development and decreased TSS levels when applied near *veraison*. Early CEPA treatments have been associated to grape and bunch necrosis. ABA also has differential effects depending on application time, showing an increase in skin tannin content with early applications, but reduction after *veraison* applications.

The comparison of *veraison* CEPA and ABA treatment effects on cvs Carmenère and Cabernet Sauvignon shows some similarities like the effects on acid and some phenolic compound concentrations (*i.e.* titratable acidity, pH, total phenol index and anthocyanin concentration). However, effects on wine alcohol content show interesting differences. CEPA treatment increases or decreases the wine alcohol content depending if the cv. is Cabernet Sauvignon or Carmenère respectively, whereas ABA treatment decreases only cv. Carmenère wine alcohol content. Another important difference is the effect on tannin concentration increase that was achieved only by ABA treatment on cv. Carmenère and only by CEPA treatment on cv. Cabernet Sauvignon. Finally, the wine sensory attributes are affected only by ABA treatment on cv. Carmenère.

In this thesis project a comparison between doses was performed in cv. Carmenère, showing a large reduction of treatment effects with half doses. The lowest dose tested in this work had some effects on grape quality (*i.e*: increase of anthocyanin content with CEPA), but with very limited effects on wine composition.

The projections of these treatments have applicability in the wine industry. They can influence wine quality without affecting vineyard crop yield. Determination of the effect of different environmental and vine balance conditions on hormonal balance in PGR-treated grapes is required to determine the factors modulating the effect of these treatments leading to the presence or absence of effects on wine

composition and quality. As a continuation of the fundamental studies of PGR application effects, it would be important to test in controlled conditions the differential CEPA and ABA effects on the internal hormonal balance and on phenolic compound accumulation, with extreme environmental and vine balance conditions: effect of sunlight exposure, effect of water status and effect of fruit-to-leaf ratio.