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# ABA and $GA_3$ regulate the synthesis of primary and secondary metabolites related to alleviation from biotic and abiotic stresses in grapevine

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ABSTRACT

Plants are able to synthesize a large number of organic compounds. Among them, primary metabolites are known to participate in plant growth and development, whereas secondary metabolites are mostly involved in defense and other facultative processes. In grapevine, one of the major fruit crops in the world, secondary metabolites, mainly polyphenols, are of great interest for the wine industry. Even though there is an extensive literature on the content and profile of those compounds in berries, scarce or no information is available regarding polyphenols in other organs. In addition, little is known about the effect of plant growth regulators (PGRs), ABA and $GA_3$ (extensively used in table grapes) on the synthesis of primary and secondary metabolites in wine grapes. In table grapes, cultural practices include the use of $GA_3$ sprays shortly before veraison, to increase berry and bunch size, and sugar content in fruits. Meanwhile, ABA applications to the berries on pre-veraison improve the skin coloring and sugar accumulation, anticipating the onset of veraison.

Accordingly, the aim of this study was to assess and characterize primary and secondary metabolites in leaves, berries and roots of grapevine plants cv. Malbec at veraison, and changes in compositions after ABA and $GA_3$ aerial sprayings. Metabolic profiling was conducted using GC-MS, GC-FID and HPLC-MWD. A large set of metabolites was identified: sugars, alditols, organic acids, amino acids, polyphenols (flavonoids and non-flavonoids) and terpenes (mono-, sesqui-, di- and triterpenes). The obtained results showed that ABA applications elicited synthesis of mono- and sesquiterpenes in all assessed tissues, as well as L-proline, acidic amino acids and anthocyanins in leaves. Additionally, applications with $GA_3$ elicited synthesis of L-proline in berries, and mono- and sesquiterpenes in all the tissues. However, treatment with $GA_3$ seemed to block polyphenol synthesis, mainly in berries. In conclusion, ABA and $GA_3$ applications to grapevine plants cv. Malbec influenced the synthesis of primary and secondary metabolites known to be essential for coping with biotic and abiotic stresses.



## 1. Introduction

Grapevine is one of the major fruit crops in the world, considering its global distribution and its high economic value. Grapes are used not only for wine, but also as fresh and dried fruit, and for juice production (Bouquet et al., 2006; Vivier and Pretorius, 2002). Moreover, grapevine leaves are used in several Mediterranean dishes (Harb et al., 2015).

Plants primary metabolites regulate plant nutrition and essential biochemical processes, whereas their secondary metabolites are mostly involved in defense and other facultative processes, such as biotic and abiotic stress responses (Berli et al., 2010; Croteau et al., 2000; Escoriaza et al., 2013; Gil et al., 2012; Grassmann et al., 2002; Nagegowda, 2010). However, several studies

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confirmed that changes in primary metabolite levels may contribute to an elevated resistance to stress as well (Conde et al., 2014; Figueiredo et al., 2008; Harb et al., 2015). In addition, primary and secondary metabolites in grapevine berries are key factors for the organoleptic properties of grapes and wines (Conde et al., 2007).

Polyphenols are defined as natural products which contain one or more hydroxyl groups covalently linked to a benzene ring (Croteau et al., 2000). They have different functions, such as defense against herbivores and pathogens, mechanical support (lignin), pollinator attractants, UV-B damage amelioration and allelopathic effects (Taiz and Zeiger, 1998). In grapevine, polyphenols are divided into two main groups: non-flavonoids (hydroxybenzoic and hydroxycinnamic acids and their derivatives, stilbenes and phenolic alcohols) and flavonoids (anthocyanins, flavanols, flavonols and dihydroflavonols) (Fanzone et al., 2012). The latter are of particular interest, since they define organoleptic and nutritional characteristics of grape berries (González et al., 2015); moreover, flavonoids act as potent antioxidants, helping plants cope with abiotic stress. In accordance with this, previous results in berries showed that combined treatments with UV-B and exogenous ABA (**1**) (Fig. 1) changed the profile of anthocyanins and non-anthocyanin phenolics, increasing the proportion of phenols with high antioxidant capacity (Berli et al., 2011). In addition, physical factors other than UV-B radiation, such as water stress (Castellarin et al., 2007), light intensity and quality (Azuma et al., 2012; González et al., 2015), as well as high temperatures (Mori et al., 2007), induced polyphenol biosynthesis and the accumulation of these metabolites.

Even though there is a vast amount of work on environmental regulation of polyphenol metabolism, the knowledge of the effects of plant growth regulators (PGRs) in grapevine plants, principally gibberellins, is still scarce. In fact, only a few papers analyze the content and profile of these secondary compounds in tissues different from grapevine berries (Berli et al., 2010; Harb et al., 2015).

Terpenes are the most representative variety of compounds within natural products (approximately 25,000 compounds, Croteau et al., 2000). All terpenes are derived from isoprene, and they are classified in hemi- (5 C), mono- (10), sesqui- (15 C), di- (20 C), tri- (30 C) and tetraterpenes (40 C), according to their carbon number (Croteau et al., 2000). Terpenes synthesis starts with the precursor isopentenyl diphosphate, or its isomer dimethylallyl diphosphate. Two metabolic pathways are involved in the formation of these compounds in higher plants, the mevalonic acid pathway (MVA), occurring in the cytosol, and the methylerythritol phosphate pathway (MEP), active in plastids (Lichtenthaler et al., 1997). The MVA pathway provides precursors for sesquiterpene and sterol synthesis, whereas the MEP pathway provides precursors for mono-, di- and tetraterpene synthesis (Hampel et al., 2005; McGarvey and Croteau, 1995; Tholl, 2006).

It has been found that these secondary metabolites act as protective molecules against biotic and abiotic stresses. In this regard, Escoriaza et al. (2013) reported the synthesis of nerolidol (sesquiterpene) in response to a fungal infection affecting grapevines. Additionally, treatment with UV-B radiation induced synthesis of mono- and sesquiterpenes (Alonso et al., 2015; Gil et al., 2012). Furthermore, plant growth promoting rhizobacteria (PGPR), isolated from a commercial vineyard, also elicited synthesis of mono- and sesquiterpenes as a response to coping with abiotic stress (Salomon et al., 2014). In this regard, there is information on terpene synthesis and environmental control, while there is scarce or no information on ABA (**1**) and $GA_3$ (**2**) regulation of terpene metabolism in grapevine plants as a protection and as a defense against environmental stresses. These phytohormones are extensively used in table grapes to enhance color and sugar accumulation in berries (Ban et al., 2003; Fidan et al., 1981). Moreover, ABA (**1**) and $GA_3$ (**2**) treatments increased the content of sugars in berries of *Vitis vinifera* L. cv. Malbec (Moreno et al., 2011; Murcia et al., 2016).

Up to now, little is known about the effect of these PGRs on the synthesis of primary and secondary metabolites in wine grapes. It can be considered, however, that ABA (**1**) and $GA_3$ (**2**) applications on grape plants may be a suitable strategy to prepare plants to cope with biotic and abiotic stresses, thereby increasing yield and quality of fruits at harvest.

In this study, it is hypothesized that aerial sprayings with ABA (**1**) and $GA_3$ (**2**) to grape plants cv. Malbec could enhance the content of primary and secondary metabolites related to stress alleviation, not only in aerial parts (leaves and berries), but also in the roots (systemic response).

## 2. Results and discussion

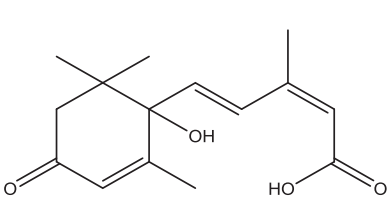
In the current study, the principal organic compounds (Fig. 1) present in different tissues (leaf, berry and root) of *Vitis vinifera* L. cv. Malbec were characterized at the stage of veraison, and their variation was correlated to applications with growth regulators (ABA (**1**) and $GA_3$ (**2**)). Also, *VvNCED1* gene expression and ABA (**1**) concentration in leaves and berries of ABA (**1**) and $GA_3$ (**2**) treated plants were assessed.

