Brassinosteroids as a tool for color improvement in red table grapes (*Vitis vinifera* L.)

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El origen de la estrella

Jimena Muñoz pone un cubo de hielo tras el lóbulo de la oreja izquierda de su hijo de 13 años.

Le dice que no tenga miedo, que con frío no se siente nada y con un pequeño aro de oro sin punta le perfora la oreja. No sale sangre.

Jimena le dice a su hijo que se ve lindo.

El adolecente corre al espejo, se mira, sonríe, agradece y besa a su madre.

Así comienza mi vida como estrella de rock y así termina mi madre de parirme.

Pablo Paredes

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Chapter 1

Hormonal control and participation of brassinosteroids during the ripening of grape berries

1.- Introduction

The development of grape berries involves the coordination of numerous events such a increase in berry size, loss in firmness and acidity, increase in sugar content, etc. In this sense, the increase in berries size over time is adjusted to a double sigmoid curve (Figure 1). This curve has been traditionally divided into three phases or stages of development (Coombe, 1995; Coombe and McCarthy, 2000).

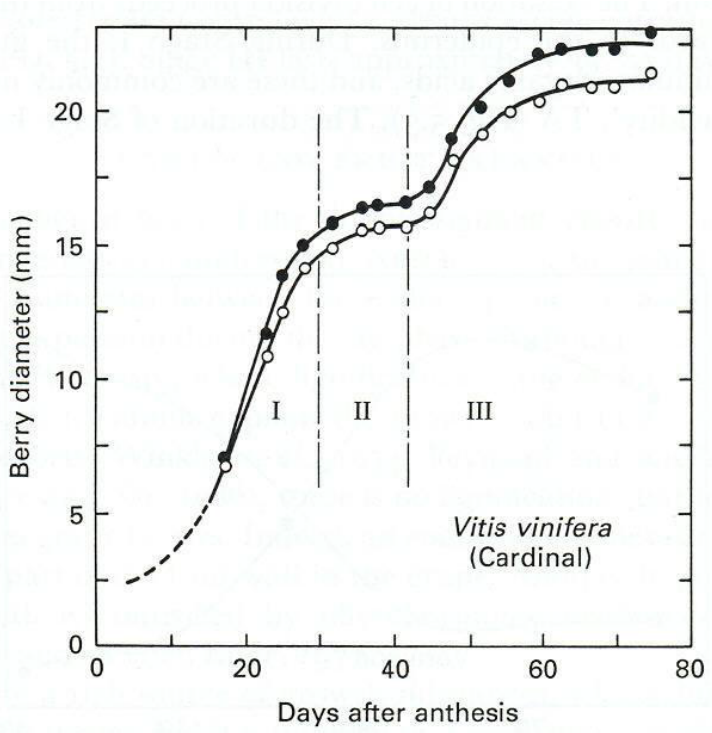

Figure 1. Growth of grape berries from anthesis to harvest. The three stages of berry development are indicated as I, II and III.

In the first phase, rapid growth of the berries is observed, which is explained by the cellular division that is typical in newly formed berries (Coombe and McCarthy, 2000). It has been described that during this stage there is a large accumulation of organic acids (Muñoz-Robredo et al., 2011; Sweetman et al., 2009; Wen et al., 2014), and embryogenesis occurs (Keller, 2010).

During the second stage of berry development the veraison (also called “pinta” in Spanish) occurs and is characterized by a slow growth rate of berries. It is during this period where the embryo has its highest growth rate, reaching its final size and physiological maturity (Adams, 2006; Keller, 2010; Pratt, 1971; Ristic and Iland, 2005), and accumulation of sugars, change of berries color and loss of total acidity of the berries also begins (Coombe and McCarthy, 2000; Keller, 2010).

Ripening processes occur during the third and final stage of berry development. This phase is characterized by a rapid increase in berry size (Figure 1), which then slows down towards the end of ripening. Although these processes begin during fruit set, it is during the last phase of berry development when the accumulation of anthocyanins and of sugars, and degradation of organic acids were observed (Coombe, 1995; Coombe and McCarthy, 2000; Keller, 2010).

2.- Changes experienced by berries during ripening

2.1.- Synthesis of anthocyanins and color development

Anthocyanins are synthesized by the phenylpropanoid pathway (Figure 2), which has been characterized in different species, particularly in vines, where the expression of genes related to the synthesis of flavonoids (particularly anthocyanins and protoanthocyanidins) has been studied in berries and seeds, both colored and white cultivars (Bogs et al., 2005; Boss et al., 1993; Castellarin et al., 2007; Matus et al., 2009; Sparvoli et al., 1994).

The first key step of anthocyanin synthesis pathway is the generation of cinnamic acid from the amino acid phenylalanine, a reaction catalyzed by the enzyme phenylalanine ammonium lyase (PAL). The fourth reaction of the route is catalysed by the enzyme chalcone synthase (CHS) and constitutes the second point of regulation of the synthesis of phenolic compounds since from here the formation of flavonoid compounds is diverted. The enzyme chalcone isomerase (CHI) will later generate a flavonone, which will ultimately give rise to dihydroflavonols. These will be precursors of the synthesis of flavonols, flavan-3-oles or anthocyanidins. The last stage and third regulatory point of anthocyanin synthesis is the glycosylation of the 3'-hydroxyl group of the anthocyanidins. This step is essential to confer stability and greater solubility to anthocyanins for their transport and confinement in vacuoles as

well as their chromophore behaviour (Marrs et al., 1995; Matus et al., 2009; Wang et al., 2001). The last reaction is catalysed by the enzyme UDP-glucose flavonoid 3-O-glucosyltransferase (UFGT).

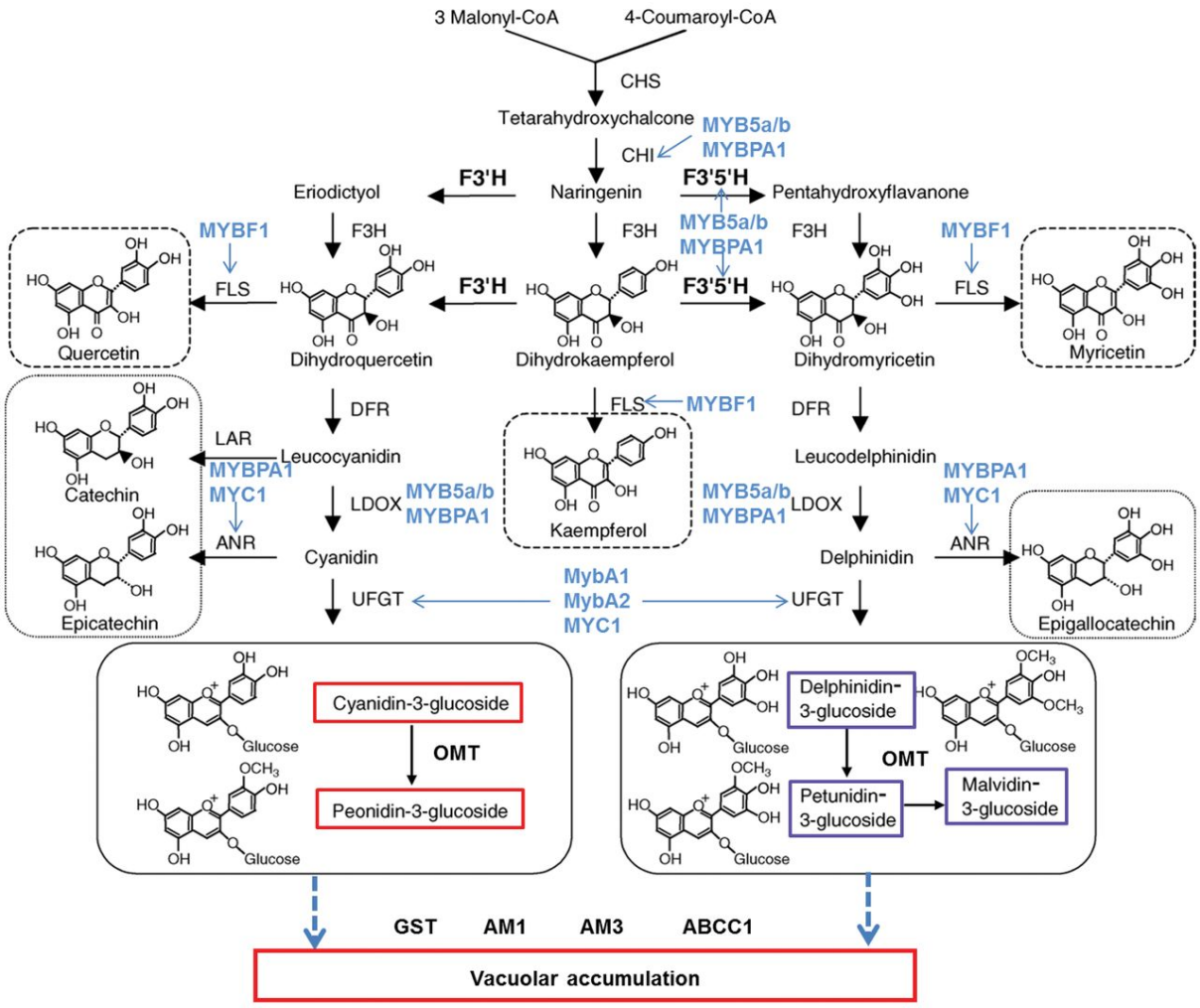

Figure 2. Simplified diagram of the anthocyanin synthesis pathway. The acronyms of the enzymes shown in the figure are the following: CHS, chalcone synthase; F3'H, flavonoids-3-hydroxylase; F3'5H, flavonoids-3'5'-hydroxylase, FLS1, flavonol synthase; BAN, anthocyanidin reductase; DFR, dihydroflavonol reductase; LDOX, leucoanthocyanidin oxidase; LAR, leucoanthocyanidin reductase; UFGT, UDP-glucose flavonoids-3-O-glucosyltrasferase; OMT, O-methyl transferase; GST, glutathione S-transferase. The transcription factors that control the expression of the genes that code for the enzymes are indicated in blue. Source: Kuhn et al. (2014).

Color is the main indicator of quality on which acceptance of the fruit by the consumer is based (Clydesdale, 1993). Although the anthocyanins are the main pigments responsible for berry color in coloured varieties, its development is evaluated, at the commercial level, through the use of hedonic scales, which allow a visual evaluation of color coverage of cluster, berries and color intensity (INIA, 2017). Evaluation of color intensity and berries color are the parameters that are under a greater subjectivity.

A more objective method to evaluate the color intensity of berries is colorimetry. Carreño et al. (1995) developed a colour index for red berries (CIRG; Colour Index for Red Grapes) based on the CIELAB color space system, using the parameters L^* (luminosity), H^* (hue angle) and C (chroma) and defined as $CIRG = (180 - H^*)\cdot(L^* + C)^{-1}$. The CIRG exhibits a strong correlation with the external colour of berries (Figure 3A) and is able to distinguish between different color groups (Carreño et al., 1996). Therefore, the different color classifications that the International Organization of Vine and Wine (IOVW) have established are associated with a CIRG value (Figure 3B) and, in turn, with a concentration of total anthocyanins (Figure 3C).

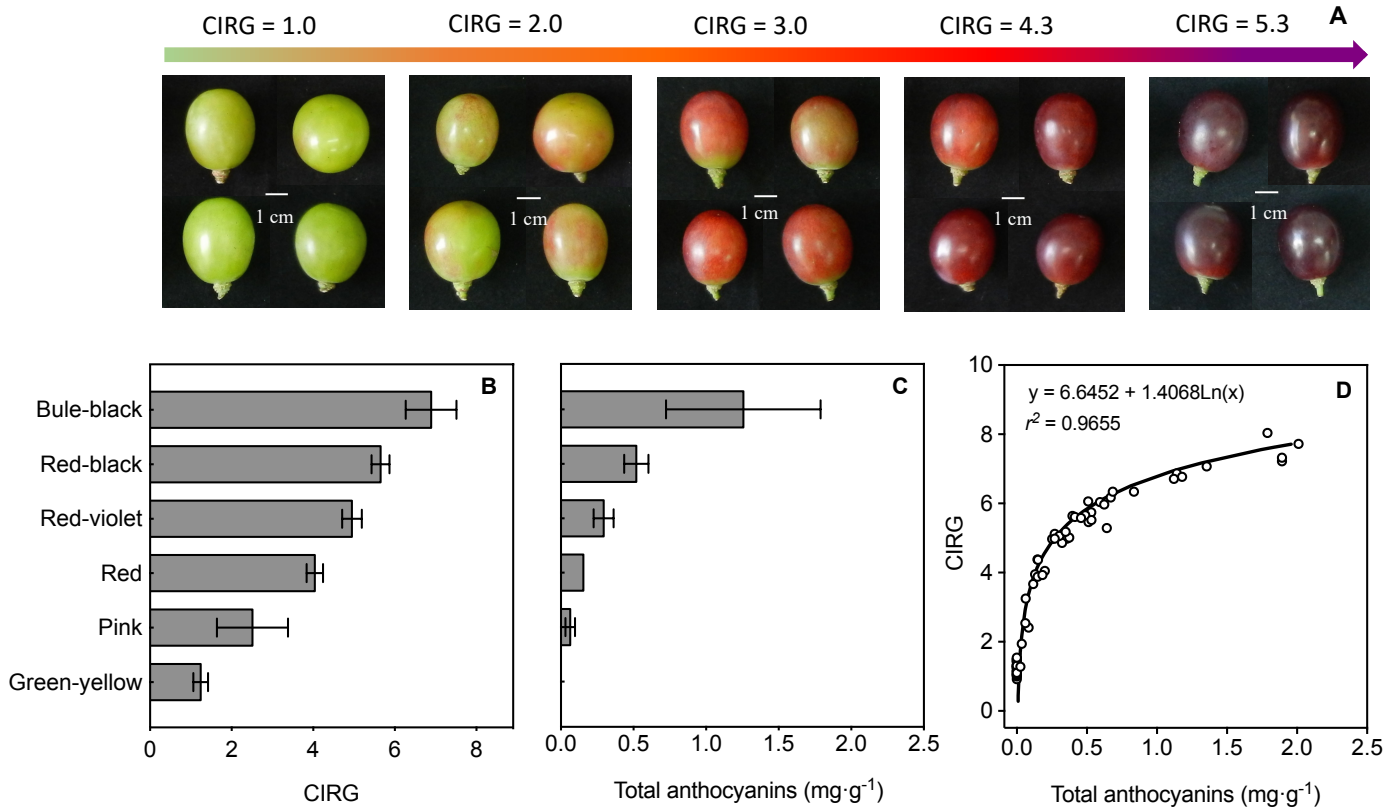

Figure 3. 'Redglobe' berry appearance for CIRG values from 1 to 5.3 (A), CIRG values (B) and anthocyanin concentration (C) for each of IOVW color categories and relationship between total contraction of anthocyanins and color of berries expressed as CIRG and the regression between both parameters (D). In (B) and (C) the error bars represent ± one standard deviation. (A) adapted form Vergara et al. (2018), (B), (C) y (D) adapted from Carreño et al. (1996).

The manifestation of berries color intensity (expressed as CIRG) depends on factors such as type anthocyanin and its concentration. Dihydroxylated anthocyanins (cyanidin-3-glucoside and peonidin-3-glucoside) are associated with pink and red color, and trihydroxylated anthocyanins (delphinidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside) are associated with darker colours (Fernández-López et al., 1998). Table grape varieties, such as 'Redglobe', 'Crimson Seedless' and 'Flame Seedless', predominantly contain dihydroxylated anthocyanins (Carreño et al., 1997; Vergara et al., 2018), while varieties such as 'Black Seedless' and wine-make varieties predominantly contain trihydroxylated anthocyanates (Castellarin et al., 2007; Xi et al., 2013). The relationship between concentration of anthocyanins and color of berries expressed as CIRG is reported as logarithmic (Figure 3D). Therefore, berry color response to changes in anthocyanin concentration decreases as anthocyanin concentration increases, so, once CIRG values close to 6.0 have been reached, an increase in anthocyanins does not necessarily mean a noticeable increase in the color of berries.

2.2.- Sugars accumulation the during ripening of berries

The process by which sugars accumulate during the ripening of grape berries has been widely described (Coombe and McCarthy, 2000; Keller, 2010); nevertheless, the limiting steps and the molecular mechanisms that control the final sugar concentration in berries are still unknown. According to Agasse et al. (2009), the

compartmentalization and enzymatic events that carry sucrose from the sieve tubes of the phloem to hexoses inside the vacuoles of the mesocarp cells involve an apoplastic unloading of the phloem, action of sucrose and monosaccharide transporters and acidic invertase of wall and vacuole (Figure 4).