### 2.1. Principal component analysis (PCA) of metabolites

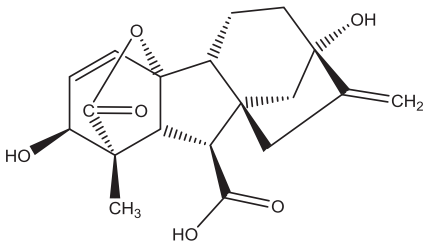
For an overall interpretation of the results obtained, a PCA was used (Fig. 2). The matrix of the analysis consisted of 3 cases corresponding to the treatments (control, ABA (**1**) and $GA_3$ (**2**)), and 23 variables (main groups of metabolites). 3 PCA *biplots* graphics in relation to each tissue assessed are reported. Fig. 2a shows the *biplot* corresponding to leaves, in which the CP1 explained 65.1% of the variance, and separated the treatments ABA (**1**) and $GA_3$ (**2**) from the control. CP2 explained 34.9% of the variance, and separated the ABA (**1**) and $GA_3$ (**2**) treatments. The cyclic amino acid, L-proline (**3**), acidic amino acids (AAs), petunidins, delphinidins, cyanidins and monoterpenes were associated with ABA (**1**) treatment, whereas sucrose (**4**), organic acids, flavanols, malvidins and sesquiterpenes were associated with $GA_3$ (**2**). On the other hand, monosaccharides, alditols, aliphatic AAs, basic AAs, aromatic AAs, hydroxycinnamic acids, flavonols, peonidins and di- and triterpenes were associated with the control treatment.

In berries (Fig. 2b), the CP1 explained 52.8% of the variance, and separated the treatments $GA_3$ (**2**) and control from ABA (**1**), whereas the CP2 explained 47.2% of the variance, and separated $GA_3$ (**2**) from the control. In this case, alditols, organic acids, basic AAs, aromatic AAs, acidic AAs, flavanols, OH-tyrosol (**5**), peonidins, cyanidins, delphinidins, petunidins, monoterpenes and sesquiterpenes were associated with the treatment ABA (**1**). Moreover, sucrose (**4**), sulfur AAs, aliphatic AAs, hydroxybenzoic acids and malvidins were associated with $GA_3$ (**2**) treatment. On the other hand, monosaccharides, flavonols and di- and triterpenes were associated with control treatment.

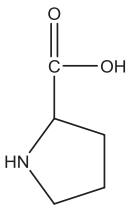
In roots (Fig. 2c), the CP1 explained 69.7% of the variance and separated the treatments ABA (**1**) and $GA_3$ (**2**) from the control, whereas the CP2 explained 30.3% of the variance and separated the $GA_3$ (**2**) from ABA (**1**). Acidic AAs and flavonols were associated with ABA (**1**) treatment. Likewise, sucrose (**4**), alditols, aromatic AAs and sesquiterpenes were associated with $GA_3$ (**2**) treatment, while monosaccharides, organic acids, L-proline (**3**), sulfur AAs, aliphatic AAs, hydroxybenzoic acids, hydroxycinnamic acids, OH-tyrosol (**5**) and di- and triterpenes were associated with the control.



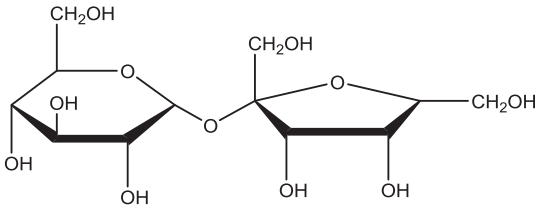

ABA (**1**)



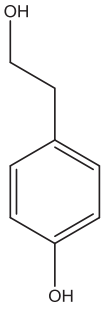

$GA_3$ (**2**)



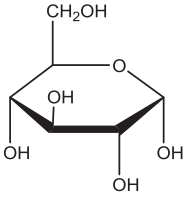

L-proline (**3**)



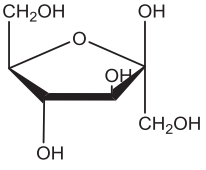

Sucrose (**4**)



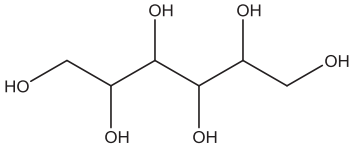

OH-tyrosol (**5**)



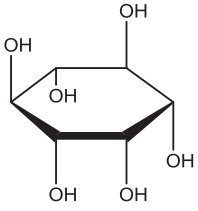

Glucose (**6**)




Fructose (**7**)




Sorbitol (**8**)




Inositol (**9**)

**Fig. 1.** Chemical structures of sugars, alditols, organic acids, amino acids, polyphenols and terpenes identified by GC-MS, GC-FID and HPLC-MWD in leaves, berries and roots of *Vitis vinifera* L. cv. Malbec and chemical structures of plant growth regulators.

### *2.2. ABA (1) and $GA_3$ (2) affect the biosynthesis of ABA (1) in grapevine leaves and berries*

Applications with ABA (**1**) caused an increment in ABA (**1**) content in both leaves and berries of grapevine plants cv. Malbec, as expected (Fig. 3). However, the relative expression level of *VvNCED1*, a key gene in the ABA (**1**) biosynthetic pathway, was highly down-regulated in ABA (**1**) treated leaves, likely due to an ABA (**1**) homeostasis (Fig. 4a). Surprisingly, there were no significant differences in the expression of *VvNCED1* between ABA (**1**) treatments and control in berry tissues (Fig. 4b). Apparently, the regulation of ABA (**1**) biosynthesis by exogenous ABA (**1**) is tissue dependent, and may involve an autocatalytic mechanism, similarly to ethylene in climacteric fruits, as described by Wheeler et al. (2009). Another explanation of our findings could be a reduced catabolism of ABA (**1**), as suggested by Castellarin et al. (2016), who observed an increase of ABA (**1**) concentration in grapevine berries at the onset of ripening with no concomitant increase in *VvNCED1* expression. On the other hand, $GA_3$ (**2**) applications induced a reduction in ABA (**1**) concentration in leaves, which was correlated with a tenfold down regulation of *VvNCED1* compared to the control (Figs. 3a and 4a). Nevertheless, an increment in ABA (**1**) content was recorded in $GA_3$ (**2**) treated berries, correlated with a ~twofold up-regulation of the *VvNCED1* gene (Figs. 3b and 4b). The latter

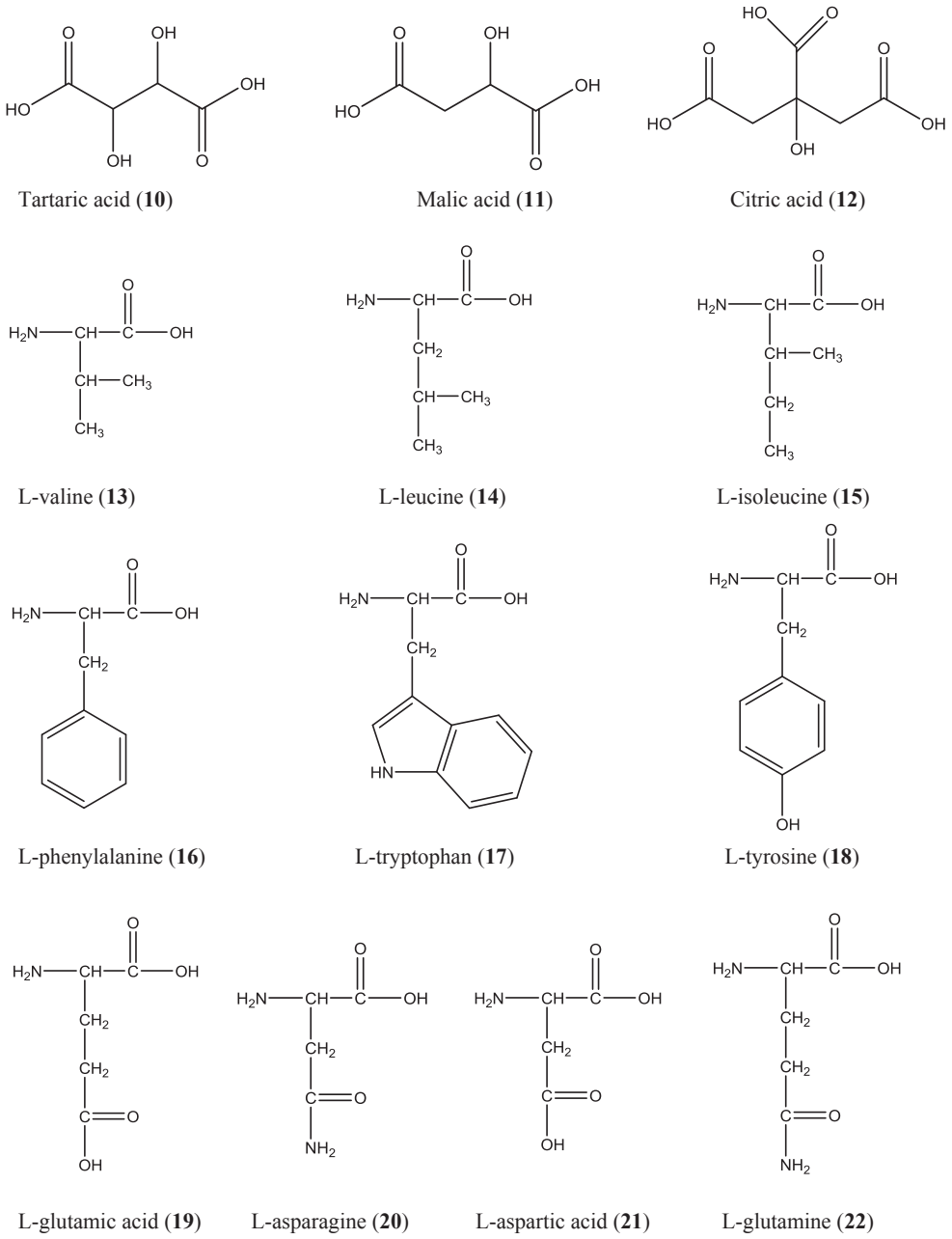



**Fig. 1.** (*continued*).

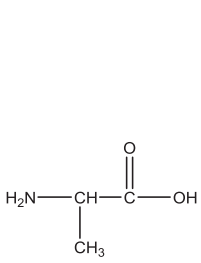
results suggest that the regulation of ABA (**1**) biosynthesis by $GA_3$ (**2**) is tissue dependent, but the explanation of this mechanism remains unclear.

### 2.3. ABA (1) and $GA_3$ (2) modify primary metabolite levels in grape leaves, berries and roots

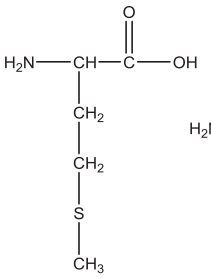
#### 2.3.1. Sugars

ABA (**1**) and $GA_3$ (**2**) spraying reduced the content of the monosaccharides, glucose (**6**) and fructose (**7**), in grape leaves as compared to the control, whereas the sucrose (**4**) concentration increased with both PGR treatments (approximately two times, Table 1 and Supplementary Table S1). In higher plants, photoassimilates are translocated either as sucrose (**4**) or alditols. In the Rosaceae, a family including the genera Pyrus, Malus and Prunus, the polyol sorbitol (**8**) is the main translocated photoassimilate (Bielesk, 1982; Moing et al., 1997; Zimmermann and Ziegler, 1975), while in grapevine sucrose (**4**) is the main sugar transported throughout the phloem (Swanson and Elshishiny, 1958). The results presented here show an increase of sucrose (**4**) amounts in leaves of ABA (**1**) and $GA_3$ (**2**) treated plants, in accordance with Murcia et al. (2016), who demonstrated that such an increase was related to an improvement of phloem loading, leading to high sugar transport to stems and berries. In addition, in leaves of plants treated with both PGRs, glucose (**6**) and fructose (**7**) may be used as carbon skeletons, or as an energy source for the synthesis of sucrose (**4**) and secondary metabolites.

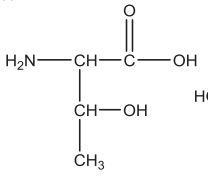
In berries (Table 2 and Supplementary Table S2), the contents of sucrose (**4**), glucose (**6**), and fructose (**7**) between $GA_3$ (**2**) treated plants and control were similar, while ABA (**1**) treatment decreases



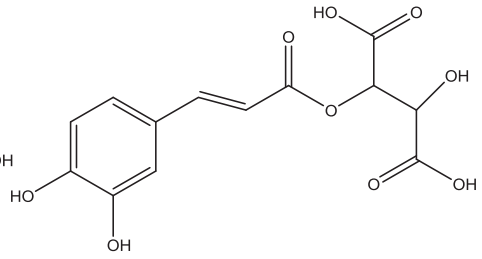
L-alanine (**23**)



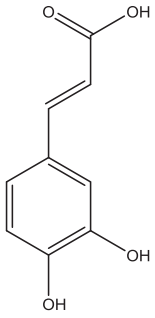
L-methionine (**24**)



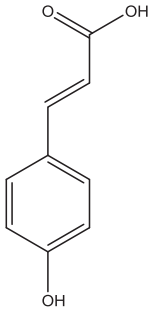
L-threonine (**25**)



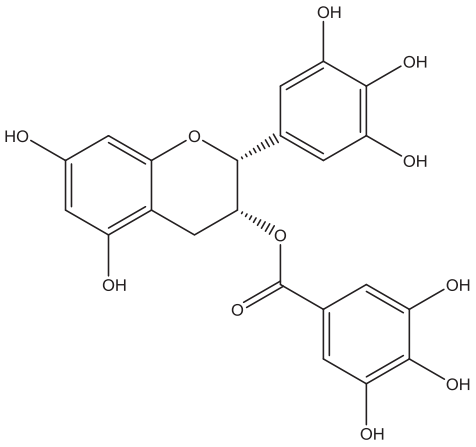
Caftaric acid (**26**)



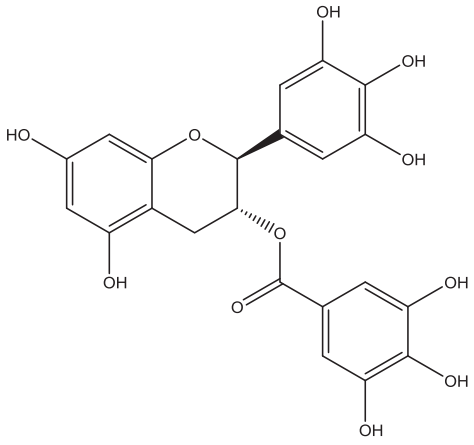
Cafeic acid (**27**)



*p*-coumaric acid (**28**)



(-)-epigallocatechin gallate (**29**)

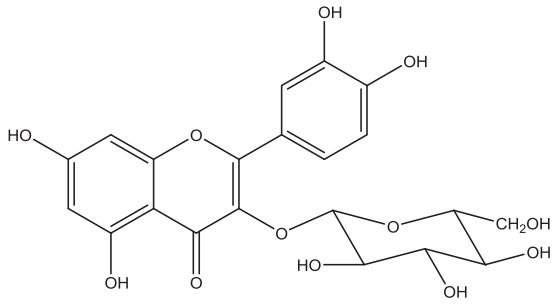


(-)-gallocatechin gallate (**30**)