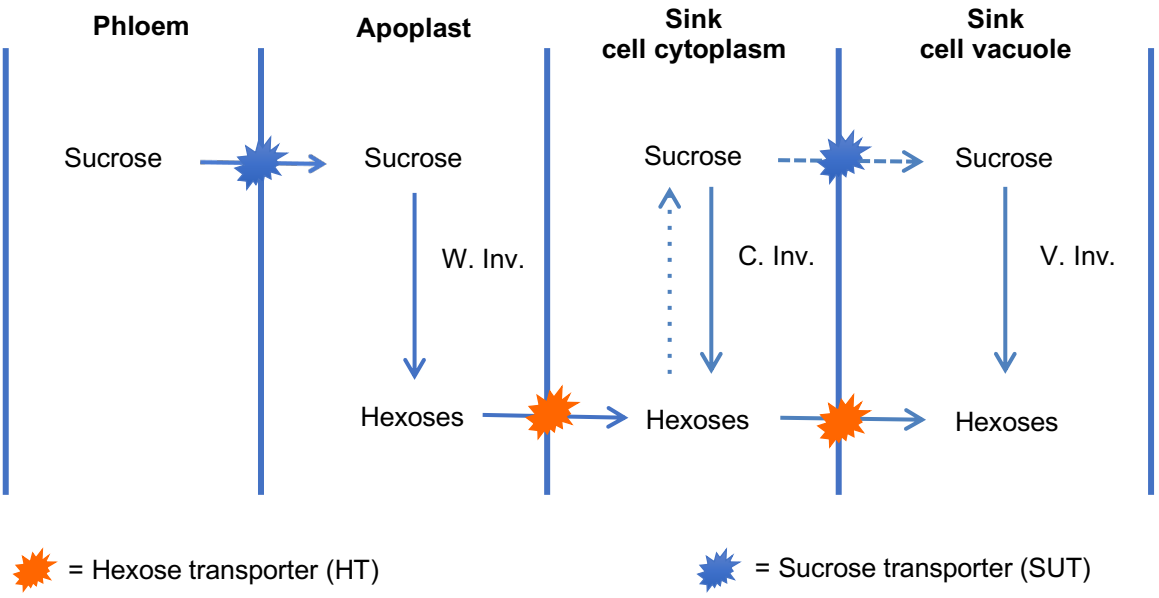

Figure 4. Representation of phloem unloading and sugars loading in sink cells during ripening of berries. Phloem cells, apoplastic space, cytoplasm and vacuole of sink cells, wall invertase (W. Inv), cytoplasmic invertase (C. Inv) and vacuole invertase (V. Inv) and sucrose and hexose transporters are indicated. The arrows of solid lines show the main direction in which the reactions or transport occur, while the arrows of dashes lines show the least predominant direction in which the reactions or transport take place.

The sucrose unloading from the phloem into apoplastic space, in which it is degraded into glucose and fructose by the cell wall invertase (Agasse et al., 2009). This process maintains a constant gradient of sucrose from the phloem to the apoplastic space. Then, hexoses are transported from the apoplastic space to the cytoplasm of the sink cell and from there to the vacuole. This process is mediated by the action of hexose transporters (Agasse et al., 2009; Conde et al., 2006; Hayes et al., 2007), which maintains the gradient of hexoses from the apoplastic space to the cytoplasm and vacuole. This model has been validated by Zhang et al. (2006), who using a variety of techniques, such as electron microscopy, enzymatic activity assays and transport studies with carboxyfluorescein (a simplistic transport tracer), accompanied by the expression of a viral movement protein (as a simplistic marker), allowed to establish that, before veraison, both carboxyfluorescein and the viral movement protein are released from the phloem bundles, but remained confined in the vascular bundles after veraison. This shift from simplistic to apoplastic transport, which occurs immediately before or shortly after veraison, is accompanied by an increase in the expression and activity of cell wall invertases (Zhang et al., 2006).

Regarding to sugars composition, two types of grapevines have been identified: the hexose accumulators, in which the ratio between glucose/(fructose + sucrose) is greater than 0.8, and sucrose accumulators, in which the ratio is less than 0.8. The hexose and sucrose accumulation traits are related to differences in genetics and regional origin. Thus, the composition of sugars mainly depends on the genetic background of the cultivar (Shiraishi et al., 2010), while the concentration mainly

depends on environmental conditions and technical management (Dai et al., 2011; Kuhn et al., 2014).

2.3.- Loss of acidity during the ripening of berries

Another important aspect related to ripening of berries is loss of total acidity. This phenomenon, in combination with the accumulation of sugars, constitutes one of the factors witch determinate the organoleptic quality of the fruit (Muñoz-Robredo et al., 2011; Shiraishi et al., 2010; Sweetman et al., 2009). During the ripening of berries not only a decrease in total acidity is observed, but also a change in distribution of acids, that explain the final acidity of fruit. Thus, during the earliest stages of berry ripening, malic acid is the predominant acid, followed by tartaric and citric acid. However, at harvest and at least in table grape, this distribution changes; tartaric acid predominates, followed by citric acid and malic acid (Muñoz-Robredo et al., 2011). This change in the distribution of acids indicates that the decrease in total acidity is not only explained by a dilution effect, given by the increase in berry size during ripening. In this sense, it is known that the loss of total acidity of berry is explained by the decrease in the concentration of malic acid (Sweetman et al., 2009). Malic acid reaches its maximum concentration just before the beginning of veraison (Muñoz-Robredo et al., 2011; Sweetman et al., 2009). The malic acid tracing during berry ripening is complex since it is involved in several metabolic processes, nevertheless it is known that its decrease is associated, mainly, with mitochondrial

oxidation. Based on mass sequencing results, the decrease in malic acid in the post-veraison period results from lower expression of cytoplasmic malate dehydrogenase and genes related to its synthesis at the mitochondrial level. In addition, changes in the expression of phosphoenolpyruvate carboxylase and kinase would shift the reaction catalysed by malate dehydrogenase towards the catabolism of malic acid (de Angeli et al., 2013; Regalado et al., 2013; Sweetman et al., 2009).

3.- Hormonal control of berries ripening

Although the ripening of grape berries is controlled by the plant development programme, it responds to environmental factors, which can alter the dynamics of berry ripening. Some of these environmental factors, whose effects have been extensively studied are exposure to solar radiation (Berli et al., 2010; Matus et al., 2009), water status (Deis et al., 2011; Deluc et al., 2009) and temperature (Cohen et al., 2012; Pillet et al., 2012; Spayd et al., 2002). As an example, a moderate water deficit and low temperatures are capable of increasing the total soluble solid and anthocyanin contents (Berli et al., 2010; Castellarin et al., 2007; Mori et al., 2005), while high temperatures, shade and viral infections delay or even inhibit the adequate ripening of berries (Carbonell-Bejerano et al., 2013; Greer and Weston, 2010; Lorrain et al., 2012; Mori et al., 2007; Pillet et al., 2012; Vega et al., 2011). The modulatory effects of these environmental factors on ripening are essentially mediated by altering the hormonal balance. For example, the levels of abscisic acid

(ABA) increase in response to water stress and low temperatures (Deluc et al., 2009; Yamane et al., 2006; Zarrouk et al., 2012), explaining the way in which environmental factors are modulating the berry ripening dynamics.

In many studies ABA was been reported as the signal that triggers the ripening of the berries. In addition, a significant increase in the levels of this hormone is observed immediately after of color turnaround period and in the first stage of berry development (Giribaldi et al., 2010; Sun et al., 2010; Wheeler et al., 2009). In this sense, exogenous applications of ABA increases the weight of the berries (Peppi et al., 2008), decreases the total acidity (Peppi and Fidelibus, 2008) and increases the total anthocyanin concentration (Berli et al., 2010; Gambetta et al., 2010; Hiratsuka et al., 2001; Jeong et al., 2004; Wheeler et al., 2009). In the case of ethylene, an increase in the levels of this hormone in berries just before veraison has been described, and that this increase precedes the increase in ABA levels (Chervin et al., 2004; Sun et al., 2010). On the other hand, the application of ethephon (2-chloroethyl phosphonic acid, an ethylene releaser) at vearaison, increases the concentrations of anthocyanins (El-Kereamy et al., 2003). Meanwhile, the application of 1-MCP (1-methylcyclopropane), a specific inhibitor of the ethylene receptor, decreases the diameter of the berries and the total anthocyanin concentration (Chervin et al., 2008), and also It has been described that this compound applied shortly before veraison, delays the increase in ABA observed in this state of berry development (Sun et al., 2010). Thus, this suggests that the effect of ethylene could be partly mediated by ABA.

Another important hormonal group during the development of berries and which has been widely described are auxins. During the earliest stages of berry development, auxins levels are high; then, they decrease to very low levels near veraison and remain at those levels throughout the remainder of berry development (Böttcher et al., 2010). This decrease is associated with the process of auxin conjugation, because the highest levels of the conjugated forms of these hormones are detected in the presence of low levels of free auxins (Böttcher et al., 2010). In general, auxins delay the ripening process of berries (Böttcher et al., 2011); in fact, the application of BOTA (benzothiazolic acid-2-oxyacetic acid), an artificial auxin, delays the increase in ABA concentrations by two weeks (Davies et al., 1997). On the other hand, transcriptomic studies of berries treated with NAA (1-naphthaleneacetic acid, an artificial auxin) one week before veraison, show a repression of genes associated with ABA synthesis, while the expression of genes related to ethylene synthesis are induced (Ziliotto et al., 2012). Based on these results, auxins negatively regulate the ripening processes associated with ABA.

3.1.- Brassinosteroids during the ripening of the berries

Brassinosteroids (BRs) are a group of hormones originally discovered in the pollen extract of *Brassica napus* L. in 1970 (Mitchell et al., 1970). The isolation of its most active form, brassinolide (BL) (Grove et al., 1979), as well as its receptor (Wang et

al., 2001), allowed the identification of BRs. On the other hand, these hormones have been attributed roles in seed germination, stem elongation, rhinogenesis, flowering, senescence and are considered as a hormones with a pleiotropic effect. (Vidya Vardhini and Rao, 2002).

BRs synthesis has been described as a complex and highly regulated process. A summary of the pathways of BR synthesis is presented in Figure 5. Briefly, this process begins with the generation of campesterol from 24-methylcholesterol. Campesterol is then transformed to campestanol. If at this point the oxidation of carbon 6 (C6) occurs, giving rise to 6-deoxocampestanol, BL is reached by the so-called early C-6 oxidation pathway. Meanwhile, if C6 oxidation occurs once campestanol is converted to 6-deoxocastasterol (6-DeonoCS), BL is produced via the late C-6 oxidation pathway (Nomura et al., 2001).

In the vine, Symons et al. (2006) reported a rapid increases in the levels of castasterone (CS) and its precursor (6-deoxoCS) near veraison in 'Cabernet Sauvignon'. On the other hand, the expression levels of VvDWF1, a BRs synthesis gene, are high in berries two weeks after anthesis, and then steadily decrease until veraison, increasing again, and staying constant in the later stages of berry development. This same pattern of expression was observed by Castellarin et al. (2007). For its part, *VvBR6OX1* (responsible for CS synthesis) is expressed at low levels during flowering and the first stages of berry development. Afterwards, the

expression of this gene increases progressively, reaching its maximum levels during veraison and then rapidly decreasing (Castellarin et al., 2007; Pilati et al., 2007; Symons et al., 2006). The negative correlation between the levels of the *VvBR6OX1* transcript and the amount of available enzyme substrate, suggest that this gene has a negative feedback regulation system, which has been described in other species (Goda, 2002; Nomura et al., 2001, 2002).

Regarding the signalling pathway, Wang et al. (2001) described and characterized the function of BRI1, the critical component of the receptor for BRs in the membrane. Symons et al. (2006) functionally described and characterized the homologue of this receptor in grapevine. The authors observed that *VvBRI1* is expressed constitutively in berries throughout their development, presenting an increase in expression in the early stages of newly set fruits, in veraison and in the first stages of ripening. These results suggest that the BRs participate in ripening of berries and that they would be one of the first signs that trigger their ripening (Ziliotto et al., 2012) and participate in various steps throughout this process.

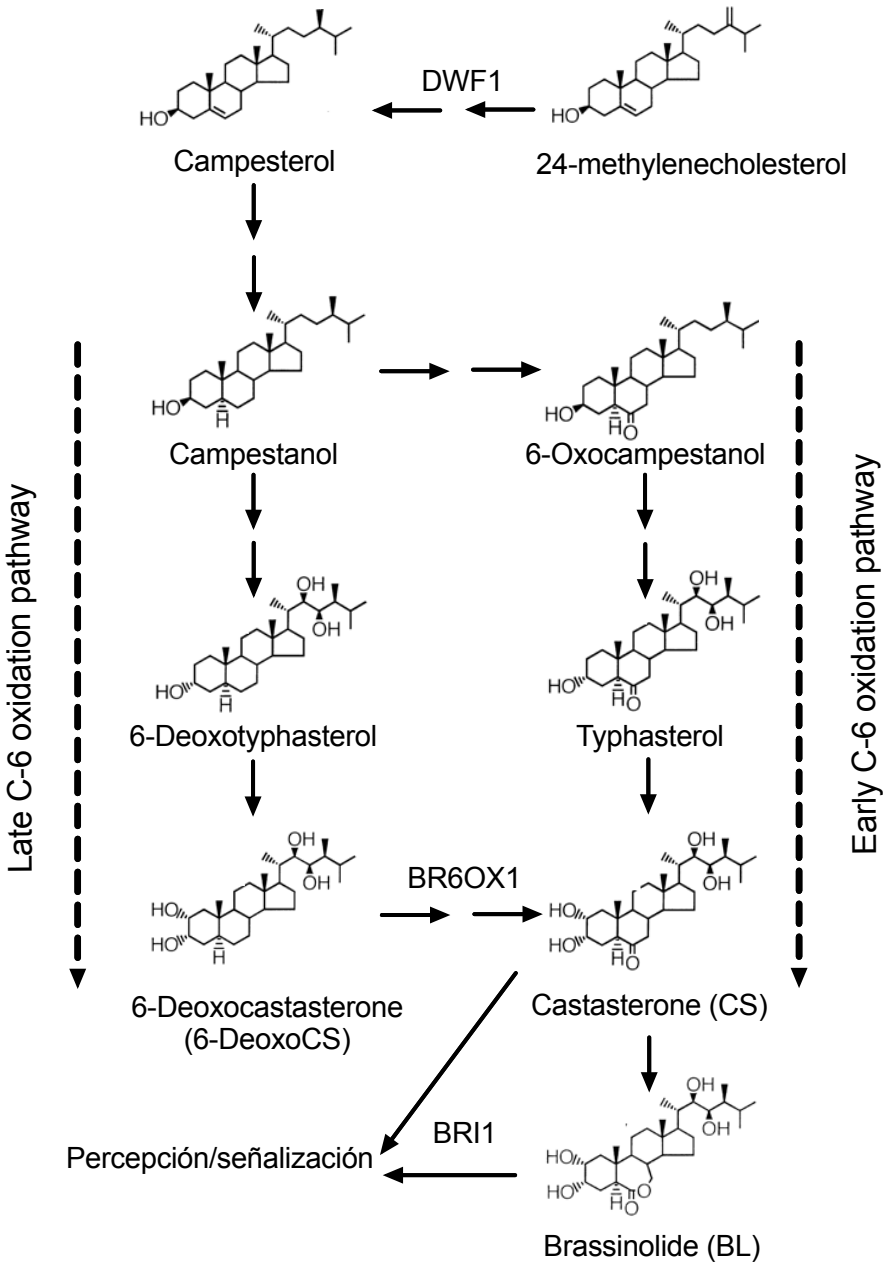

Figure 5. Simplified representation of synthesis pathway of brassinosteroids, where the route of early C- oxidation (right) and late C-6 oxidation (left) is appreciated. In addition, the genes of synthesis, DWF1 and BR6OX1, and of perception, BRI1, are shown. Adapted from Nomura et al. (2001) and Symons et al. (2006).

This role has been partially verified by the exogenous application of 24-epibrasinolide (an analogue of BRs), resulting in an acceleration in color development of berries, while the application of brassinazole (an inhibitor of endogenous synthesis of BRs) delayed the ripening of 'Cabernet Sauvignon' berries (Symons et al., 2006). In other studies, exogenous application of the same analogues increased the accumulation of anthocyanins. This increase is explained by induction of expression of regulatory and structural genes involved in the phenylpropanoid pathway as a consequence of BRs application (Luan et al., 2013). Although the direct mechanism by which BRs control anthocyanin synthesis is unknown, this hormone interacts intimately with ethylene and ABA (Choudhary et al., 2012; Zhang et al., 2009), indicating that this increase is at least partially mediated by these two hormones. Additionally, in 'Cabernet Sauvignon', the application of BRs during veraison increases the total sugar content by inducing the expression and activity of hexose transporters and invertase, to which is added a decrease in acidity of the berries (Xi et al., 2013; Xu et al., 2015). While all these studies were developed in a wine grape variety ('Cabernet Sauvignon'), where the growth potential of berries is lower, Xi et al. (2013) reported a discrete increase in the weight of the berries. While berry growth has been classically associated with gibberellic acid (GA) (Kumar et al., 2014), BRs have been reported as a master regulator of GA synthesis in *Arabidopsis thaliana* (Unterholzner et al., 2015). Additionally, in studies conducted in our laboratory, the applications of GA that are

usually made for berry growth in 'Thompson Seedless' were partially replaced by applications of BRs, suggesting that the increase in berry size is mediated by GA.