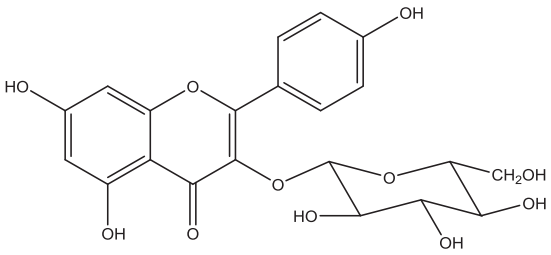
**Fig. 1.** (*continued*).

the concentration of monosaccharides and sucrose (**4**) relative to the control. In grapevine plants, the sucrose (**4**) transport from leaf to berry depends on the difference of turgor pressure between them. Moreover, the turgor pressure in the phloem zone corresponding to a certain organ is directly related to the sugars concentration (Keller, 2010). Therefore, the lower sucrose (**4**) content observed in berries of ABA (**1**) treated plants lead to a higher difference in turgor pressure between leaves and fruits, hastening berry ripening (63 DAA), and confirming the results obtained by Murcia et al. (2016). The same effect may be attributable to the lower glucose (**6**) and fructose (**7**) concentration found in berries of ABA (**1**) treated plants, because those sugars are redirected to synthesize protective metabolites, mainly phenolics and terpenes, as discussed later.

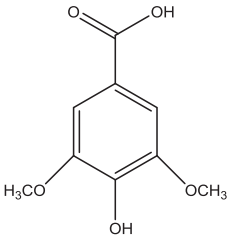
In roots (Table 3 and Supplementary Table S3) as in leaves, treatments with both PGRs reduced the content of glucose (**6**) and fructose (**7**). As it was explained above, these monosaccharides could be redirected to synthesize protective metabolites, in this case only terpenes. Moreover, a decrease in sucrose (**4**) concentration was recorded in roots of plants treated with ABA (**1**).



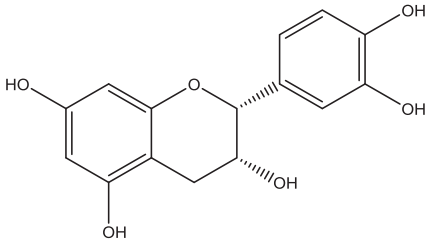
Quercetin-3-glucoside (**31**)



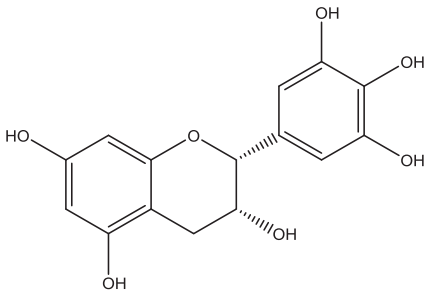
Kaempferol-3-glucoside (**32**)



Syringic acid (**33**)



(-)-epicatechin (**34**)



(-)-gallocatechin (**35**)

**Fig. 1.** (*continued*).

*2.3.2. Alditols*

A major content of alditols (sorbitol (**8**) and inositol (**9**)) was observed in leaves of control plants, where the level of inositol (**9**) was higher than that of sucrose (**4**) (Table 1 and Supplementary Table S1). Conde et al. (2014) and Pillet et al. (2012) claimed that alditols protect cells against stress caused by osmotic and metabolic imbalances. Thus, the high inositol (**9**) levels found in leaves, independently from the treatment may be related to overcoming water stress instead of being connected to photoassimilate translocation.

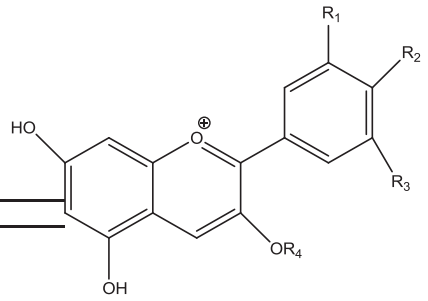
In berries, a low content of alditols was observed in all treatments, with non-statistically relevant differences among them (Table 2 and Supplementary Table S2). On the other hand, a low content of alditols was also observed for all treatments in roots; in particular, the inositol (**9**) concentration was higher with $GA_3$ (**2**) applications, while sorbitol (**8**) was reduced by both ABA (**1**) and $GA_3$ (**2**) (Table 3 and Supplementary Table S3).

*2.3.3. Organic acids*

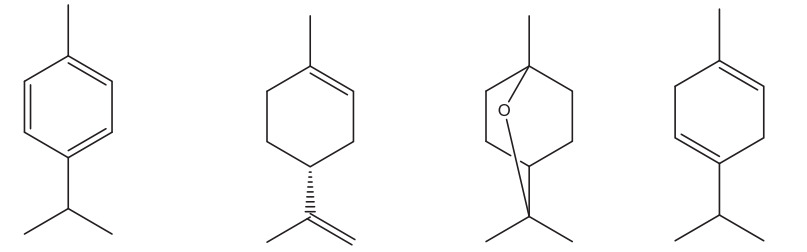
Kliewer (1966) demonstrated that the main organic acids present in grapevine are tartaric (**10**) and malic acids (**11**), followed by citric acid (**12**) in lower concentrations, as confirmed by the results here (Tables 1–3 and Supplementary Tables S1, S2 and S3). In leaves, there were no statistically significant differences among the treatments as regards tartaric (**10**) and malic acid (**11**) contents, although the concentration of citric acid (**12**) was increased by $GA_3$ (**2**) treatment (Table 1 and Supplementary Table S1). The higher content of citric acid (**12**) may be connected to the biosynthesis of secondary compounds, such as mono- and sesquiterpenes, derived from molecules synthesized during the Citric Acid Cycle.

In berries, plants treated with ABA (**1**) had the highest content of malic acid (**11**), while no significant differences in the concentration of organic acids were observed between control and $GA_3$ (**2**) treated plants (Table 2 and Supplementary Table S2). According to this, a higher biosynthesis rate of acids from sucrose (**4**) may be involved in ABA (**1**) treated plants, as suggested by Possner and Kliever (1985) and Coombe (1992). This trait is crucial for the wine industry, since a high content of organic acids in grapes is important for the wines organoleptic features (Conde et al., 2007).

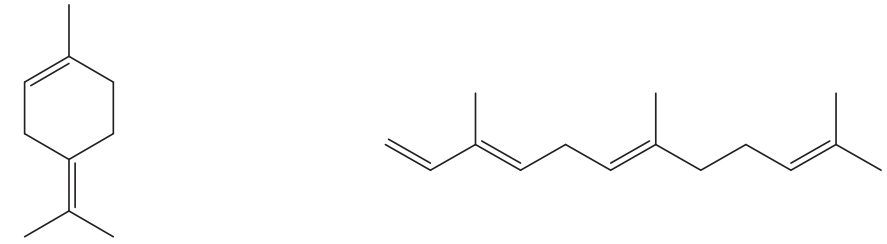
In roots, the concentration of organic acids decreased with $GA_3$ (**2**) application. An increment in malic acid (**11**) in control plants, and of tartaric (**10**) and citric acids (**12**) in control and ABA (**1**)




| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| Malvidin 3-O-glucoside (**36**) | $OCH_3$ | OH | $OCH_3$ | glucose |
| Peonidin 3-O-glucoside (**37**) | $OCH_3$ | OH | H | glucose |
| Delphinidin 3-O-glucoside (**38**) | OH | OH | OH | glucose |
| Cyanidin 3-O-glucoside (**39**) | OH | OH | H | glucose |
| Petunidin 3-O-glucoside (**40**) | $OCH_3$ | OH | OH | glucose |
| Malvidin 3-O-*p*-coumaroylglucoside (**41**) | $OCH_3$ | OH | $OCH_3$ | *p*-coumaroyl-glucose |
| Cyanidin 3-O-*p*-coumaroylglucoside (**42**) | OH | OH | H | *p*-coumaroyl-glucose |
| Peonidin 3-O-*p*-coumaroylglucoside (**43**) | $OCH_3$ | OH | H | *p*-coumaroyl-glucose |
| Malvidin 3-O-acetylglucoside (**44**) | $OCH_3$ | OH | $OCH_3$ | acetyl-glucose |
| Cyanidin 3-O-acetylglucoside (**45**) | OH | OH | H | acetyl-glucose |
| Petunidin 3-O-acetylglucoside (**46**) | $OCH_3$ | OH | OH | acetyl-glucose |
| Petunidin 3-O-*p*-coumaroylglucoside (**47**) | $OCH_3$ | OH | OH | *p*-coumaroyl-glucose |