4.- Conclusions and perspectives

The ripening of the berries consists of a series of complex and coordinated changes, and although it is subject to exogenous factors, it is the endogenous ones that are responsible for controlling the ripening dynamics. Therefore, hormones such as ABA and ethylene are the main factors promoting the ripening of berries, which has been confirmed in various studies. Additionally, BRs actively participate in the ripening of berries, particularly in the synthesis and/or accumulation of anthocyanins. While the direct mechanisms by which BRs control the synthesis and/or accumulation of anthocyanins or other processes of ripening are unknown, this process is likely mediated by other hormones, such as ethylene, ABA or GA.

Although the effects of exogenous applications of BRs on the ripening of grape berries have only been tested in wine-make varieties, the use of these applications in table grapes production constitutes an opportunity to be addressed in order to generate a new alternative when application programs for growth regulators are designing, so it is necessary to establish the effects of these applications under productive contexts and in varieties for fresh consumption.

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Chapter 2

Applications of a commercial formulation of brassinosteroid increase the content of anthocyanins in red table grape varieties (*Vitis vinifera* L.)

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1.- Introduction

The assessment of table grape quality is traditionally based on sensory attributes such as flavor, aroma, texture and color. Numerous studies together with demographic observations have demonstrated the importance of color as the main quality indicator on which consumer acceptance of fruit is based (Clydesdale, 1993).

Often, minimum quality standards can be obtained by performing good management practices in the vineyard (Cameron 1994; Schrader et al. 1994), but in the case of grape varieties such as 'Redglobe' and 'Crimson Seedless', which have large-sized berries and clusters, these practices can easily lead to overproduction. Under these conditions, berries of red table grape varieties fail to develop suitable red color (Kliewer and Weaver 1971; Schrader et al; 1994, Kliewer and Dokoozlian 2005). In addition, the production of table grapes in Chile is conducted in areas with warm climatic and/or microclimatic conditions. These high temperature conditions reduce the accumulation of anthocyanins (Mori et al., 2005), negatively affecting the development of berry color (Spayd et al. 2002; Mori et al. 2005; Kuhn et al. 2014). So, a series of techniques tending to correct and/or prevent these problems have been implemented, such as reducing the number and dose of gibberellic acid (GA) treatments to promote berry growth in cv. 'Crimson Seedless' as compared with 'Thompson Seedless'. These techniques also include the use of growth regulators such as ethephon and/or abscisic acid (ABA) during veraison, widely used on

varieties such as ‘Redglobe’ and ‘Crimson Seedless’ (Peppi et al., 2007, 2008). However, softening problems have been reported in berries in these two varieties as a consequence of ethephon and ABA applications, especially at high doses. In addition, the results of such treatments are not consistent between growing seasons, and these treatments can lead to the expression of darker colors in berries (Peppi et al., 2006, 2007, 2008; Peppi and Fidelibus, 2008), which renders fruit berries less attractive in their target markets (Clydesdale, 1993). The side effects associated with the use of ethephon and/or ABA can be reduced by the use of lower doses of these compounds; however, those lower doses are also less effective at increasing anthocyanin content and berry color (Peppi et al., 2006, 2007, 2008; Peppi and Fidelibus, 2008).

From this point of view, the alternatives to growth regulators for preventing and/or correcting color development problems appear to be limited. Therefore, new growth regulators must be identified and characterized for inclusion in management programs. In this sense, brassinosteroids (BRs) have been associated with the ripening of berries (Symons et al., 2006), and exogenous application of 24-epibrassinolide (a BR analog) increases the accumulation of phenolic compounds (Luan et al. 2013; Xi et al. 2013) such as anthocyanins, which are secondary metabolites that determine the color of the berries.

BRs represent a group of hormones first isolated from pollen extracts of *Brassica napus* L. (Mitchell et al., 1970). The isolation of brassinolide, the most active of these hormones (Grove et al., 1979), and the identification of its receptor (Wang et al., 2001) made possible the study of this hormone in various species, including grapes. Symons et al. (2006) reported that BRs are involved in the ripening of 'Cabernet Sauvignon' berries. Recent studies show that BRs have an effect on the accumulation of sugar during the ripening of 'Cabernet Sauvignon' (Xu et al., 2015). Exogenous applications of 24-epibrassinolide during veraison effectively increase sugar accumulation, reduce total acidity at harvest and significantly increase total anthocyanin content in 'Cabernet Sauvignon' (Luan et al. 2013; Xi et al. 2013). Although the observed increase in total anthocyanins may be associated with an increase in the color of grape berries, this phenomenon has not been proven in any table grape variety, especially red ones, for which berry color is one of the main quality attributes (Clydesdale, 1993). In addition, all studies related to the effect of BRs on ripening of berries have only used 24-epibrassinolide (an analogue of BRs not susceptible to use in table grape production), limiting the understanding and scope that growth regulators analogous to BRs may have within a productive context. Therefore, the objective of the present work was to evaluate the effects of applications of a commercial formulation of BR analogue on the development of color and final quality of 'Redglobe' and 'Crimson Seedless' berries.

2.- Materials and methods

2.1.- Plant material and experimental conditions

2.1.1.- Redglobe

The experiments were carried out during 2014-2015 growing season in a commercial vineyard located in Colina (Chacabuco Province, Santiago Metropolitan Region Chile; 33°08’15.5"S 70°39’59.4"W). The climate corresponding to the study site is Mediterranean, which consists of a dry season during the summer (from December to February).

Six-year-old self-rooted ‘Redglobe’ grapevines (*Vitis vinifera* L.) of similar vigor, health and fruit load (38 ± 1.1 clusters·plant^{-1}) were used in the study. The vines were spaced 3.5 m within rows and 3.5 m between rows, and the rows were oriented in southwest-northeastern direction. The vines were trained on an overhead trellis (‘parronal español’) with four main arms and were pruned on a spur-cane system; each cane contained six to seven buds. Plants were under commercial management and subjected to a plant growth regulator application program. However, the plants used in the experiments did not receive the application of ethephon and/or ABA, thus avoiding its interference on the development of fruit color.

2.1.2.- Crimson Seedless

The experiments were carried out during 2014-2015 growing season in a commercial vineyard located in Buin (Maipo Province, Santiago Metropolitan Region Chile; 33°46’31.0"S 70°47’54.3"W). The climate corresponding to the study site is Mediterranean, which consists of a dry season during the summer (from December to February).

Ten-year-old self-rooted ‘Crimson Seedless’ grapevines (*Vitis vinifera* L.) of similar vigor, health and fruit load (44 ± 1.1 clusters·plant^{-1}) were used in the study. The vines were spaced 3.5 m within rows and 3.5 m between rows, and the rows were oriented in southwest-northeastern direction. Plants were trained on an overhead trellis (‘parronal español’) with four main arms and were pruned on a spur-cane system; each cane contained six to seven buds. Plants were under commercial management and subjected to a plant growth regulator application program. However, the plants used in the experiments did not receive the application of ethephon and/or ABA, thus avoiding interference on fruit color development.

2.2.- Treatments

A commercial formulation of BRs, (B-2000®; IONA, Chile), with active ingredient (25R)-3β-5α-dihydroxy-spirostan-6-one, at concentration of 0.06 $mg \cdot L^{-1}$ (equivalent to 60 $mg \cdot ha^{-1}$ using 1000 L of $solution \cdot ha^{-1}$, i.e., dose recommended by manufacturer) was applied with a backpack sprayer to clusters during berry softening at the beginning of veraison (January 19, 2015 in 'Redglobe' and January 20, 2015 for 'Crimson Seedless') and 7 days after veraison (dav) until runoff ensuring that all berry surfaces were covered (approximately 1.2 $L \cdot plant^{-1}$). A wetting agent (Break®, BASF, Germany) was added at a rate of 0.2 $mL \cdot L^{-1}$ of solution. Details of the treatments are shown in Table 1, and the chemical structure of BR analog is shown in Figure 1.

Table 1. Details of the treatments applied to. 'Red Globe' and 'Crimson Seedless' grapevines.

Treatment	Concentration (mg·L-1)	Application moment	Application date	
			'Redglobe'	'Crimson Seedless'
B60	0.06	Veraison	January 19	January 20
B60 + B60	0.06 + 0.06	Veraison + 7 dav	January 19 and 26	January 20 and 28
Control*	0.00	Veraison	January 19	January 20

* The control treatment consisted in the application of water with a wetting agent.

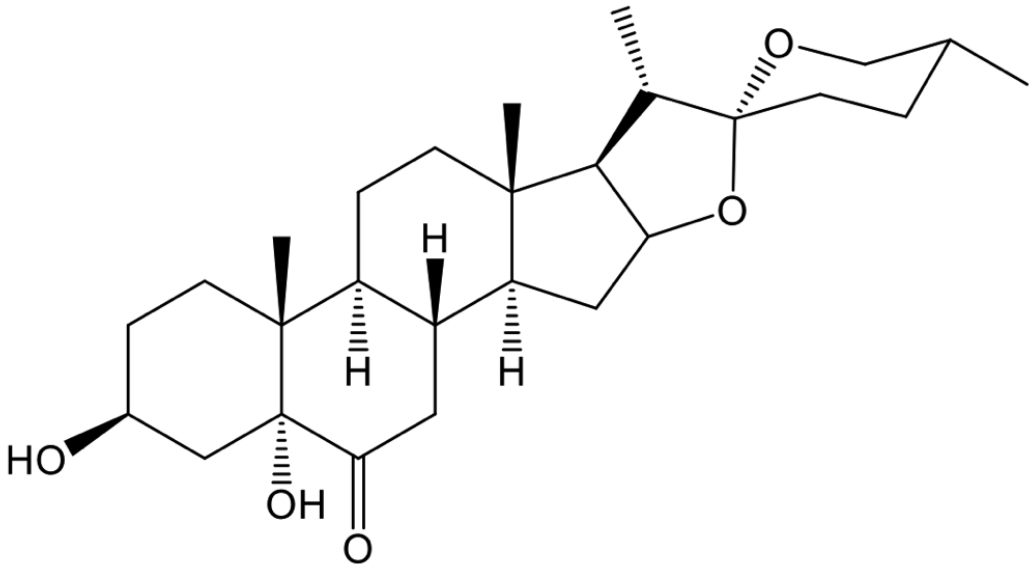

Figure 1. Chemical structure of BR analog (active ingredient) present in the commercial formulation.

2.3.- Preharvest and harvest evaluations

The evolution of berry skin color, equatorial diameter and soluble solids were measured using 30 berries per plant; berries were sampled from at least 10 clusters. Sampling was performed from treatments application until harvest (42 and 49 days after veraison (dav) for 'Redglobe' and 'Crimson Seedless', respectively).

Berry skin color was evaluated using the color index for red grapes (CIRG), which is based on the CIELAB parameters L^* (lightness), H (hue angle) and C (chroma) (Carreño et al., 1995) measured with a Chroma Meter CR-400 (Konica Minolta, Japan) and is calculated as $(180 - H) / (L^*+C)$. The CIRG exhibits strong relation with the visual color of berries and can distinguish between sample groups of different external color (Carreño et al., 1995). CIRG was measured on 8-mm-diameter circles (50.26 mm^2) on two opposite sides of the equatorial zone of berries. For this evaluation, all berries were cleaned with a cotton cloth to remove dust and prevent irregularities in the measurements. Berry diameter at the equatorial zone was measured with a digital caliper. Finally, the juice of 10 berries was used for determining the soluble solids concentration (°Brix), using a temperature-compensated hand refractometer.

At harvest, four clusters per plant were sampled. Fifty berries were collected from each cluster and were used for the evaluation previously described (30 for berry skin color,

diameter and soluble solids). In addition, total acidity (expressed as tartaric acid) and total anthocyanins were evaluated using 10 whole berries pert plant.

Total acidity was measured using the mixed juice of 10 berries; juice was titrated with NaOH (0.1 N) until pH 8.2 was reached using an automatic titrator (HI 901 Tiratatro, Hanna Instrument, USA).

The total concentration ($mg \cdot g^{-1}$ fresh weight (FW)) and total content ($mg \cdot berry^{-1}$) of anthocyanins were evaluated using a spectrophotometric method described by Iland et al. (2004). Briefly, 10 whole berries were ground and macerated with 50% ethanol (v/v) at pH 2.0 for 24 h in darkness at room temperature. Afterward, the samples were centrifuged. The supernatants were recovered, and their absorbance at 520 nm was measured with a UV-visible spectrophotometer (Nanodrop 2000, Thermo Fisher, USA) to determine total anthocyanin concentrations (Iland et al., 2004).

2.4.- Experimental design and statistical analysis

A randomized complete block design was used in both varieties. Treatments were sorted in 6 blocks within 2 rows of plants; this design left one untreated plant as a border between adjacent experimental units (a single plant with all its clusters). Differences among treatment means of preharvest and harvest parameters were evaluated by analysis of variance (ANOVA), and significant differences were

subjected to Tukey-Kramer honestly significant difference (HSD) multiple comparison test ($p \leq 0.05$).

3.- Results

3.1.- Quality parameters

At harvest, in 'Redglobe', no significant differences were observed in equatorial diameter, total acidity, soluble solids content and the weight of berries and clusters (Table 2). In 'Crimson Seedless', significant differences were only found in equatorial diameter, for which treatments B60+B60 and B60 presented higher values than control (Table 3).

3.2.- Color of berries

In both varieties the color of berries (expressed as CIRG) increased from the time of treatments application (veraison) to harvest (Figure 2).

In 'Redglobe', from day 14th after application of the treatment (14 dav) until harvest, the berries treated with BR showed higher CIRG values in respect to the Control.

However, only at 35 dav and harvest significant differences were observed among treatments. At 35 dav, the B60 treatment presented statistically higher CIRG values than the Control treatment, while B60+B60 treatment remained similar to the Control (Figure 2A). On the other hand, at time of harvest, both treatments with BR application showed statistically higher CIRG values than the control treatment, in which the B60 showed the highest CIRG values (Figure 2B). Similarly, 'Crimson Seedless' also presented an increase in CIRG values from application of treatments until harvest. Although sampling times were limited, a stabilization of CIRG values at 7 dav values was observed (Figure 2C). At harvest time, no significant differences were observed among treatments (Figure 2D).

3.3.- Total anthocyanins

Results from statistical analysis of concentration ($mg \cdot g^{-1}$ fresh weight (FW)) and total anthocyanins content ($mg \cdot berry^{-1}$) at harvest (Figure 3 and 4) revealed that anthocyanins in grapes that received BR treatments were significantly greater than in grapes that received the control treatment. In the case of 'Redglobe', B60 treatment presented statistically higher values of concentration and content of anthocyanins than the control treatment, while B60+B60 treatment was not different from the control treatment (Figure 3).

On the other hand, in ‘Crimson Seedless’, it was the B60+B60 treatment that showed statistically higher concentration and content of anthocyanins than the control treatment. Meanwhile, the B60 treatment remained not different from the control treatment (Figure 4).

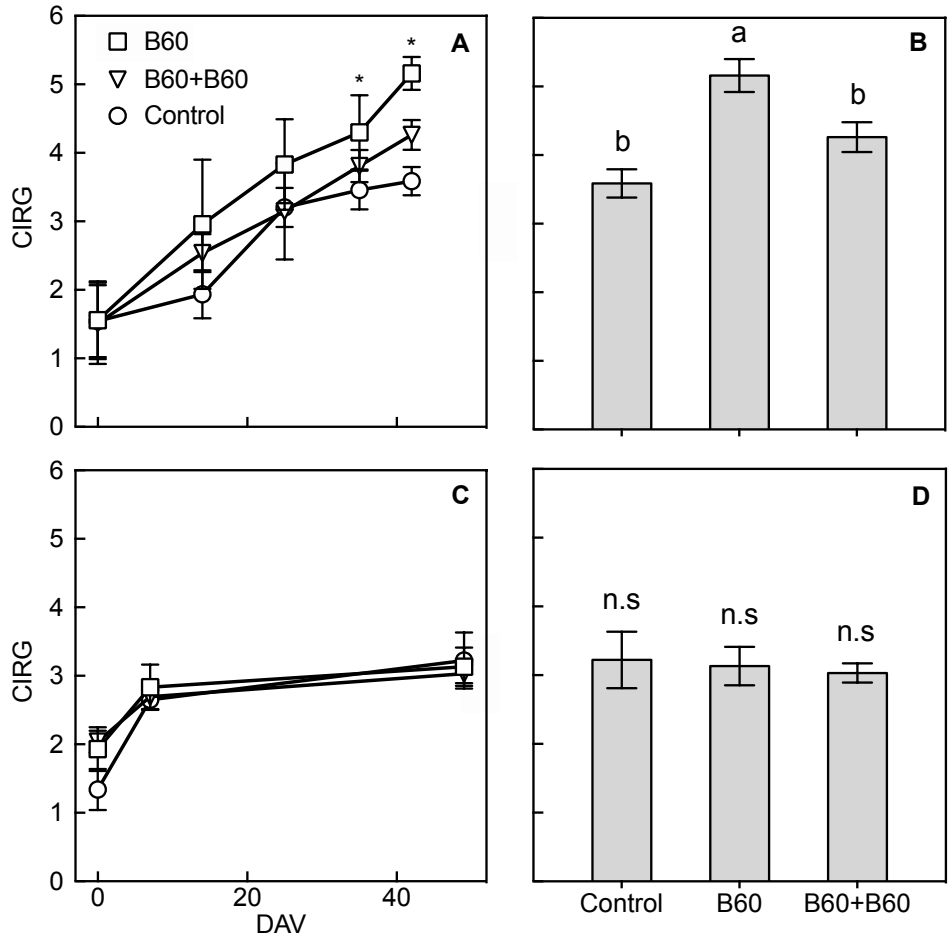

Figure 2. Effect of BR treatments on evolution of CIRG from their application until harvest (A and C) and effect of BR treatments on CIRG at harvest (B and D) in 'Redglobe' (A and B) and 'Crimson Seedless' (C and D). Each point (A and C) or bars (B and D) indicates the mean of six repetitions and error lines indicate ± one standard deviation. Asterisks (A and C) indicate significant differences among treatments during the evolution of the CIRG for at p <0.05 according to the Tukey-Kramer HSD test. Different letters (B and D) indicate significant differences among treatments at p < 0.05 according to the Tukey–Kramer HSD test. n.s = no statistical difference.