Cymene (**48**) D-limonene (**49**) Eucaliptol (**50**) γ-terpinene (**51**)



Terpinolene (**52**) α-farnesene (**53**)

**Fig. 1.** (*continued*).

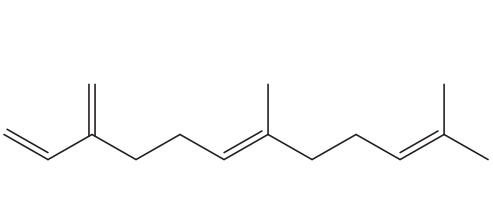
treatments was observed (Table 3 and Supplementary Table S3).

### 2.3.4. Abundance of sugars, alditols and organic acids

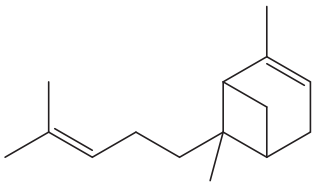
When the results were expressed as a percentage of the principal groups (monosaccharides, disaccharides, alditols and organic acids), it was observed that organic acids were the most abundant compounds in leaves, whereas monosaccharides and sucrose (**4**) were the most abundant compounds in berries and roots, respectively (Supplementary Tables S1, S2 and S3). The percentage of sucrose (**4**) in ABA (**1**) and $GA_3$ (**2**) treated leaves was higher compared to the control, thus reducing the percentage of monosaccharides in both treatments (Supplementary Table S1). In addition, alditols, especially inositol (**9**), were highly represented among the primary metabolites of leaves (Supplementary Table S1). In berries, ABA (**1**) treatment increased the percentage of organic acids as well as decreasing the percentage of sucrose (**4**) and monosaccharides compared to control and $GA_3$ (**2**) treatment (Supplementary Table S2). In roots, even though the percentage of monosaccharides was diminished in plants treated with ABA (**1**) and $GA_3$ (**2**), the abundance of sucrose (**4**) was higher than in the control (Supplementary Table S3).

### 2.3.5. Free amino acids

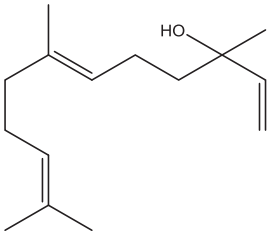
Supplementary Tables S4, S5 and S6 show the free amino acids (AAs) identified in leaf, berry and root respectively. In leaves, within the group of aliphatic AAs, the contents of the amino acids L-valine (**13**), L-leucine (**14**) as well as L-isoleucine (**15**) was decreased by ABA (**1**) and $GA_3$ (**2**) treatments (Supplementary Table S4). The same pattern was observed for aromatic AAs, where the contents of L-phenylalanine (**16**), L-tryptophan (**17**) and L-tyrosine (**18**) were



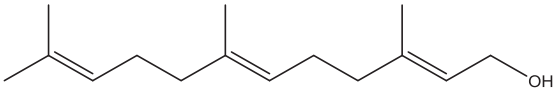
β-farnesene (**54**)



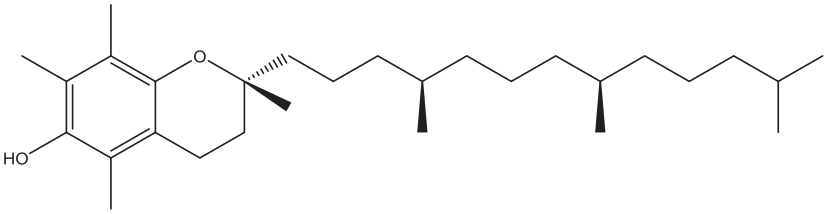
Bergamotene (**55**)



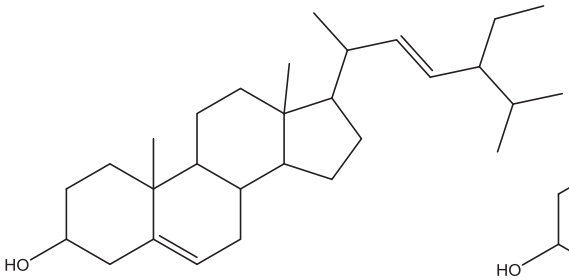

Nerolidol (**56**)



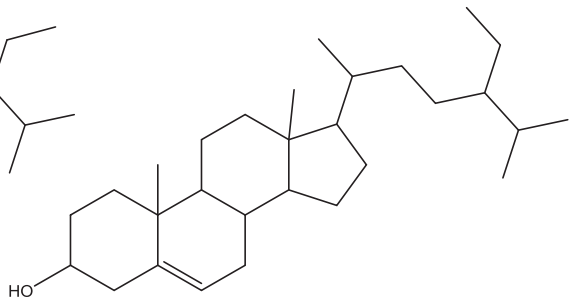

Farnesol (**57**)




α-tocopherol (**58**)



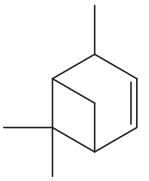

Stigmasterol (**59**)



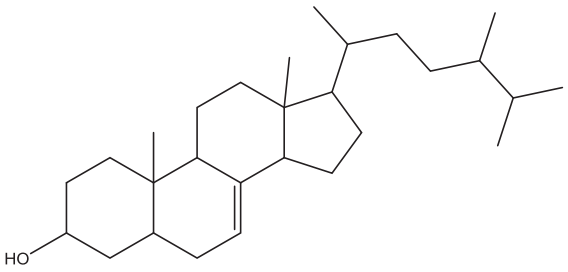

γ-sitosterol (**60**)

**Fig. 1.** (*continued*).

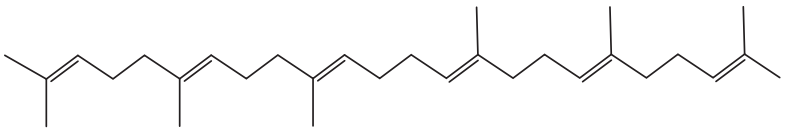
highly reduced by ABA (**1**) and $GA_3$ (**2**) treatments. Moreover, ABA (**1**) treated leaves displayed the lowest concentration of the aromatic AA, L-phenylalanine (**16**) (Table 1 and Supplementary Table S4). This result was correlated with higher anthocyanin contents (Table 1 and Supplementary Table S10); in fact both L-phenylalanine (**16**) and, in a lower proportion, L-tyrosine (**18**) serve as precursors of those secondary metabolites (Dias, 2003; Liang et al., 2011). On the other hand, applications with ABA (**1**) showed an increase in the content of the acidic AAs, L-glutamic (**19**) acid and L-asparagine (**20**), while there were no statistically significant differences in the rest of the acidic AAs. AAs such as L-glutamic acid (**19**), L-asparagine (**20**), L-aspartic acid (**21**), and L-glutamine (**22**) increased in amounts during water deficit stress and cold acclimation (Dionne et al., 2001; Harb et al., 2015; Thakur and Rai, 1982). Additionally, L-glutamic acid (**19**) is involved in the redox balance of the cells due to its role as a precursor of glutathione, L-proline (**3**) and polyamines, well known ROS scavengers (Kocsy et al., 2000; Kovács et al., 2011). Concerning the amino acid L-proline (**3**), the highest concentration was observed after ABA (**1**) treatment (Table 1 and Supplementary Table S4). It has been stated



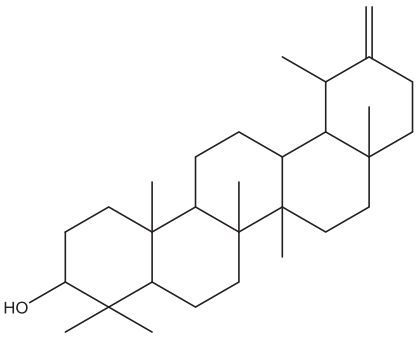
Pinene (**61**)



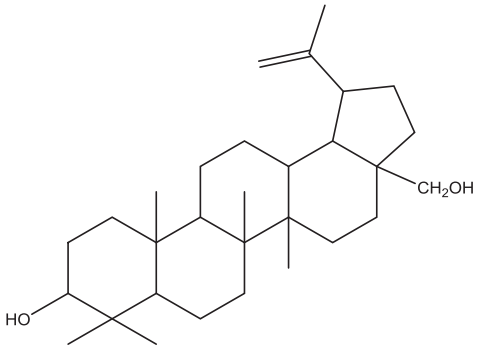

Ergostenol (**62**)



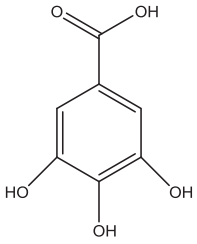
Squalene (**63**)



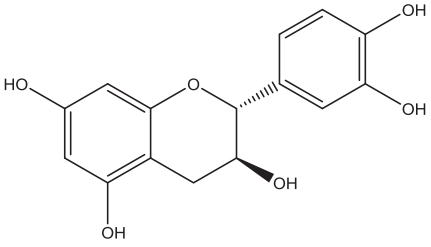

Taraxasterol (**64**)




Betuline (**65**)




Gallic acid (**66**)

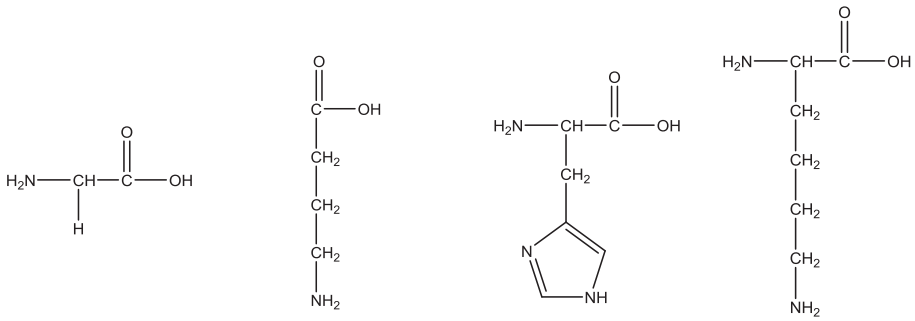



(+)-catechin (**67**)

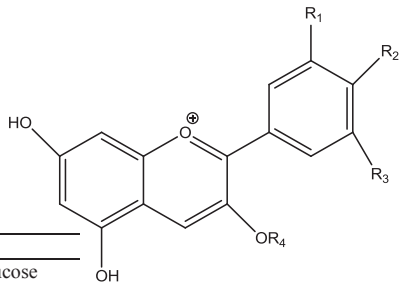
**Fig. 1.** (*continued*).

that the accumulation of L-proline (**3**), the amino acid related to water stress, may have a key role in plant tolerance (Joshi et al., 2010). In this sense, L-proline (**3**) can be mobilized for the synthesis of new proteins needed to cope with water deficit or alternatively serve as osmotic adjuster (Campalans et al., 1999).

Opposite to leaves, in berries, the content of aliphatic AAs, L-valine (**13**), L-leucine (**14**), L-isoleucine (**15**) and L-alanine (**23**) was highly increased by $GA_3$ (**2**) (Supplementary Table S5). Furthermore, a higher concentration of the aromatic AA, L-tyrosine (**18**), was observed in $GA_3$ (**2**) treated berries. This result was related to a low anthocyanin concentration in berries of $GA_3$ (**2**) treated plants (Table 2 and Supplementary Table S11), as it was explained above for ABA (**1**) treated leaves. Also, $GA_3$ (**2**) augmented the content of L-glutamic acid (**19**), L-asparagine (**20**) and L-aspartic acid (**21**) (Table 2 and Supplementary Table S5). Those amino acid concentrations increased as a consequence of elevated content of ABA (**1**) in $GA_3$ (**2**) treated berries due to an increase in relative expression of *VvNCED1* (Figs. 3b and 4b). *VvNCED1* is a key gene in the ABA (**1**) biosynthetic pathway, which codes for the enzyme 9-*cis*-epoxycarotenoid dioxygenase. This enzyme cleaves either 9-*cis*-neoxanthin or 9-*cis*-violaxanthin or both to produce xanthoxin, the direct C15 precursor of ABA (**1**) (Schwartz et al., 2003). On the other



L-glycine (**68**) GABA (**69**) L-histidine (**70**) L-lysine (**71**)



| Compound | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| Delphinidin 3-O-acetylglucoside (**72**) | OH | OH | OH | acetyl-glucose |
| Peonidin 3-O-acetylglucoside (**73**) | $OCH_3$ | OH | H | acetyl-glucose |
| Delphinidin 3-O-*p*-coumaroylglucoside (**74**) | OH | OH | OH | *p*-coumaroyl-glucose |

**Fig. 1.** (*continued*).

hand, the highest contents of the sulfur-containing AA L-methionine (**24**) and the cyclic AA, L-proline (**3**), were registered after $GA_3$ (**2**) treatment (Supplementary Table S5). The latter result could be due to an up-regulation of *VvNCED1* and its concomitant ABA (**1**) increase (Figs. 3b and 4b).

In roots, it was observed that both PGRs decreased the content of aliphatic AAs (L-valine (**13**), L-leucine (**14**), L-isoleucine (**15**), L-alanine (**23**), and L-threonine (**25**)) (Supplementary Table S6), as noted also for leaves. Moreover, the concentration of the aromatic AA, L-phenylalanine (**16**), was also reduced by ABA (**1**) and $GA_3$ (**2**) treatments. The same pattern was shown for acidic AAs (L-asparagine (**20**) and L-aspartic acid (**21**)), sulfur AA (L-methionine (**24**)) and the cyclic AA, L-proline (**3**) (Supplementary Table S6). These results suggest that ABA (**1**) and $GA_3$ (**2**) may induce translocation of organic nitrogen compounds to the aerial part or may inhibit hydrolysis of proteins by proteases in roots.

The highest content of free AAs in leaves was found in plants treated with ABA (**1**), whereas the highest content in berries and roots was observed in $GA_3$ (**2**) and control treatments, respectively (Supplementary Tables S4, S5 and S6). The high free amino acids levels in leaves of plants treated with ABA (**1**), may be the result of protein degradation by proteases (Good and Zaplachinski, 1994), which are activated during water deficit (Less and Galili, 2008) to produce osmolytes (Rai, 2002). However, the high amino acid contents in berries of plants treated with $GA_3$ (**2**) may be related to the high ABA (**1**) concentration, due to an increase in relative expression of *VvNCED1* (Figs. 3b and 4b). When the data were expressed as percentage, the main group present in all tissues was the acidic AAs (Supplementary Tables S4, S5 and S6). However, the contents of L-proline (**3**) and aliphatic AAs were also important in berry and root tissues, respectively (Supplementary Tables S5 and S6). In fact, the high abundance of L-proline (**3**) in grapevine berries at veraison may be related to a peak in ABA (**1**) concentration (Fillion et al., 1999). The acidic AAs were higher in ABA (**1**) treated plants, independent of tissue (Supplementary Tables S4, S5 and S6). It was also observed that applications with ABA (**1**) increased the proportion of L-proline (**3**) in leaves, while in berries and roots a decrease in L-proline (**3**) level was recorded in relation to the other treatments (Supplementary Tables S4, S5 and S6). Moreover, the percentage of aromatic AAs was enhanced in berries treated with ABA (**1**) (Supplementary Table S5). On the other hand, the control plants had the highest percentage of aliphatic AAs in leaves and roots (Supplementary Tables S4 and S6). Grapevine plants showed the highest percentage of aliphatic AAs in their berries after $GA_3$ (**2**) treatment, (Supplementary Table S5).