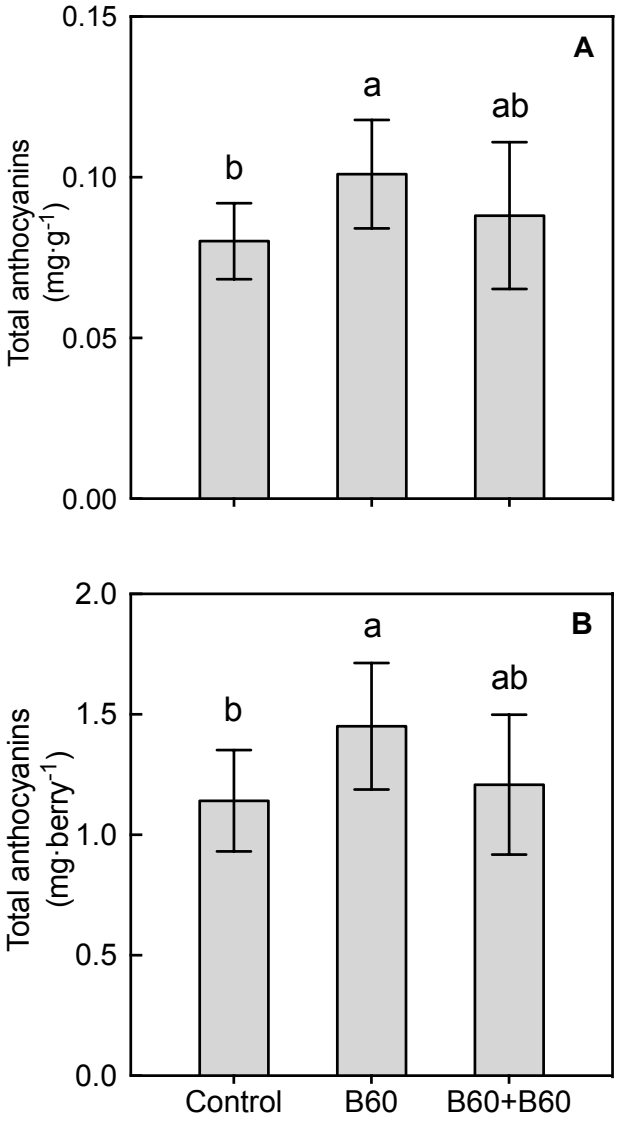

Figure 3. Effect of BR treatments on total anthocyanins content at harvest in 'Redglobe', expressed as milligram per gram of fresh weight (A) or milligram per berry (B). The bars indicate means of six replicates and error lines indicate ± one standard deviation. Different letters indicate significant differences among treatments at $p < 0.05$ according to the Tukey–Kramer HSD test.

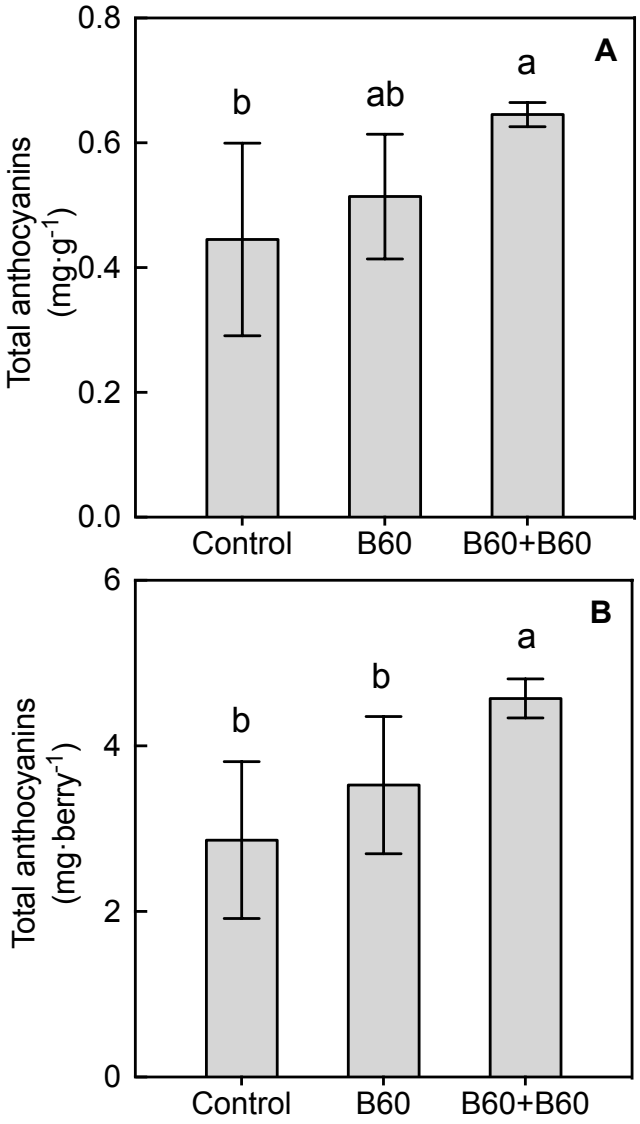

Figure 4. Effect of BR treatments on the total anthocyanin content at harvest in 'Crimson Seedless', expressed as milligram per gram of fresh weight (A) or milligram per berry (B). The bars indicate means of six replicates and error lines indicate ± one standard deviation. Different letters indicate significant differences among treatments at $p < 0.05$ according to the Tukey–Kramer HSD test.

Table 2. Effects of BR treatments on total soluble solids, total acidity, weight of cluster and berries and equatorial diameter of 'Redglobe' berries. Each value indicates the mean of six replicates ± 1 standard deviation. n.s = no statistical difference.

Treatments	Soluble solids (Brix)		Total acidity ($g \cdot L^{-1}$)		Cluster weight (g)		Berry weight (g)		Equatorial diameter (mm)	
B60	18.0 ± 1.20	n.s	3.8 ± 0.25	n.s	990.7 ± 113.06	n.s	14.3 ± 0.63	n.s	27.6 ± 0.38	n.s
B60+B60	18.1 ± 0.72	n.s	3.7 ± 0.35	n.s	1010.9 ± 169.45	n.s	13.8 ± 1.17	n.s	27.0 ± 0.76	n.s
Control	18.1 ± 0.92	n.s	3.8 ± 0.23	n.s	1021.6 ± 140.75	n.s	14.2 ± 1.44	n.s	26.9 ± 0.96	n.s

Table 3. Effects of BR treatments on total soluble solids, total acidity, weight of cluster and berries and polar and equatorial diameter of berries 'Crimson Seedless'. Each value indicates the mean of six replicates ± 1 standard deviation. Different letters indicate significant differences among treatments at $p < 0.05$ according to the Tukey–Kramer HSD test. n.s = no statistical difference.

Treatments	Soluble solids (Brix)		Total acidity ($g \cdot L^{-1}$)		Cluster weight (g)		Berry weight (g)		Polar diameter (mm)		Equatorial diameter (mm)	
B60	16.5 ± 0.42	n.s	7.4 ± 0.39	n.s	725.5 ± 44.31	n.s	6.8 ± 0.61	n.s	27.5 ± 0.55	n.s	18.6 ± 0.16	b
B60+B60	16.3 ± 0.44	n.s	7.8 ± 0.22	n.s	665.0 ± 176.55	n.s	7.0 ± 0.36	n.s	27.9 ± 0.56	n.s	18.9 ± 0.25	a
Control	16.7 ± 0.64	n.s	7.8 ± 0.74	n.s	688.6 ± 54.37	n.s	6.4 ± 0.47	n.s	26.7 ± 1.06	n.s	17.7 ± 0.28	c

4.- Discussion

Exogenous application of growth regulators is an important tool for improving quality parameters of grape berries, including size, organoleptic characteristics, and color, among others (El-Kereamy et al., 2003; Han and Lee, 2004; Jeong et al., 2004; Symons et al., 2006; Fanzone et al., 2010; González et al., 2012; Xi et al., 2013). However, even when BRs are a group of hormones that participate in the ripening of grape berries (Symons et al., 2006; Xu et al., 2015; Vergara et al., 2018), their use as a plant growth regulator in table grape production has not been widely studied.

In the case of 'Redglobe', although no changes occurred in most quality (total acidity, berry size and soluble solids content) and yield parameters (berry and cluster weight) at harvest time (Tables 1), a marked effect of BR treatments on berry color (Figure 2 A and B) and anthocyanin concentration (Figure 3) was observed. While in 'Crimson Seedless', significant increases in equatorial diameter (Table 2), content and concentration of anthocyanins were observed with respect to the control (Figure 4).

Although the first published work associated BRs with cell elongation in stems of bean seedling (Mitchell et al., 1970), most of the published work on grapes do not report similar changes on berry expansion and size; yet, Xi et al. (2013) reported an

increase in the weight of berries as a consequence of 24-epibrassinolide treatments. In our work, the treatments of BR generated a significant increase in equatorial diameter of 'Crimson Seedless' berries. This could be related to the absence of seeds in this variety, which could make the berries more prone to be enlarged by exogenous applications of plant growth regulators, such as BRs, GA and cytokinins (CKs). In addition, normally in 'Crimson Seedless', in order to avoid deficient color development in the berries, GA doses to promote berry growth are lower than in other seedless varieties, such as 'Thompson Seedless'. Regarding BRs, there are no precedents that explain the mechanism by which they control the growth of grape berries. Classically GAs have been associated with the growth of grape berries and other fleshy fruits (Kumar et al., 2014). In fact, in the production of table grapes, GA applications are made during the first phase of growth of the berries to increase their size. With respect to BRs and GA, it has been reported that these hormones participate together in the control of seed germination (Small et al., 2019; Steber and Mccourt, 2001), which would be mediated by the interplay between the signalling pathways of these two hormones (Gallego-bartolomé et al., 2012; Unterholzner et al., 2015). In addition, it is known that BRs generate higher levels of GA_1, by promoting a greater expression of *18D/GA3OX-2*, during cell elongation in rice seedlings (Tong et al., 2014). In this way, it is possible that the increase in berry size induced by the application of BR could be mediated by GA. In fact, previous work carried out by our laboratory have shown that the BR analog used in the present work, would be able to replace, at least partially, GA applications to increase berry size in 'Thomson Seedless' variety (unpublished data). These applications of GA are usually made before veraison, a time at which the endogenous levels of GA are

relatively high. There is also an increase of GA levels during veraison and at the beginning of the second phase of growth (Mcatee et al., 2013; Pérez et al., 2000), during this stage also increases the endogenous levels of BRs, which are maintained throughout the development of the berries (Symons et al., 2006). This indicates that BRs participate in the changes experienced by berries during their maturation, including the increase in size, thus exogenous applications of BR analogs could potentiate this increase. In ‘Red Globe' no similar effect was observed. This could be explained by the presence of seeds in this variety and the commercial applications of GA for berry growth, which would make them close to their potential size, leaving little opportunity for BRs to promote greater berry growth.

On the content of anthocyanins, treatment B60 for ‘Redglobe’ and B60+B60 for ‘Crimson Seedless’, yielded the highest total anthocyanin content and concentration relative to the control values (Figures 3 and 4). These results agree with previous work regarding the role of this hormone in grape berry ripening (Luan et al., 2013; Symons et al., 2006; Xi et al., 2013; Xu et al., 2015), suggesting a potential role for BRs analog growth regulators as a table grape production management tool, especially considering the results obtained regarding berry coloration (Figure 2). This greater accumulation of anthocyanins in berries treated with BR, could be explained by a higher expression of structural genes of the phenylpropanoid pathway, or transcription factors related with it. In this sense, a study published in 2013 pointed out that exogenous applications of 24-epibrasinolide (a non-commercial analog of BRs), in ‘Cabernet Sauvignon’, during veraison, increased the expression of genes

related to the phenylpropanoid pathway, especially flavanone-3-hydroxylase (*F3H*), flavonoid-3'-hydroxylase (*F3'H*), flavonoid-3',5'-hydroxylase (*F3'5'H*) and dihydroflavonol-4-reductase (*DFR*), as well as transcription factors *MYBA1* and *MYBA2* (Luan et al., 2013). Furthermore, in another study published in 2013, a greater enzymatic activity of phenylalanine ammonia-lyase (PAL) and leucoanthocyanidin dioxygenase (LDOX) was observed in the skin of 'Cabernet Sauvignon' and 'Yan 73' berries treated with 24-epibrasinolide (Luan et al., 2013).

Although anthocyanin accumulation is responsible for red coloration in berries, the relationship between them is not linear, as it is shown in Figure 5A (Carreño et al., 1996). However, in the case of 'Redglobe', the increase in anthocyanins was also reflected as increase in the color of berries, expressed as CIRG. In this sense, both treatments with BRs showed statistically higher CIRG values than the control treatment, in which B60 promoted the highest CIRG values (Figure 2B). On the other hand, in 'Crimson Seedless', the increase in anthocyanin content was not reflected in an increase in the color of the berries expressed as CIRG (Figure 2D). As the accumulation of anthocyanins is confined to the skin of berries (Jeong et al., 2004; Zhang et al., 2006), the effect of BRs treatments on berry color can be attenuated by the increase in their skin surface due to increased berry volume. In this way, anthocyanin increment observed in 'Crimson Seedless' was not reflected in berry color partially due to the larger berry size generated by BRs treatments. In addition, and as mentioned above, the relationship between color of berries and content or concentration of anthocyanins is logarithmic. Thus, when anthocyanin contents are

low, the CIRG response rate to anthocyanin changes is very high. While when the anthocyanin contents are high, this response rate is lower (Figure 5B). So, given that the magnitudes associated to CIRG response curve to anthocyanins content may be different for each variety (Carreño et al., 1996, 1997; Fernández-López et al., 1998), if this curve were constructed for 'Crimson Seedless' variety, its anthocyanin values observed in this study would probably be in the low response zone of CRIG to anthocyanins. While the anthocyanin values for 'Redglobe' would be in the high response zone of CIRG to anthocyanins.

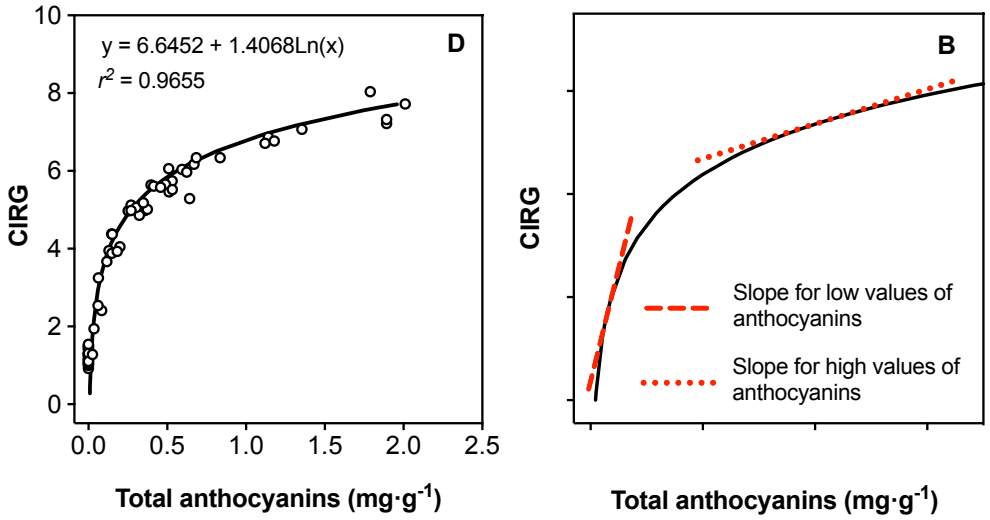

Figure 5. Relationship between the concentration of anthocyanins (expressed in mg per gram of fresh weight) and the color of berries (expressed as CIRG) and regression between both parameters (A). Schematic representation of relationship between anthocyanin content and CIRG, and slopes associated with low (cut line) and high (dotted line) anthocyanin content (B). Adapted from Carreño et al. (1996).

5.- Conclusions

Although there were differences between the doses used, in general terms the BR analog used in this study generated an increase in anthocyanin content in the two varieties of table grapes used. In 'Redglobe', the increase of anthocyanins was able to generate a greater color of the berries, while in 'Crimson Seedless' at least allowed to maintain it, even though there was an increase in the diameter of the berries, which could have generated a decrease in color, just as happens with other growth regulators such as GA or CK. Finally, the BR analog used in this study could be used to increase the size of the berries and/or promote the development of color or at least maintain it, when applied at veraison, time in which other growth regulators (i. e. GA and CK) are not usually used.

6.- Author contributions

AV and AP-D designed the research. AV and MT performed the research. AV, AP-D, MT and JA analysed the data and results. AV wrote the paper

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Chapter 3

Exogenous applications of brassinoesteroids improve the color of red table grapes (*Vitis vinidera* L. Cv. 'Redglobe`) berries

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1.- Introduction

The assessment of table grape quality is traditionally based on sensory attributes such as flavor, aroma, texture and color. Numerous studies together with demographic observations have demonstrated the importance of color as the main quality indicator on which consumer acceptance of fruit is based (Clydesdale, 1993).

Often, minimum quality standards can be obtained by performing good management practices in the vineyard (Cameron 1994; Schrader et al. 1994), but in the case of grape varieties such as 'Redglobe', which has large-sized berries and clusters, these practices can easily lead to overproduction. Under these conditions, 'Redglobe' berries fail to develop suitable red color (Kliewer and Weaver 1971; Schrader et al; 1994, Kliewer and Dokoozlian 2005). In addition, the production of table grapes in Chile is conducted in areas with warm climatic and/or microclimatic conditions. These high temperature conditions inhibit the accumulation of anthocyanins (Mori et al., 2005), negatively affecting the development of berry color (Spayd et al. 2002; Mori et al. 2005; Kuhn et al. 2014). Thus, a series of techniques that tend to correct and/or prevent these problems have been implemented. These techniques include the use of growth regulators such as ethephon and/or abscisic acid (ABA) during veraison; these techniques have been widely used on varieties such as 'Flame Seedless' and 'Redglobe'. However, softening problems have been reported in 'Redglobe' berries as a consequence of ethephon and ABA applications, especially at higher doses. In addition, the results of such treatments are not consistent between growing seasons, and these treatments can lead to the expression of

darker colors in berries, which renders ‘Redglobe’ berries less attractive in their target markets (Peppi et al. 2006; Peppi 2007; Peppi and Fidelibus 2008). The side effects associated with the use of ethephon and/or ABA can be reduced by the use of lower doses of these compounds; however, those lower doses are also less effective at increasing the anthocyanin content (Peppi et al., 2007) and berry color.

From this point of view, the alternatives to growth regulators for preventing and/or correcting color development problems appear to be limited. Therefore, new growth regulators must be identified and characterized for inclusion in management programs. In this sense, brassinosteroids (BRs) are associated with the ripening of berries (Symons et al., 2006), and exogenous application of 24-epibrassinolide (a BR analog) increases the accumulation of phenolic compounds (Luan et al. 2013; Xi et al. 2013) such as anthocyanins, which are secondary metabolites that determine the color of the berries.

BRs represent a group of hormones first isolated from pollen extracts of *Brassica napus* L. (Mitchell et al., 1970). The isolation of brassinolide, the most active of these hormones (Grove et al., 1979), and the identification of its receptor (Wang et al., 2001) made it possible to study this hormone in various species, including grape species. Symons et al. (2006) reported that BRs are involved in the ripening of ‘Cabernet Sauvignon’ berries. Recent studies have shown that BRs are involved in the accumulation of sugar during the ripening of ‘Cabernet Sauvignon’ (Xu et al., 2015). On the other hand, exogenous applications of 24-epibrassinolide during veraison are effective at increasing sugar accumulations, reducing total acidity at

harvest and significantly increasing the total anthocyanin content in ‘Cabernet Sauvignon’ (Luan et al. 2013; Xi et al. 2013). Although the observed increase in total anthocyanins may be associated with an increase in the color of grape berries, this phenomenon has not been proven in any table grape variety, especially red ones, for which berry color is one of the main attributes of quality (Clydesdale, 1993). In addition, all studies related to the ripening of berries and to the effect of BRs on ripening have used only one analog (24-epibrassinolide), limiting the understanding and scope that growth regulators analogous to BRs may have within a productive context. Therefore, the objective of the present work was to evaluate the effects of the exogenous applications of different BR analogs on the development and final quality of ‘Redglobe’ berries.

2.- Materials and methods

2.1.- Plant material and experimental conditions

The experiments were carried out during 2014-2015 and 2015-2016 growing seasons in a commercial vineyard located in Santa María (Aconcagua Province, Valparaíso Region, Chile; 32°43’00.9"S 70°37’56.7"W). The climate corresponding to the study site is Mediterranean, which consists of a dry season during the summer (from December to February).

Sixteen-year-old self-rooted ‘Redglobe’ grapevines (*Vitis vinifera* L.) of similar vigor, health and fruit load (38 ± 1.1 clusters·plant^{-1}) were used in the study. The vines were spaced 3.5 m within rows and 3.5 m between rows, and the rows were oriented in southwest-northeastern direction. The vines were trained on an overhead trellis (parronal español) with four main arms and were pruned on a spur-cane system; each cane contained six to seven buds. In both seasons plants were under commercial management and subjected to plant growth regulator application program showing in Table 1. However, the plants used in the experiments did not receive the application of ethephon, thus avoiding its interference on the development of fruit color.

Table 1. Detail of the application program of plant growth regulators in the commercial field in which the study was conducted.

Date	Objective	Commercial product	Active ingredient (ai)	Dose ($g \cdot ha^{-1}$)
06-10-14	Shoot elongation	Splendor ®	Thidiazuron, 5.0% (p/v)	2.05
11-11-14	Thinning	Proggib ®	Gibberellic acid, 3.2% (p/v)	1.85
22-11-14	Berry growth *	Splendor ®	Thidiazuron, 5.0% (p/v)	0.40
		Proggib ®	Gibberellic acid, 3.2% (p/v)	37.49
27-11-14	Berry growth *	Biozime TF ®	Gibberellic acid, 0.036% (p/v)	0.05
			Indoleacetic acid, 0.036% (p/v)	0.05
			Zeatin, 0.0094% (p/v)	0.14
02-12-14	Berry growth*	Proggib ®	Gibberellic acid, 3.2% (p/v)	37.49
22-01-15	Color uniformity **	Ethrel 48 ®	Ethephon, 48.0% (p/v)	239.92

* Growth regulator applications were applied as a total volume of 1000 $L \cdot ha^{-1}$.

** Not applied to the plants used in this study.

2.2.- Treatments

In the season 2014 – 2015 three analogs of BRs, 24-epibrassinolide (E; Phyto Technology Laboratories®, USA), 3 α-22(*S*), 23-trihydroxy-24-nor-5 α-cholan-6-one (Triol; T), and 3 α-22(*S*), 23-trihydroxy-24-nor-5 α-cholan-6-one (Lactone; L) at concentrations of 0.0 (control), 0.4, and 0.8 $mg \cdot L^{-1}$ as well as a commercial formulation of BRs, B-2000® (B-2000; IONA, Chile), at concentration of 0.06 $mg \cdot L^{-1}$ (equivalent to 60 $mg \cdot ha^{-1}$ using 1000 L of $solution \cdot ha^{-1}$, i.e., dose recommended by manufacturer) were applied with a backpack sprayer to 'Redglobe' clusters during berry softening at the beginning of veraison (January 7, 2015) at a rate of approximately 1.2 $L \cdot plant^{-1}$ until runoff, ensuring that all berry surfaces were covered. The details of the treatments are shown in Table 2, and the chemical structures of the analogs are shown in Figure 1. The side chain of the structure of the analog Lactone is not shown, as it may be patented and/or protected by copyright; hence, the side chain was replaced with an R.

Table 2. Details of the treatments applied in growing season 2014 - 2015 and/or 2015 - 2016.

Treatment	Active ingredient	Concentration ($mg \cdot L^{-1}$)
E-0.4*	24-epibrassinolide	0.4
E-0.8		0.8
T–0.4*	3 α-22(*S*), 23-trihydroxy-24-nor-5 α-cholan-6-one (Triol)	0.4
T–0.8		0.8
L-0.4*	3 α-22(*S*), 23-trihydroxy-24-nor-5 α-cholan-6-one (Lactone)	0.4
L-0.8		0.8
B-2000*	(25R)-3β-5 α-dihydroxy-spirostan-6-one (B-2000)	0.06
Control*	----------	0.0

E-0.4: 24-epibrassinolide applied at 0.4 $mg \cdot L^{-1}$; E-0.8: 24-epibrassinolide applied at 0.8 $mg \cdot L^{-1}$; T-0.4: Triol applied at 0.4 $mg \cdot L^{-1}$; T-0.8: Triol applied at 0.8 $mg \cdot L^{-1}$; L-0.4: Lactone applied at 0.4 $mg \cdot L^{-1}$; L-0.8: Lactone applied at 0.8 $mg \cdot L^{-1}$; B-2000: B-2000 applied at 0.06 $mg \cdot L^{-1}$; Control: control treatment. The treatments were applied to cluster at beginning of veraison at a rate close to 1.2 $L \cdot plant^{-1}$. * Treatments evaluated in a second season (2015 - 2016).

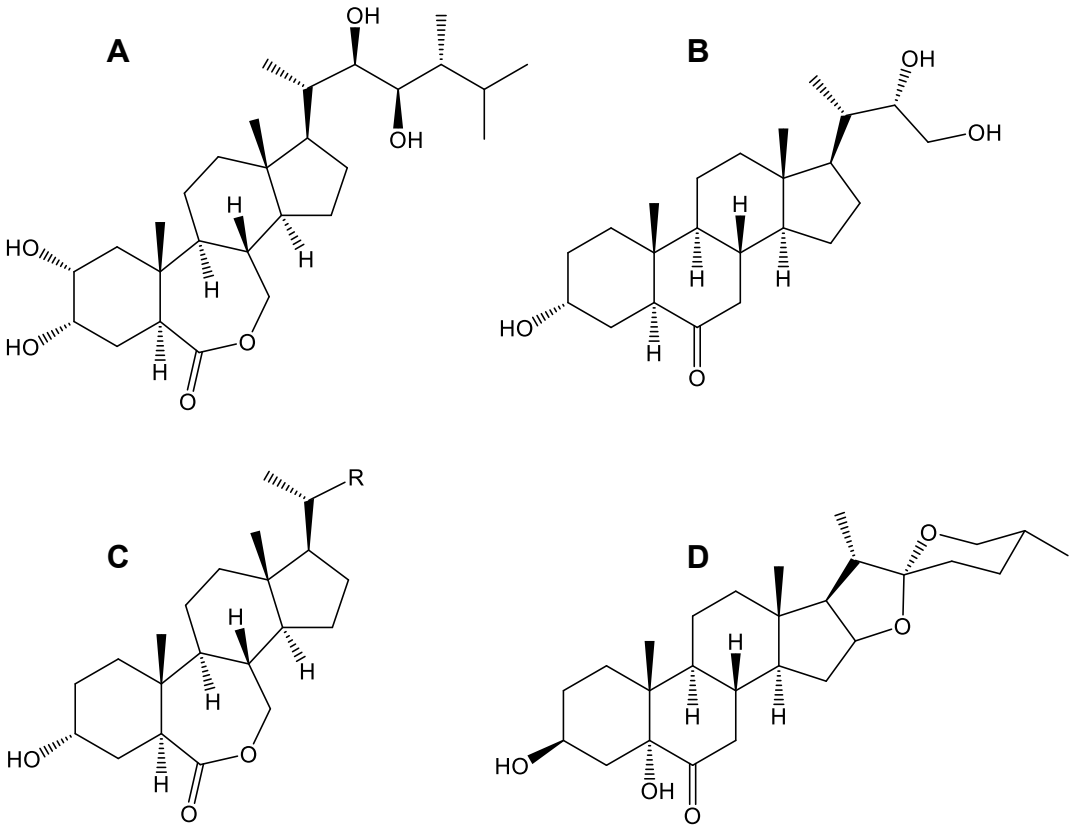

Figure 1. Chemical structure of the different BR analogs used in this study: 24-epibrassinolide (A), 3 α-22(*S*), 23-trihydroxy-24-nor-5 α-cholan-6-one (B, Triol), 3 α-22(*S*), 23-trihydroxy-24-nor-5 α-cholan-6-one (C, Lactone) and 25R)-3β-5 α-dihydroxy-spirostan-6-one (D, commercial formulation, B-2000®).

The solutions of BR analogs were prepared by dissolving 4 or 8 mg in 100 mL of 98% ethanol (v/v) and bringing each solution to a final volume of 10 L with water. The control solution was prepared adding water to 100 mL of 98% ethanol (v/v) until reaching 10 L, and the commercial formulation solution was prepared as specified by the manufacturer. A wetting agent (Break®, BASF, Germany) was added at a rate of 0.2 $mL \cdot L^{-1}$ to all solutions.

Additionally, and using the same methodology described above, at the beginning of veraison of 2015 – 2016 growing season (January 28, 2016), the same analogues were tested, but only at 0.4 $mg \cdot L^{-1}$. The commercial formulation at 0.06 $mg \cdot L^{-1}$ was also tested.

2.3.- Preharvest and harvest evaluations

The evolution of berry skin color, equatorial diameter and soluble solids were measured using 30 berries per plant; berries were sampled from at least 10 clusters. Sampling was performed at 20-day intervals after treatment application until harvest (57 and 61 days after veraison (dav) for 2014 - 2015 and 2015 - 2016 seasons respectively).

Berry skin color was evaluated using the color index for red grapes (CIRG), which is based on the CIELAB parameters L^* (lightness), H (hue angle) and C (chroma) (Carreño et al. 1995) measured with a Chroma Meter CR-400 (Konica Minolta, Japan) and is calculated as $(180 - H) / (L^*+C)$. The CIRG exhibits strong linearity with the visual color of berries and can distinguish between sample groups of different external color (Carreño et al., 1995). The CIRG was measured on 8-mm-diameter circles (50.26 mm^2) on two opposite sides of the equatorial zone of berries. For this evaluation, all berries were cleaned with a cotton cloth to remove dust and prevent irregularities in the measurements. Berry diameter at the equatorial zone was measured with a digital caliper. Finally, the juice of 10 berries was used for determining the soluble solids concentration (°Brix), using a temperature-compensated hand refractometer.

At harvest, four clusters per plant were sampled. Fifty berries were collected from each cluster and were used for the evaluation previously described (30 for berry skin color, diameter and soluble solids); in addition, total acidity (expressed as tartaric acid), total anthocyanins and the profiles of anthocyanin groups were evaluated using 10 whole berries pert plant.

Total acidity was measured using the mixed juice of 10 berries; juice was titrated with NaOH (0.1 N) until pH 8.2 was reached using an automatic titrator (HI 901 Tiratatro, Hanna Instrument, USA).

The total concentration ($mg \cdot g^{-1}$ fresh weight (FW)) and total content ($mg \cdot berry^{-1}$) of anthocyanins were evaluated using a spectrophotometric method described by Iland et al. (2004). Briefly, 10 whole berries were ground and macerated with 50% ethanol (v/v) at pH 2.0 for 24 h in darkness at room temperature. Afterward, the samples were centrifuged. The supernatants were recovered, and their absorbance at 520 nm was measured with a UV-visible spectrophotometer (Nanodrop 2000, Thermo Fisher, USA) to determine total anthocyanin concentrations (Iland et al., 2004).

To determine the profiles of the different groups of anthocyanins, the extracts obtained in the previous step were subjected to HPLC analysis in accordance with the methodology described by González et al. (2012). HPLC-diode-array detection (DAD) analysis was performed using 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, 4.6 mm i.d., 5 µm) at 25°C; the detection was carried out at 520 nm. The elution gradient consisted of two solvents: solvent A consisted of 90:10 water:formic acid (v/v), and solvent B consisted of acetonitrile (Fanzone et al., 2010). Aliquots of 200 µL of grape extract were injected after being filtered through a 0.45-µm-pore size membrane. To determine the groups of anthocyanins (dihydroxylated (2-OH), trihydroxylated (3-OH), methylated (Met) and non-methylated (Non-met) groups), standard solutions of delphinidin-3-glucoside, petunidin-3-glucoside, malvidin-3-glucoside, cyanidin-3-glucoside and peonidin-3-glucoside (Extrasynthese, France) were used as standards.