### *2.4. ABA (1) and $GA_3$ (2) modify secondary metabolites in grapevine leaves, berries and roots*

#### *2.4.1. Low molecular weight polyphenols (LMWPPs)*

Polyphenols are involved in abiotic stress alleviation, especially in coping with increased ROS and harmful UV-B radiation levels (Berli et al., 2010; Frohnmeyer and Staiger, 2003). In addition, it has been found in grapevine leaves that genes involved in the phenylpropanoid biosynthetic pathway were up-regulated immediately after treatment with UV-B (Pontin et al., 2010). There is also evidence that ABA (**1**) induces the non-enzymatic defense system, increasing the level of antioxidant molecules (Jiang and Zhang, 2002).

Supplementary Tables S7, S8, and S9 show LMWPPs contents in leaves, berries and roots of grapevine plants, respectively. The LMWPPs were divided into 5 groups named: hydroxybenzoic acids, hydroxycinnamic acids, flavanols, flavonols and other compounds (OH-tyrosol (**5**)). In leaves, no hydroxybenzoic acids were detected (Supplementary Table S7). However, within the group of hydroxycinnamic acids, applications with $GA_3$ (**2**) reduced the contents of caftaric acid (**26**), caffeic acid (**27**) and *p*-coumaric acid (**28**) compared to the control (Supplementary Table S7). Furthermore, the concentration of the flavanol, (−)-epigallocatechin gallate (**29**), was highly increased by $GA_3$ (**2**) treatment, whereas it was

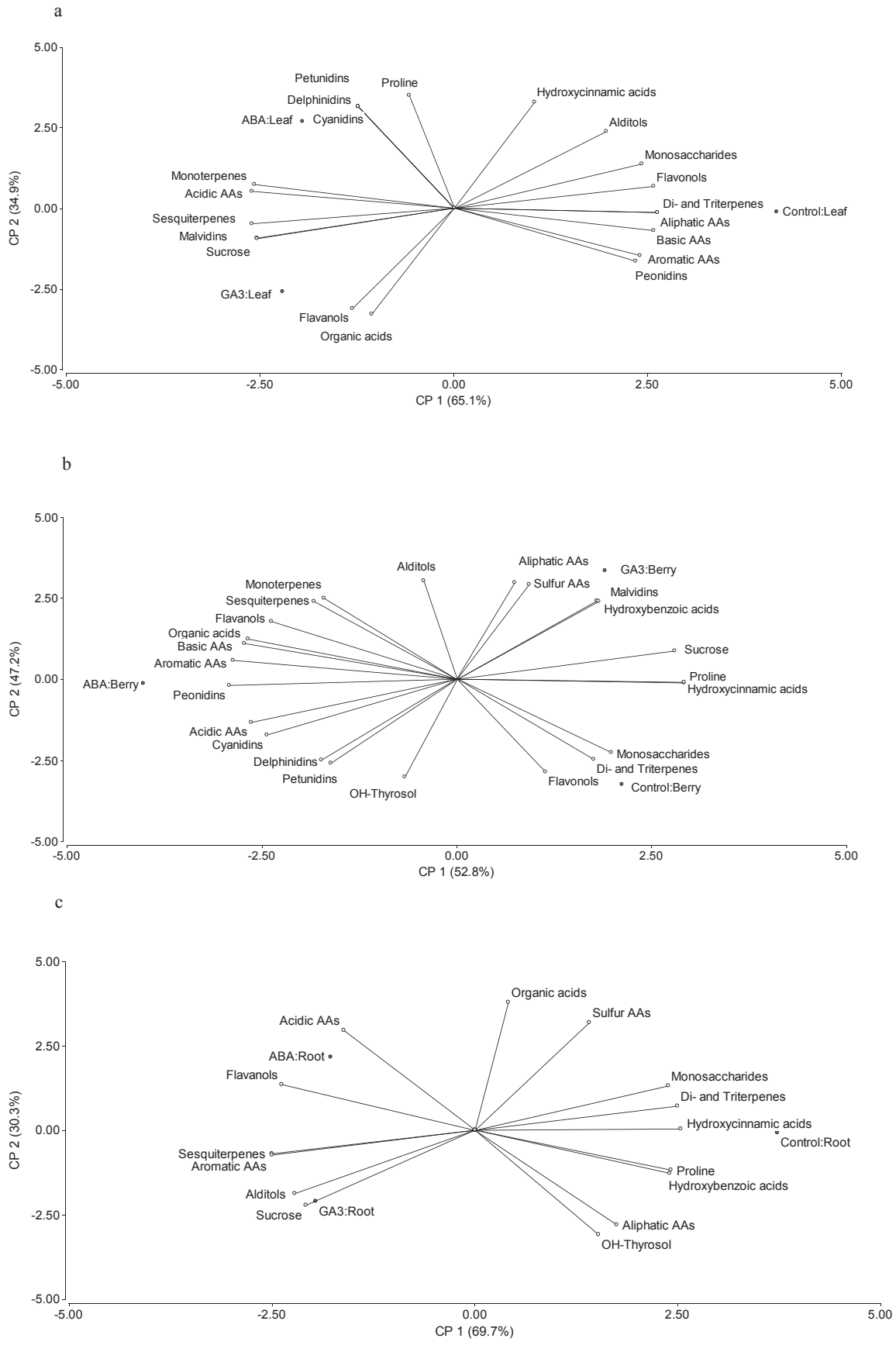



**Fig. 2.** Biplot display of the principal component analysis (PCA) of the main groups of metabolites analyzed in leaves (a), berries (b) and roots (c) of *Vitis vinifera* cv. Malbec at full veraison (70 DAA in control plants, 63 DAA in ABA (**1**) plants and 75 DAA in $GA_3$ (**2**) plants).

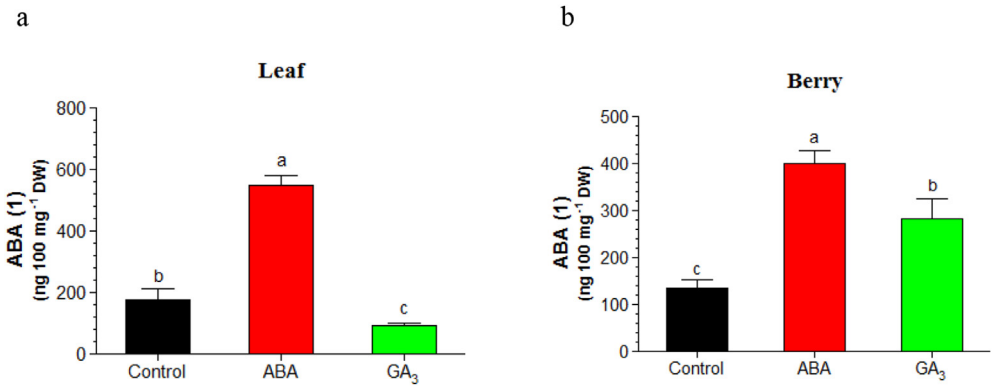



**Fig. 3.** ABA (**1**) content assessed by GC-MS in leaves (a) and berries (b) of *Vitis vinifera* cv. Malbec at full veraison (70 DAA in control plants, 63 DAA in ABA (**1**) plants and 75 DAA in $GA_3$ (**2**) plants). Values are means ± SE, n = 4. Different letters indicate significant differences ($p < 0.05$).