2.4.- Experimental design and statistical analysis

A randomized complete block design was used for both growing seasons. Treatments were sorted in 5 blocks within 2 rows of plants; this design left one untreated plant as a border between adjacent experimental units (a single plant with all its clusters). Differences among treatment means of preharvest and harvest parameters were evaluated by the analysis of variance (ANOVA), and significant differences were subjected to the Tukey-Kramer honestly significant difference (HSD) multiple comparison test ($p \leq 0.05$). In addition, correlation analysis were performed to determine the correlation strength between the different evaluated parameters

3.- Results

3.1.- Quality parameters

At harvest, in both seasons, no significant differences were observed in equatorial diameter, total acidity, or weight of the berries or clusters (Supplementary table 1 and 2). However, significant differences were found in the soluble solids content of berries (Figure 2A and 2B). On average, compared with the control treatment, BR applications led to higher soluble solids content in berries. In the season 2014 - 2015

the soluble solids content of berries that received treatments E-0.4, B-2000, T-0.8, E-0.8 and T-0.4 were significantly different from that of the control berries, whereas treatments L-0.4 and L-0.8 did not significantly differ respect to control treatment in this parameter (Figure 2A). These results were consistent with those observed in the 2015 - 2016 season, in which the treatments E-0.4 and B-2000 presented values statistically higher than those of control treatment.

3.2.- Color of berries

In both seasons an increase in the values of CIRG was observed from the moment of the application of the treatments until the harvest (data not shown). However, significant differences in CIRG values were observed only at harvest (Figure 3A - 3C).

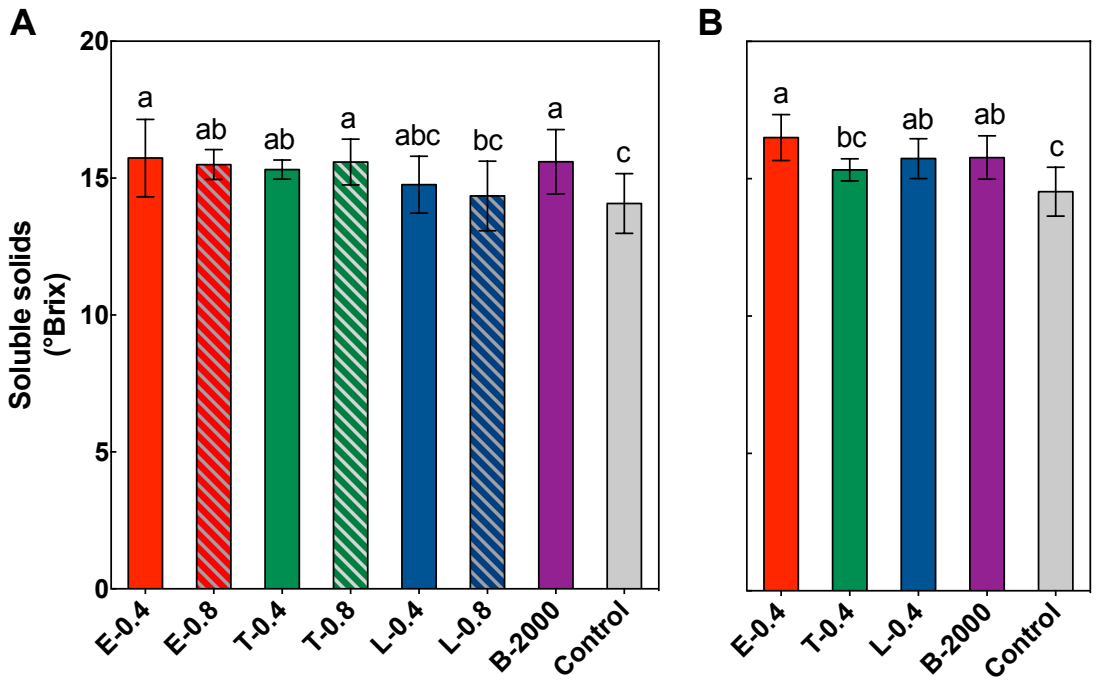

Figure 2. Soluble solids content, expressed as °Brix, at harvest for the growing seasons 2014 - 2015 (A) and 2015 - 2016 (B). Each bar indicates the mean of five replicates and error lines correspond to standard deviations. Different letters indicate significant differences at $p < 0.05$ according to the Tukey-Kramer HSD test (n=5).

In the season 2014 - 2015, ANOVA results and subsequent multiple comparison analyses showed that treatments E-0.4, T-0.4, T-0.8 and B-2000 resulted in significantly higher CIRG values than the control treatment, whereas the remaining treatments resulted in CIRG values that were similar to those of the control treatment (Figure 3 A). However, analysis of covariance indicated a significant effect of soluble solids content on CIRG value. Nevertheless, and considering the effect of soluble solids content, treatments E-0.4, T-0.8 and L-0.8 still had a statistically significant effect on berry color (Figure 3 B). On the other hand, the interaction between treatment and soluble solids content with respect to the CIRG was not significant, indicating that effects of treatments on CIRG value did not vary with the level of soluble solids. Although in the 2015 - 2016 season no significant effect of soluble solids content was observed on the CIRG values, a significant increase in the values of CIRG was observed in treatments E-0.4, T-0.4 and B- 2000 with respect to those of control treatment (Figure 3C).

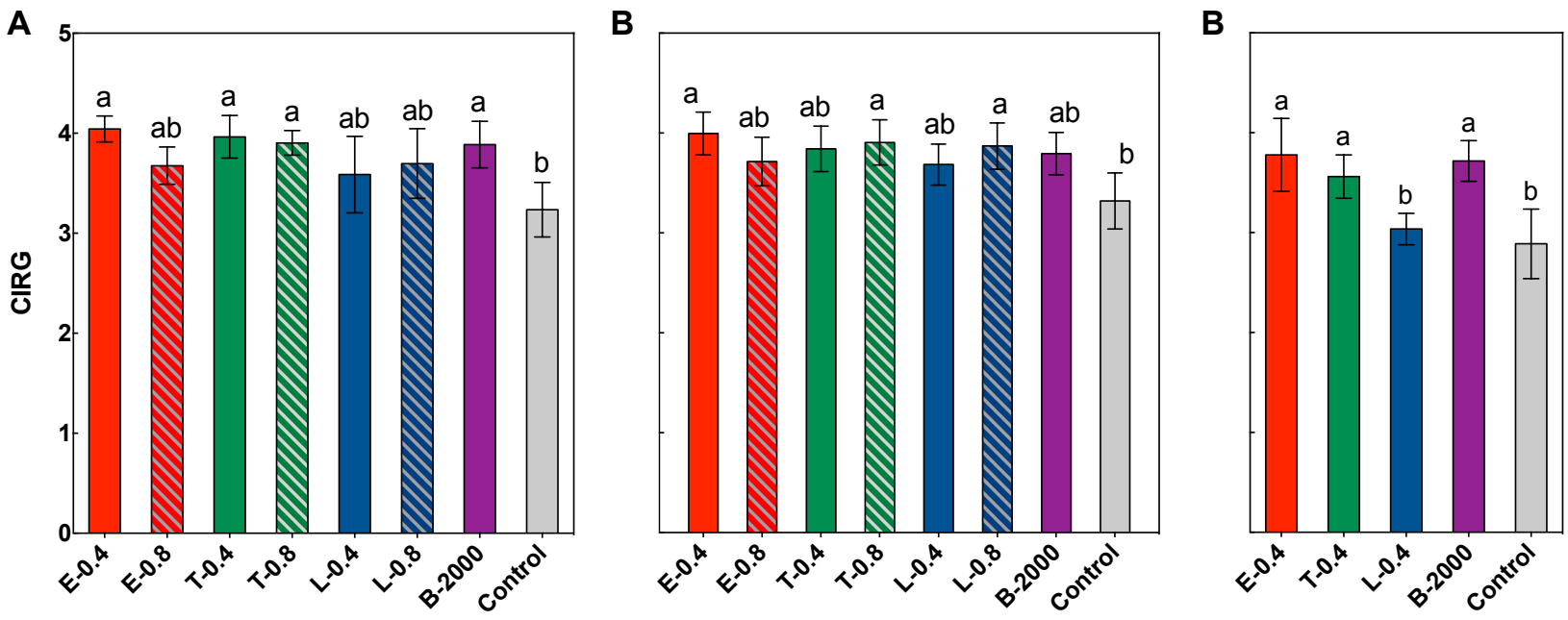

Figure 3. Effect of BRs treatments on CIRG at harvest for the growing seasons 2014 - 2015 (A) and BRs effect in growing season 2014 - 2015 considering the soluble solids effect (B BRs effect on CIRG at harvest in growing season 2015 - 2016 (C). Each bar indicates the mean of five replicates and error lines correspond to standard deviations. Different letters indicate significant differences at $p < 0.05$ according to the Tukey-Kramer HSD test (n=5).

3.3.- Total anthocyanins and anthocyanin groups

The results of the statistical analysis of total concentration and content of anthocyanins at harvest revealed that in season 2014 - 2015 the anthocyanin in grapes that received treatments B-2000, E-0.4, T-0.4 and L-0.8 were significantly higher than those in grapes that received the control treatment; although the other treatments (E-0.8, T-0.8 and L-0.4) resulted in average values that were higher than those of the control, these differences were not statistically significant (Figure 4A and AC). Despite the fact that in 2015 - 2016 season the concentration and content of anthocyanins were lower than in the 2014 - 2015 season (Figure 4), a significant increase in the concentraction and anthocyanin content was observed in the treatments E-0.4, T-0.4 and B-2000 with respect to the control treatment (Figure 4B and 4D), which is consistent with the results of the previous season.

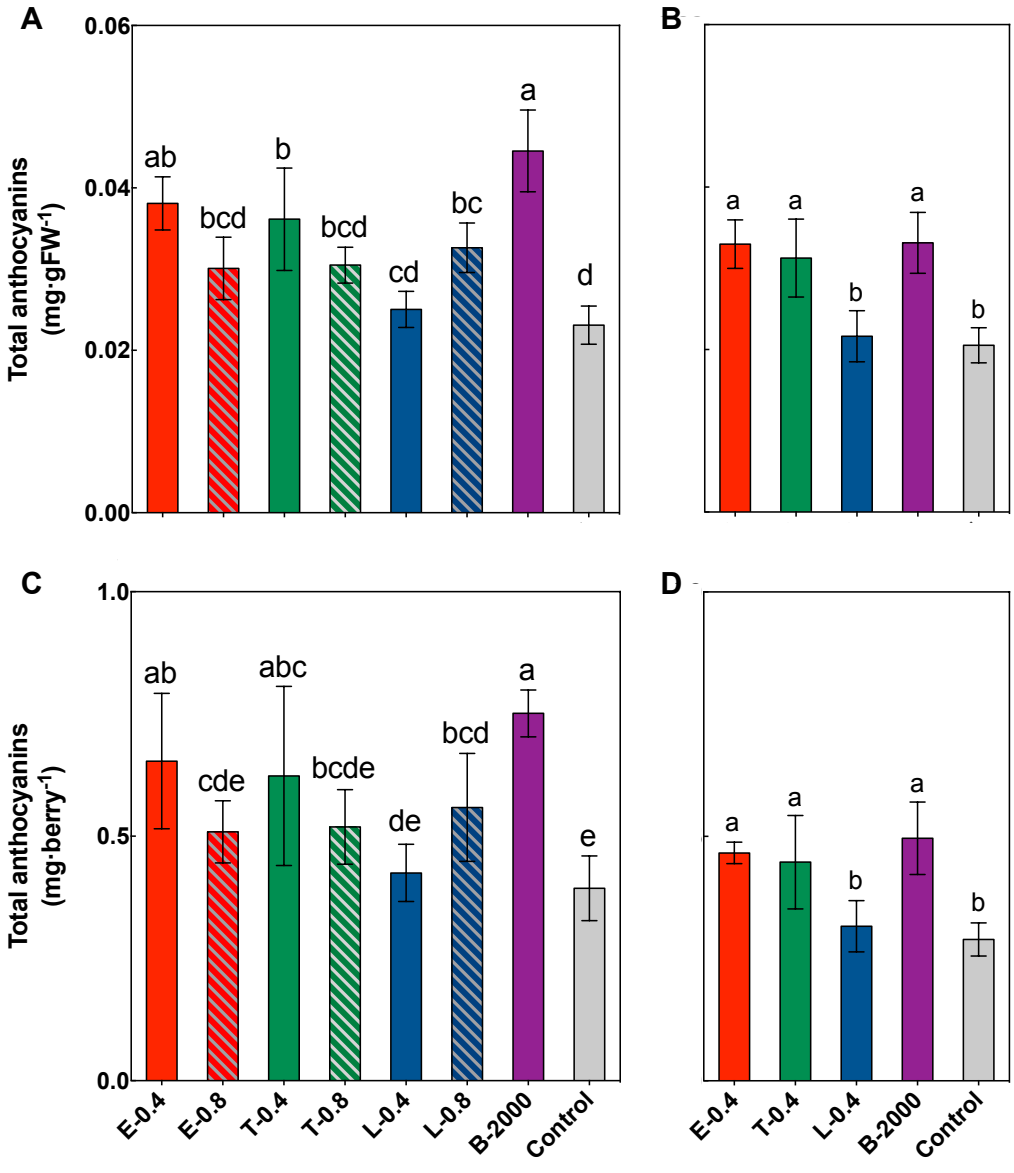

Figure 4. Effect of BR treatments on the total anthocyanin content at harvest, expressed as milligram per gram of fresh weight (A and B) or milligram per berry (C and D), for the growing seasons 2014 - 2015 (A and C) and 2015 - 2016 (B and D). The bars indicate means of five replicates and error lines show their standard deviation. Different letters indicate significant differences at $p < 0.05$ according to the Tukey-Kramer HSD test (n=5).

The observed changes in total anthocyanin concentration and content were also reflected in the concentration and content of specific groups of anthocyanins (Table 3 and 4). The predominant anthocyanins in all of the samples of both seasons were 2-OH anthocyanins (cyanidin-3-glucoside and peonidin-3-glucoside) rather than 3-OH anthocyanins (delphinidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside). In the case of 2014 - 2015 season, ANOVA results revealed that all treatments yielded 2-OH anthocyanins levels that were significantly higher than those of 3-OH anthocyanins, whereas no significant difference in the levels of 2-OH and 3-OH anthocyanins was observed in the control berries (Table 3). Compared with the control treatment, treatments B-2000, E-0.4, T-0.4, L-0.8 and T-0.8 resulted in a higher content of 2-OH anthocyanins in berries, whereas the level of 2-OH anthocyanins in berries that received the L-0.4 treatment was the same as in berries that received the control treatment. With respect to 3-OH anthocyanins, only treatments T-0.4, B-2000 and T-0.8 resulted in levels that were significantly higher than those from the control treatment (Table 3). On the other hand, in the 2015 - 2016 season, treatments E-0.4, T-0.4 and B-2000 exhibited statistically higher values of 2-OH anthocyanins than the control treatment; whereas L-0.4 treatment remained not different from the control treatment (Table 4

Table 3. Effects of BR treatments on the abundance of dihydroxylated, trihydroxylated, methylated, and non-methylated anthocyanins as well as the dihydroxylated:trihydroxylated anthocyanin and the methylated:non-methylated anthocyanin ratios for season 2014 - 2015.

Treatments	Anthocyanins (mg·berry^{-1})								2-OH·3-OH^{-1}		Met·Non-met^{-1}	
	2-OH		3-OH		Met		Non-met					
E-0.4	0.439 ± 0.1209*	ab	0.214 ± 0.0296	abc	0.451 ± 0.1037*	ab	0.203 ± 0.0359	b	2.052 ± 0.4687	a	2.205 ± 0.1677	a
E-0.8	0.299 ± 0.0547*	cde	0.210 ± 0.0204	abc	0.319 ± 0.0396*	cde	0.190 ± 0.0284	b	1.426 ± 0.2572	b	1.698 ± 0.1799	c
T-0.4	0.378 ± 0.0933*	bc	0.245 ± 0.0948	ab	0.416 ± 0.1101*	abc	0.208 ± 0.0761	b	1.597 ± 0.2381	ab	2.083 ± 0.3377	ab
T-0.8	0.292 ± 0.0456*	cde	0.227 ± 0.0337	ab	0.310 ± 0.0377*	de	0.209 ± 0.0392	ab	1.294 ± 0.1259	b	1.496 ± 0.1143	c
L-0.4	0.243 ± 0.0475*	de	0.182 ± 0.0148	bc	0.267 ± 0.0474*	de	0.159 ± 0.0143	bc	1.331 ± 0.1940	b	1.679 ± 0.2036	c
L-0.8	0.333 ± 0.0702*	bcd	0.226 ± 0.0410	ab	0.362 ± 0.0656*	bcd	0.197 ± 0.0450	b	1.471 ± 0.0913	ab	1.861 ± 0.0949	abc
B-2000	0.507 ± 0.0413*	a	0.243 ± 0.0218	a	0.481 ± 0.0379*	a	0.270 ± 0.0123	a	2.097 ± 0.2376	a	1.783 ± 0.0933	bc
Control	0.215 ± 0.0381	e	0.179 ± 0.0346	c	0.253 ± 0.0433*	e	0.140 ± 0.0258	c	1.217 ± 0.1963	b	1.815 ± 0.1813	bc

2-OH: dihydroxylated anthocyanins; 3-OH: trihydroxylated anthocyanins; Met: methylated anthocyanins; Non-met: non-methylated anthocyanins. Each value represents the mean of five replicates. Different letters indicate significant differences among treatments for the same type of anthocyanins at $p < 0.05$ according to the Tukey-Kramer HSD test (n=5). Asterisks indicate significant differences within the same treatment for different groups of anthocyanins (2-OH vs. 3-OH or Met vs. Non-met) at $p < 0.05$ according to the Tukey-Kramer HSD test (n=5).

Table 4. Effects of BR treatments on the abundance of dihydroxylated, trihydroxylated, methylated, and non-methylated anthocyanins as well as the dihydroxylated:trihydroxylated anthocyanin and the methylated:non-methylated anthocyanin ratios for season 2015 - 2016.

Treatments	Anthocyanins (mg·berry^{-1})								2-OH·3-OH^{-1}		Met·Non-met^{-1}	
	2-OH		3-OH		Met		Non-met					
E-0.4	0.280 ± 0.0250*	a	0.101 ± 0.0120	abc	0.286 ± 0.0268*	ab	0.095 ± 0.0208	a	2.829 ± 0.5982	a	3.169 ± 0.8671	ab
T-0.4	0.236 ± 0.0450*	a	0.112 ± 0.0208	ab	0.257 ± 0.0306*	b	0.091 ± 0.0333	a	2.139 ± 0.3646	ab	3.099 ± 0.9255	ab
L-0.4	0.145 ± 0.0275*	b	0.090 ± 0.0056	bc	0.168 ± 0.0202*	c	0.067 ± 0.0132	a	1.616 ± 0.2933	b	2.570 ± 0.3718	b
B-2000	0.293 ± 0.0578*	a	0.121 ± 0.0122	a	0.325 ± 0.0442*	a	0.088 ± 0.0240	a	2.413 ± 0.3481	a	3.802 ± 0.6181	a
Control	0.131 ± 0.0340*	b	0.084 ± 0.0145	c	0.154 ± 0.0265*	c	0.065 ± 0.0113	a	1.631 ± 0.4067	b	2.440 ± 0.6471	b

2-OH: dihydroxylated anthocyanins; 3-OH: trihydroxylated anthocyanins; Met: methylated anthocyanins; Non-met: non-methylated anthocyanins. Each value represents the mean of five replicates. Different letters indicate significant differences among treatments for the same type of anthocyanins at $p < 0.05$ according to the Tukey-Kramer HSD test (n=5). Asterisks indicate significant differences within the same treatment for different groups of anthocyanins (2-OH vs. 3-OH or Met vs. Non-met) at $p < 0.05$ according to the Tukey-Kramer HSD test (n=5).

In both seasons, the analysis of the 2-OH:3-OH anthocyanin ratio (2-OH·3-OH^{-1}) indicated that only treatments B-2000 and E-0.4 yielded a ratio that was significantly greater than that found in control berries (Table 3 and 4).

Compared with the control treatment, in 2014 - 2015 season, treatments B-2000, E-0.4, T-0.4 and L-0.8 resulted in the highest levels of Met anthocyanins (peonidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside); the levels resulting from the remaining treatments did not significantly differ from those from the control treatment (Table 3). These results were consistent with those observed in the 2015 - 2016 season (Table 4).

In the case of Non-met anthocyanins (cyanidin-3-glucoside and delphinidin-3-glucoside), in season 2014 - 2015, all treatments, with the exception of L-0.4, produced significantly higher values than the control treatment (Table 3). In relation to the ratio between these two groups of anthocyanins, only E-0.4 significantly increased compared to the control treatment, while the rest of treatments remained not different from the control treatment (Table 3). While in 2015-2016 season, no significant differences were observed in the content of Non-met anthocyanins, and only the B-2000 treatment presented ratio values of these two groups statistically higher than the control (Table 4). Correlation analyzes revealed that berry color (expressed as CIRG) observed in this work (Figure 5), are explained by the content and concentration (Table 5) of total anthocyanins. On the other hand, the correlation

between the content and concentration of the different groups of anthocyanins and CIRG turned out to be significant, especially for the 2-OH anthocyanins expressed as mg·berry^{-1} (Table 5), while the correlation between CIRG and anthocyanins 3-OH was significant only when these were expressed as mg·g^{-1} (Table 5).

Table 5. Details of correlation between the color of the berries (expressed as the CIRG) and the different groups of anthocyanins and the total anthocyanins, expressed as mg·berry^{-1} or mg·g FW^{-1}.

Anthocyanins	mg·berry^{-1}		mg·g FW^{-1}	
	p	R	*p*	R
2-OH	0.0062*	0.4252	0.0002*	0.5555
3-OH	0.3009	0.1676	0.0079*	0.4138
Met	0.0151*	0.3814	0.0002*	0.5533
Non-met	0.0317*	0.3401	0.0007*	0.5131
Total anthocyanins	0.0153*	0.3804	0.0001*	0.5659

2-OH: dihydroxylated anthocyanins; 3-OH: trihydroxylated anthocyanins; Met: methylated anthocyanins; Non-met: non-methylated anthocyanins. The asterisks denote *p*-values that indicate statistical significance (*n*=40).

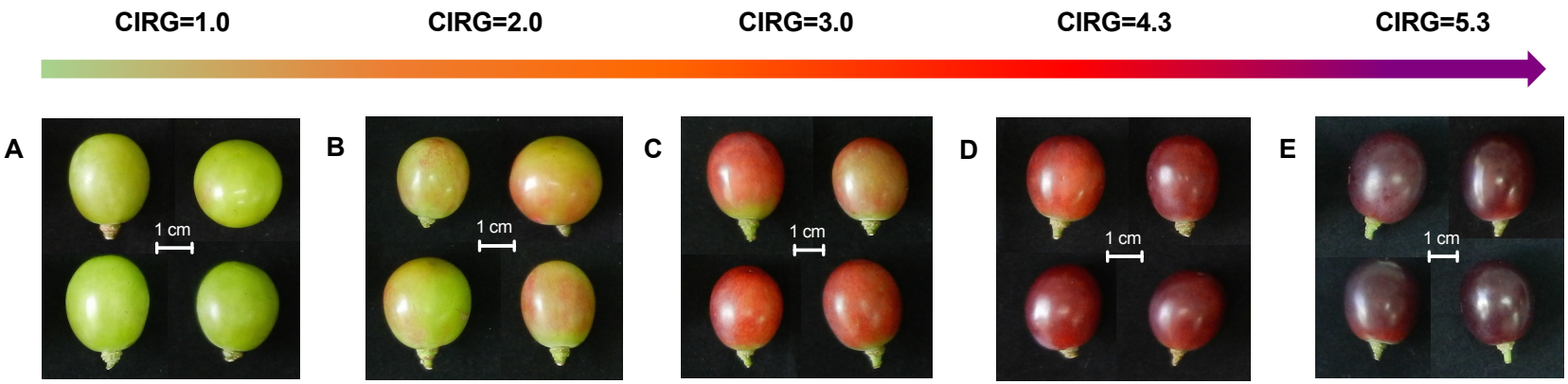

Figure 5. Visual appearance of berries with different average CIRG values, from 1.0 (A) to 5.3 (E). In each image, the average CIRG value from the four berries shown is indicated.

4.- Discussion

Exogenous application of growth regulators is an important tool for improving the quality parameters of grape berries, including size, organoleptic characteristics, and color, among others (El-Kereamy et al. 2003; Han and Lee 2004; Jeong et al. 2004; Symons et al. 2006; Fanzone et al. 2010; González et al. 2012; Xi et al. 2013). BRs compose a class of hormones involved in regulating berry ripening (Symons et al., 2006). However, the effects of exogenous applications of this type of growth regulator to table grapes have never been described. In fact, most studies on BRs as growth regulators have been performed on grapes for wine-make and involve the testing of only one BR analog, 24-epibrassinolide (Symons et al. 2006; Luan et al. 2013; Xi et al. 2013; Xu et al. 2015), thus limiting the understanding of the scope of this potential tool in a productive context in the case of table grapes.

Exogenous application of growth regulators is an important tool for improving the quality parameters of grape berries, including size, organoleptic characteristics, and color, among others (El-Kereamy et al. 2003; Han and Lee 2004; Jeong et al. 2004; Symons et al. 2006; Fanzone et al. 2010; González et al. 2012; Xi et al. 2013). BRs compose a class of hormones involved in regulating berry ripening (Symons et al., 2006). However, the effects of exogenous applications of this type of growth regulator to table grapes have never been described. In fact, most studies on BRs as growth regulators have been performed on grapes for wine-make and involve the

testing of only one BR analog, 24-epibrassinolide (Symons et al. 2006; Luan et al. 2013; Xi et al. 2013; Xu et al. 2015), thus limiting the understanding of the scope of this potential tool in a productive context in the case of table grapes.

BRs affect the accumulation of phenolic compounds, including anthocyanins (Symons et al. 2006; Xi et al. 2013); however, the exogenous application of BR analogs has not been previously demonstrated to be associated with the color of berries or with their evolution from veraison to harvest. In our study, compared with the control treatment, treatments E-0.4, T-0.4, T-0.8 and B-2000 effectively increased CIRG at harvest (Figure 3A). However, as noted above in the results, in season 2014 - 2015, analysis of covariance revealed a significant effect of soluble solids on CIRG values. Despite these, when considering the effect of soluble solids content on CIRG, treatments E-0.4, T-0.8 and L-0.8 showed a significant direct effect in increasing CIRG, while the remains of the treatments did not show significant differences respect to control (Figure 3B). If the concentration and content of anthocyanins (Figure 4) are considered, the increase in color produced by BRs may occur on at least two levels: one level involves directly a higher levels of anthocyanins (Figure 4), and another level involving the effect of the sugar content (expressed as °Brix) on color (Figure 2A). In addition, the effects of BR applications on CIRG appear to depend on the analog and concentration used. For example, treatment T-0.4 (application of the Triol at 0.4 $mg \cdot L^{-1}$) appears to act by increasing the content of soluble solids, whereas treatment L-0.8 (application of the Lactone at 0.8 $mg \cdot L^{-1}$) appears to act by increasing the concentration of anthocyanins.

Treatment E-0.4 (application of 24-epibrassinolide at 0.4 $mg \cdot L^{-1}$) and treatment T-0.8 (application of the Triol analog at 0.8 $mg \cdot L^{-1}$) appear to act on both levels. Although a significant effect of soluble solids content on CIRG values was not observed in the 2015-2016 season, the results for treatments E-0.4, T-0.4 and B-2000 (Figure 3B) are consistent with the observed in the previous season (Figure 3A), since CIRG values were also statistically higher than those of control treatment.

Aside from the effect of the soluble solids content on the color of the berries or growing season, berries of clusters that received BR treatments, exhibited average CIRG values close to 3.9; according to Carreño et al. (1996), these values correspond to colors near red and pink (Figure 5), which are very attractive colors for 'Redglobe' in the target markets.

Treatments E-0.4, T-0.4, L-0.8 and B-2000 and E-0.4, T-0.4 and B-2000 for seasons 2014 - 2015 and 2015 - 2016 respectively, yielded the highest total anthocyanin content and concentration relative to the control values (Figure 4). These results are in agreement with those of Symons et al. (2006), Luan et al. (2013) and Xi et al. (2013) regarding the role of this hormone in grape berry ripening, and they suggest a potential role for BR analog growth regulators as a table grape production management tool, especially considering the results obtained regarding berry coloration (Figure 3 and 5). In addition, despite that ANOVA results indicate only significant effects of the treatments on content and concentration of anthocyanins, a

significant relationship was observed between soluble solids content and anthocyanins (data not shown). This type of effect has been described before, in that a higher sugar content is associated with greater synthesis and accumulation of anthocyanins (Hiratsuka et al., 2001).

The analysis of the effects of different concentrations of BR analogs (with the exception of the commercial formulation) revealed differences in CIRG values and total anthocyanin contents of berries. For example, when treatment E-0.4 (0.4 $mg \cdot L^{-1}$) was applied, a significant increase in CIRG values was observed relative to those of treatment E-0.8 (0.8 $mg \cdot L^{-1}$), which produced similar CIRG values to those of the control treatment (Figures 3 and 4). A similar behavior was observed in the case of the Triol analog, especially with respect to the effect on anthocyanin content: a concentration of 0.4 $mg \cdot L^{-1}$ (T-0.4) generated values that were significantly higher than in the control, whereas a concentration of 0.8 $mg \cdot L^{-1}$ (T-0.8) resulted in anthocyanin contents that were similar to those of the control. This type of dose-response relationship was already described by Luan et al. (2013) and Xi et al. (2013), who reported that concentrations of 24-epibrassinolide greater than 0.4 $mg \cdot L^{-1}$ produced the same levels of anthocyanins, sugars and acidity in 'Cabernet Sauvignon' plants as those found in untreated plants, suggesting the existence of an optimal concentration for stimulating the development of maturity. Interestingly, the Lactone analog generated a significantly higher CIRG values respect to control (Figure 3B) when applied at the highest concentration (L-0.8 treatment) rather than the lowest concentration (L-0.4 treatment). Compared with the control treatment, L-

0.8 treatment produced a significantly greater total anthocyanin content, whereas L-0.4 treatment produced values similar to those of control. In this way, and unlike the results of the present study and others that involved 24-epibrassinolide (Luan et al. 2013; Xi et al. 2013), these findings suggest that Lactone analog applied at concentrations greater than 0.4 $mg \cdot L^{-1}$ might be the most effective for increasing CIRG and total anthocyanin content. The differences in the effects among analogs and their concentrations could be explained by differences in the affinity of the receptor or some of its components. In this sense, Clouse (2010) and Codreanu and Russinova (2011) reported that correct binding and/or interaction between BRs (natural or synthetic) and specific portions of the extracellular domain of the receptor is crucial for inducing a response.

A statistically significant correlation was observed between CIRG and total anthocyanin content ($mg \cdot berry^{-1}$) and concentration ($mg \cdot g\ FW^{-1}$) (Table 5). The results of a similar analysis but for different groups of anthocyanins revealed a significant correlation between the CIRG and 2-OH, Met and Non-met anthocyanins (Table 5). However, when anthocyanins are expressed as $mg \cdot gFW^{-1}$, the significance of the correlation extends to all 3-OH anthocyanins, with the exception of malvidin-3-glucoside (data not shown). This suggests that the CIRG values observed in this study (Figures 3 and 5) are associated with 2-OH anthocyanins. These results agree with those reported by Fernández-López et al. (1998), who associated the presence of 2-OH anthocyanins with grape varieties that have pink or red berries (CIRG ≈ 4.0). These authors also associated darker colors of berries

(CIRG ≥ 6.5) with a greater abundance of 3-OH anthocyanins, particularly malvidin-3-glucoside.

Interestingly, three of the four treatments that resulted in significantly greater CIRG values than those from the control (E-0.4, T-0.4 and B-2000 in both seasons), also resulted in greater values of total anthocyanins and 2-OH anthocyanins, suggesting that the increase in total anthocyanin content occurred differentially toward this group of anthocyanins, i.e., those presenting pink and red colors. In fact, in berries of clusters that received these treatments, the 2-OH:3-OH anthocyanin ratios were close to 2.0 and 2.4 for the seasons 2014 - 2015 and 2015 - 2016 respectively (Table 3 and 4). This differential accumulation is supported by the observed differences in the ratio between these two groups of anthocyanins (Table 3 and 4). This phenomenon was particularly clear in berries that received treatments E-0.4 and B-2000 (in both seasons), which significantly differed from the control treatment in their 2-OH:3-OH ratio. The effect of the differential accumulation of anthocyanins could be explained by a varietal effect. As previously indicated, the predominant anthocyanins in ‘Redglobe’ are of the 2-OH type (Carreño et al. 1997; Cantos et al. 2002; Peppi et al. 2007; Ustun et al. 2012), therefore an increase in the total anthocyanin concentration would mean an increase in the predominant type of anthocyanin present in the variety. However, a proportional increase in the amount of non-predominant anthocyanins could also occur. Nevertheless, this phenomenon was not observed in the present study, as indicated by the 2-OH:3-OH anthocyanin ratios (Table 3). Additionally, grape clusters treated with ABA exhibit increased total

anthocyanin concentrations in berries, without altering the proportion of their different groups respect to the untreated berry clusters (Peppi et al., 2007). This is consistent with the idea that exogenous applications of BRs, especially treatments E-0.4 and B-2000, cause a differential increase in the amount of 2-OH anthocyanins present in berries. This increment could be caused by altered activity, expression or post-transcriptional regulation of enzymes involved in the phenylpropanoid pathway, or in transcription factors controlling the expression of those enzymes, particularly flavonoid 3'-hydroxylase (F3'H), which is responsible for generating the precursor of cyanidin-3-glucoside (Bogs et al. 2005; Castellarin et al. 2006); methyltransferase (OMT), which is responsible for the methylation of cyanidin-3-glucoside, giving rise to peonidin-3-glucoside (Mattivi et al., 2006); and/or flavonoid 3',5'-hydroxylase, that generates the precursor of delphinidin-3-glucoside (Bogs et al. 2005; Castellarin et al. 2006). Similar alterations in the expression and/or activity of the enzymes of the phenylpropanoid pathway occur in 'Cabernet Sauvignon' as a consequence of high temperature (Mori et al., 2005), exposure to solar radiation (Matus et al., 2009) and even viral infection (Vega et al., 2011). Notwithstanding, no study has clearly indicated changes in the proportions of different groups of anthocyanins. So far, similar effects in table grapes have not been reported; thus, the present study is the first reporting changes of this type in table grapes and over two growing seasons. Nevertheless, additional studies are needed to determine how treatments E-0.4 and B-2000 give rise to this differential anthocyanin accumulation.

Although no comparisons between BRs and alternative methods for improving the berry color, such as the use of ethephon or ABA, were performed in the present study, the effect of BR treatments is attractive in a productive context given the types of colors they generate and the absence of detrimental effects on quality attributes of the treated clusters (Figures 3 and 5).

5.- Conclusions

The results of this study demonstrate that exogenous applications of different BRs analogs to 'Redglobe' grape clusters result in a significant increase in berry color, in the soluble solids content and total anthocyanins, altering the distribution of anthocyanin groups. This is the first report describing such effects on table grapes in a productive context.

The increase in the color of berries (expressed as CIRG) induced by treatment with BR analogs could be due to their effect on soluble solids content (treatments T-0.4 and B-2000), concentration of anthocyanins (treatment L-0.8), or the combination of these two effects in the berries (treatments E-0.4 and T-0.8).

The responses of CIRG and total anthocyanin content to the concentrations of the tested BR analogs show that Triols and 24-epibrassinolide have an effective

concentration at approximately 0.4 $mg \cdot L^{-1}$, whereas Lactone appears to have a greater effect at concentrations higher than 0.4 $mg \cdot L^{-1}$.

This study showed that the activity of BR analogs is not restricted to just 24-epibrassinolide, which has been previously evaluated in other investigations, and that Triol, Lactone and the commercial formulation (B-2000) were also active. The results demonstrate consistent effects of BRs on grape berries regardless of the analog used in two consecutive growing seasons.

Although several of the treatments performed in this study increased the total concentration of anthocyanins, only treatments E-0.4 and B-2000 resulted in a differential increase in 2-OH anthocyanins in two different growing seasons. This increment could chiefly explain the higher CIRG values observed in this work, which resulted in the expression of red and pink hues in berries, a color feature that is attractive for ‘Redglobe’ markets.

Finally, this work demonstrates the potential of using BRs as a management tool in viticulture, and their use could at least complement the alternatives conventionally employed.

6.- Author contributions

AV and AP designed research. AV performed research. AV, AP, and JA analyzed data and results. LE, KD and RC synthesized, characterized and provided the two novel analogs brassinosteroid. AV wrote the paper.

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9.- Conflict of interest

LE, KD and RC declare conflict of interest because one of the analogous (Lactone) could be patented and/or protected by copyright.

10.- References

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Chapter 4

Conclusions, final considerations and perspectives

The ripening of berries is a complex process, coordinated and strongly influenced by environmental and endogenous factors. Although the environmental factors are relevant, they are based or explained by their effect on the hormonal balance.

While the hormonal control of berry ripening has been traditionally attributed to abscisic acid (ABA) and ethylene, the background shows that brassinosteroids (BRs) would also be participating. Furthermore, it is known that BRs are present throughout the development of the berries, especially from veraison. Regarding this, research conducted in model species has established that BRs participate in various development processes, and that there is also an interaction between BRs and other hormones such as ABA, ethylene and gibberellic acid (GA).

Considering that all previous studies regarding the BRs in vines have been developed in wine-making varieties, the present work constitutes the first report in which the use of BRs as a tool in the production of table grapes is evaluated under productive conditions.

Under the experimental conditions tested in this work, the exogenous applications of BRs were able to increase the content and concentration of anthocyanins in the ‘Redglobe’ and ‘Crimson seedless’ varieties. In the case of ‘Redglobe’, the response of content and contraction of anthocyanin to the application of BRs was consistent between seasons and locations, and although in ‘Crimson seedless’ it was only

tested for one season, its response was consistent with what was observed in ‘Redglobe’. To this is added that, a palette of 3 analogs of experimental BRs (24-epibrassinolide, Triol and Lactone) and a commercial formulation (B-2000) were tested. Although the effect of the BRs varied by virtue of the analogue and concentration used, the overall response was consistent, demonstrating the stability of this response throughout the test seasons, the varieties used and localities.

This increase in the concentration and content of anthocyanins was reflected in a significant increase in the color of 'Redglobe' berries. This increase is repeated throughout all the experiments and seasons and although in ‘Crimson seedless’ did not observe an increase in berry color, the analysis of the data showed that this did not occur due to the concentration values of anthocyanins observed. In addition, in this variety and in an unexpected way, a significant increase in equatorial diameter of berries treated with the commercial formulation of BRs was observed. It is important to note that, this increase was not associated with any significant reduction in berry color.

In the case of trials in which different analogues of BRs were tested in ‘Redglobe’ (Chapter 3), applications of these analogs, in addition to increasing the content of anthocyanins and berry color, generated a significant increase in content of total soluble solids. In one of the seasons (2014 - 2015), soluble solids together with the treatments had a significant effect on berries color. When considering this effect, a

change in the significant differences of the treatments (analogs and/or concentration) respect to the control was observed. If significant differences in anthocyanin content and concentration are considered, this would indicate that the increase in color produced by BRs would occur, on one side, due to the higher anthocyanin content (Lactone applied at 0.8 $mg \cdot L^{-1}$), due to the higher soluble solids content (Triol applied at 0.4 $mg \cdot L^{-1}$) or both (Triol applied at 0.8 $mg \cdot L^{-1}$ and 24-epibrassinolide applied at 0.4 $mg \cdot L^{-1}$). Thus, the level at which BRs promote color development would depend on the analogue and/or the concentration used. Additionally, 'Redglobe', in both seasons, 24-epibrassinolide treatment applied at 0.4 $mg \cdot L^{-1}$, and the commercial formulation, applied according to manufacturer's recommendation (0.06 $mg \cdot L^{-1}$), generated a differential and significant increase in dihydroxylated anthocyanins (cyanidine-3-glucoside and peonidine-3-glucoside), responsible for the red and pink color.

Although establishing the mechanisms on which BRs produce their effect was not the objective of this work, and determining them in the conditions in which these trials were carried out (in commercial fields) is particularly complex, the results embodied in this work confirm what has been established in previous research in model organisms and under controlled conditions. Thus, the interaction that has been reported between BRs and ABA, especially in conditions of biotic and abiotic stress, and with ethylene, would indicate that the effect on anthocyanin content produced by BRs would be mediated by these two hormones. This is confirmed by the fact that these two hormones are routinely used to promote the berries color

development (increased anthocyanin content) in table grape production. Similarly, it has been reported that BRs are a master regulator of GA synthesis, so the increase in berry size observed in ‘Crimson seedless’ could be mediated by GA. Regarding the increase in color and size of the berries, it is important to consider that these results could be different under productive conditions in which these parameters are not limiting.

Currently, table grape production bases the application program for growth regulators on only 2 or 3 hormone analogues, which is especially risky in a context in which there is a wide range of new varieties, whose management is not entirely clear. Thus, this work establishes the bases that allow BRs to be integrated as another tool in the application programs of growth regulators, especially with regard to the berries color development in the production of table grapes.

Appendix 1

Supplementary Tables 1 and 2 of article published in Frontiers in Plant Science. (Chapter 3) (volume nº 9, Abril 2018. 1-11 pp; DOI: 10.3389/fpls.2018.00363)

Supplementary Table 1. Effects of BR treatments on the diameter of berries, weight of berries, total acidity and weight of clusters at harvest for season 2014 – 2015. Each value indicates the mean of five replicates ± its standard deviation. n.s. = no statistical differences.

Treatments	Diameter (mm)		Berry weight (g)		Total acidity (g tartaric acid·L^{-1})		Cluster weight (g)	
E-0.4	29.2 ± 1.20	n.s	17.0 ± 2.47	n.s	3.2 ± 0.28	n.s	1177.3 ± 265,05	n.s
E-0.8	29.4 ± 1.11	n.s	18.0 ± 2.13	n.s	3.4 ± 0.26	n.s	1216.4 ± 192.27	n.s
T-0.4	28.5 ± 2.01	n.s	15.3 ± 3.26	n.s	3.2 ± 0.64	n.s	1153.7 ± 183.56	n.s
T-0.8	28.6 ± 1.11	n.s	15.9 ± 2.10	n.s	2.9 ± 0.24	n.s	1089.9 ± 209.53	n.s
L-0.4	29.0 ± 1.19	n.s	16.6 ± 2.18	n.s	3.6 ± 0.30	n.s	1277.0 ± 267.08	n.s
L-0.8	27.9 ± 0.99	n.s	15.5 ± 1.85	n.s	3.5 ± 0.38	n.s	1196.3 ± 138.79	n.s
B-2000	28.7 ± 0.82	n.s	16.6 ± 1.75	n.s	3.4 ± 0.28	n.s	1282.6 ± 293.18	n.s
Control	28.5 ± 2.01	n.s	17.1 ± 2.06	n.s	3.4 ± 0.36	n.s	1169.1 ± 367.60	n.s

Supplementary Table 2. Effects of BR treatments on the diameter of berries, weight of berries, total acidity and weight of clusters at harvest for season 2015 – 2016. Each value indicates the mean of five replicates ± its standard deviation. n.s. = no statistical differences.

Treatments	Diameter (mm)		Berry weight (g)		Total acidity (g tartaric acid·L^{-1})		Cluster weight (g)	
E-0.4	27.2 ± 0.56	n.s	14.2 ± 0.68	n.s	3.4 ± 0.17	n.s	797.7 ± 143.69	n.s
T-0.4	27.2 ± 1.09	n.s	14.3 ± 1.70	n.s	3.6 ± 0.21	n.s	795.3 ± 128.65	n.s
L-0.4	27.5 ± 0.68	n.s	14.6 ± 0.72	n.s	3,4 ± 0.41	n.s	738.9 ± 73.63	n.s
B-2000	28.0 ± 0.30	n.s	14.9 ± 0.70	n.s	3.4 ± 0.31	n.s	736.1 ± 63.69	n.s
Control	27.8 ± 0.81	n.s	14.1 ± 1.21	n.s	3.3 ± 0.16	n.s	749.5 ± 126.33	n.s

Appendix 2

Complete anthocyanins profiles present by treatment according to results presented in Chapter 3

Table 1. Anthocyanins profiles present for all treatments in the 2014-2015 season.

Treatments	Types of anthocyanins											
	mg·100g^{-1}FW					mg·berry^{-1}						
	Del-3-g	Pet-3-g	Mal-3-g	Cya-3-g	Peo-3-g	Del-3-g		Pet-3-g	Mal-3-g	Cya-3-g	Peo-3-g	
E-0.4	0.411 ab	0.395 ab	0.455 ab	0.775 ab	1.773 a	0.069	ab	0.067 ab	0.078 ab	0.133 ab	0.307	a
E-0.8	0.408 ab	0.392 ab	0.444 ab	0.709 b	1.058 cd	0.069	ab	0.066 ab	0.075 ab	0.120 b	0.178	cd
T-0.4	0.459 a	0.433 ab	0.519 ab	0.739 b	1.466 ab	0.080	ab	0.075 ab	0.090 ab	0.128 b	0.250	ab
T-0.8	0.478 a	0.379 ab	0.480 ab	0.746 b	0.967 cde	0.081	a	0.064 ab	0.081 ab	0.128 b	0.165	cde
L-0.4	0.334 bc	0.368 ab	0.372 b	0.604 bc	0.823 de	0.057	c	0.062 ab	0.063 b	0.102 bc	0.141	de
L-0.8	0.394 ab	0.434 ab	0.491 ab	0.750 b	1.194 bc	0.068	abc	0.074 ab	0.084 ab	0.129 b	0.204	bc
B-2000	0.456 a	0.449 a	0.534 a	1.150 a	1.865 a	0.077	ab	0.076 a	0.091 a	0.193 a	0.314	a
Control	0.322 c	0.345 b	0.380 b	0.499 c	0.765 e	0.055	c	0.059 b	0.065 b	0.085 c	0.129	e

Table 2. Anthocyanins profiles present for all treatments in the 2015-2016 season.

Treatments	Types of anthocyanins									
	mg·100g^{-1}FW					mg·berry^{-1}				
	Del-3-g	Pet-3-g	Mal-3-g	Cya-3-g	Peo-3-g	Del-3-g	Pet-3-g	Mal-3-g	Cya-3-g	Peo-3-g
E-0.4	0.205 abc	0.308 ab	0.197 ab	0.468 a	1.514 ab	0.029 abc	0.044 ab	0.028 ab	0.066 a	0.214 ab
T-0.4	0.229 a	0.338 a	0.224 a	0.401 a	1.252 b	0.032 ab	0.048 ab	0.032 ab	0.058 a	0.178 b
L-0.4	0.168 bc	0.287 ab	0.161 b	0.288 a	0.703 c	0.024 bc	0.042 b	0.023 b	0.042 a	0.103 c
B-2000	0.229 ab	0.350 a	0.231 a	0.360 a	1.592 a	0.034 a	0.052 a	0.034 a	0.054 a	0.238 a
Control	0.160 c	0.269 b	0.164 b	0.301 a	0.653 c	0.023 c	0.038 b	0.023 b	0.042 a	0.092 c

Appendix 3

Results presented in congresses, publications and other activities developed during doctoral studies

Results presented in congress

- Can brassinoesteroids improve berry color in table grapes cv. 'Redglobe' (poster). A. Vergara, M. Torrealba, J. A. Alcalde and A. Pérez-Donoso. XI Reunión de Bilogía Vegetal, 28 noviembre – 2 diciembre 2016. Chillán, Chile.
- Effect of brassinoesteroids application on ripening and quality of table grape berries cv. Redlobe (poster). A. Vergara, M. Torrealba, J. A. Alcalde and A. Pérez-Donoso. International Plant Molecular Biology Congress, 25th - 30th October , 2015. Foz do Iguaçu, Brasil.
- Pérez-Donoso A, A Vergara, M Torrealba, JA Alcalde. 2015. Brassinosteroids effects on the ripening and quality of table grapes (poster). The 2nd World Congress on the use of Biostimulants in Agriculture, 17th -19th Noviembre. Florence, Italy.

Indexed publications

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ORIGINAL RESEARCH
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Exogenous Applications of Brassinosteroids Improve Color of Red Table Grape (*Vitis vinifera* L. Cv. "Redglobe") Berries

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Color and other quality parameters of "Redglobe" grape (*Vitis vinifera* L.) berries were evaluated after treatment with brassinosteroid (BR) analogs. Three BRs analogs (24-epibrassinolide, Triol, or Lactone) were applied at three concentrations (0.0, 0.4, or 0.8 mg·L^{-1}), at the onset of veraison. A commercial formulation (B-2000®) was also applied, at a recommended rate of 0.06 mg·L^{-1}. The tested BR analogs were effective improving berry color (evaluated as color index for red grapes, CIRG), increasing the levels of soluble solids and anthocyanins, and changing the types of anthocyanins present without altering other quality and yield parameters. The effects of BR analogs on color enhancement could be explained by an increase in soluble solids content and/or anthocyanin content. Treatment with 24-epibrassinolide (at 0.4 mg·L^{-1}) or the commercial formulation tended to favor the production of dihydroxylated anthocyanins, which are responsible for the red and pink colors of grape berries. Results indicate that the use of BRs constitutes a potential tool in the production of table grapes. This is the first report of this enhancement effect in a productive context.

Keywords: anthocyanins, berry color, brassinosteroids, color index for red grapes (CIRG), quality

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INTRODUCTION

The assessment of table grape quality is traditionally based on sensory attributes such as flavor, aroma, texture, and color. Numerous studies together with demographic observations have demonstrated the importance of color as the main quality indicator on which consumer acceptance of fruit is based (Clydesdale, 1993).

Often, minimum quality standards can be obtained by performing good management practices in the vineyard (Cameron, 1994; Schrader et al., 1994), but in the case of grape varieties such as "Redglobe," which has large-sized berries and clusters, these practices can easily lead to overproduction. Under these conditions, "Redglobe" berries fail to develop suitable red color (Kliewer and Weaver, 1971; Schrader et al., 1994; Kliewer and Dokoozlian, 2005). In addition, the production of table grapes in Chile is conducted in areas with warm climatic and/or microclimatic conditions. These high temperature conditions inhibit the accumulation of anthocyanins (Mori et al., 2005), negatively affecting the development of berry color (Spayd et al., 2002; Mori et al., 2005; Kuhn et al., 2014). Thus, a series of techniques that tend to correct and/or prevent these problems have been implemented. These techniques include the use of growth regulators such as ethephon and/or abscisic acid (ABA) during veraison; these techniques have been widely

Teaching activities

- 2014-2015: Profesor Instructor del curso de Vitucultura, prof. coordinaro: Dr. Alonso Pérez, prof. participante

- 2014-2015: Ayudante de curso de Fisiología de las Plantas Frutales, prof. Dr. José Antonio Alcalde.

- 2104: Ayudante de la asignatura Seminario de Postgrado, Prof. Dr. Tania Zaviezo.