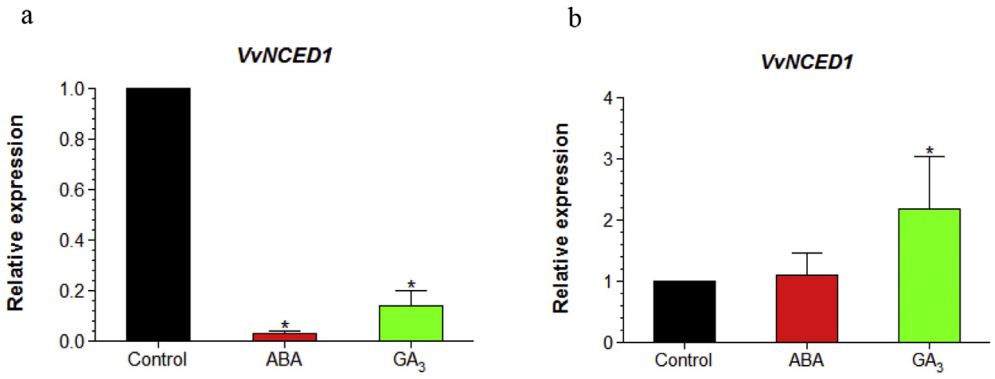



**Fig. 4.** Relative gene expression of *VvNCED1*, in leaves (a) and berries (b) of *Vitis vinifera* cv. Malbec at full veraison (70 DAA in control plants, 63 DAA in ABA (**1**) plants and 75 DAA in $GA_3$ (**2**) plants). Values are means ± SE, n = 4. Asterisks indicate statistically significant differences by the permutation test ($p < 0.05$). All values were normalized to the expression of *VvEF 1-α*.

decreased by ABA (**1**) (Supplementary Table S7). On the other hand, there were no statistically significant differences among the treatments on the content of the flavanol (−)-gallocatechin gallate (**30**). Leaves treated with $GA_3$ (**2**) had lower contents of both flavonols, quercetin-3-glucoside (**31**) and kaempferol-3-glucoside (**32**), while leaves treated with ABA (**1**) had a reduced concentration of kaempferol-3-glucoside (**32**), compared to the control (Supplementary Table S7). Berli et al. (2010) studying the effect of UV-B radiation on grapevine leaf tissues, observed an induction and accumulation of quercetin-3-glucoside (**31**) and hydroxycinnamic acids. However, these results were not confirmed in the studies here in, no modifications were found in the levels of those compounds (Supplementary Table S7). Presumably, ABA (**1**) does not act downstream in UV-B signaling in LMWPPs synthesis, or ABA (**1**) stimulates the biosynthesis of anthocyanins and terpenes in leaves (Supplementary Tables S10 and S12), instead of the one of LMWPPs.

In berries (Supplementary Table S8), there were no significant differences between PGR treatments and the control for the *p*-coumaric (**28**) and syringic acids (**33**). Applications with ABA (**1**) and $GA_3$ (**2**) decreased the content of the flavanols, (−)-epicatechin (**34**) and (−)-gallocatechin (**35**), respectively, while there were no statistically significant differences for flavonol compounds and OH-tyrosol (**5**).

In roots (Supplementary Table S9), the only statistically significant difference was in flavanol (−)-epigallocatechin gallate (**29**) levels, whose concentration increased after both PGR treatments.

Expressing the results as percentages, the most abundant groups were the hydroxycinnamic acids in leaves and the flavanols in berries and roots (Supplementary Tables S7, S8, and S9). In leaves, $GA_3$ (**2**) treatment induced an increment in the percentage of flavanols (Supplementary Table S7). In berries, ABA (**1**) applications reduced the percentage of hydroxycinnamic acids and flavonols, while increasing the flavanols (Supplementary Table S8). Furthermore, $GA_3$ (**2**) treatment reduced the proportion of flavonols, but increased the proportion of hydroxybenzoic acids, as compared to the control (Supplementary Table S8). In roots, both PGR applications increased the percentage of flavanols, while reducing the percentage of hydroxybenzoic acids and hydroxycinnamic acids (Supplementary Table S9).

### 2.4.2. Anthocyanins

Supplementary Tables S10 and S11 show the content in leaves and berries of anthocyanins divided into glycosylated, acetylated and *p*-coumarylated components. In leaves (Table 1 and Supplementary Table S10), in the control and $GA_3$ (**2**) treatment, only malvidin- (**36**) and peonidin-3-glucoside (**37**) were present, with no differences among treatments. However, ABA (**1**) treatment induced the biosynthesis of a large amount of anthocyanins: the glycosylated anthocyanins, malvidin (**36**), peonidin (**37**), delphinidin (**38**), cyanidin (**39**) and petunidin (**40**) as well as the *p*-coumarylated anthocyanins malvidin (**41**), cyanidin (**42**) and peonidin (**43**).

Liang et al. (2008) claimed that malvidin derivatives are the main anthocyanins in berries of *Vitis vinifera,* in agreement with the results here in. In that organ (Table 2 and Supplementary Table S11), $GA_3$ (**2**) applications decreased the content of most

**Table 1**
Primary and secondary metabolite levels assessed in leaves of *Vitis vinifera* L. cv. Malbec measured at the stage of full veraison (70 DAA in control plants, 63 DAA in ABA (**1**) and 75 DAA in $GA_3$ (**2**) treated plants). Values are means ± SE, n = 5. Different letters indicate significant differences ($p < 0.05$). Nd: not detected. fr. wt: fresh weight. G: glycosylated. p-cou: p-coumarilated.

| Metabolite | Leaf | | |
|---|---|---|---|
| | Control | ABA | $GA_3$ |
| **Primary metabolites** | | | |
| **Disaccharide** (mg $g^{-1}$ fr. wt) | | | |
| Sucrose (**4**) | 3.15 ± 0.52 *b* | 6.50 ± 0.09 *a* | 7.33 ± 1.39 *a* |
| **Alditol** (mg $g^{-1}$ fr. wt) | | | |
| Inositol (**9**) | 8.01 ± 0.72 *a* | 6.74 ± 0.42 *ab* | 5.42 ± 0.20 *b* |
| **Organic acid** (mg $g^{-1}$ fr. wt) | | | |
| Citric acid (**12**) | 0.16 ± 0.02 *b* | 0.13 ± 0.02 *b* | 1.05 ± 0.10 *a* |
| **Amino acids** (ng $mg^{-1}$ fr. wt) | | | |
| L-Proline (**3**) | 11.56 ± 1.13 *b* | 33.39 ± 1.07 *a* | 8.27 ± 3.50 *b* |
| L-Phenylalanine (**16**) | 39.49 ± 1.47 *a* | 6.63 ± 1.30 *c* | 18.43 ± 4.11 *b* |
| L-Tyrosine (**18**) | 11.47 ± 1.57 *a* | 3.84 ± 0.96 *b* | 4.44 ± 1.06 *b* |
| L-Glutamic acid (**19**) | 109.16 ± 16.75 *b* | 271.98 ± 40.22 *a* | 91.96 ± 26.44 *b* |
| L-Asparagine (**20**) | 2.37 ± 0.16 *b* | 4.74 ± 0.92 *a* | 1.47 ± 0.46 *b* |
| L-Aspartic acid (**21**) | 414.60 ± 24.74 *a* | 773.74 ± 138.50 *a* | 486.56 ± 119.58 *a* |
| **Secondary metabolites** | | | |
| **Anthocyanins** (μg $mg^{-1}$ fr. wt) | | | |
| Malvidin-3-G (**36**) | 0.83 ± 0.30 *b* | 4.68 ± 1.72 *a* | 1.30 ± 0.69 *b* |
| Peonidin-3-G (**37**) | 0.6 ± 0.37 *b* | 1.09 ± 0.28 *a* | 0.49 ± 0.13 *b* |
| Delphinidin-3-G (**38**) | Nd | 0.29 ± 0.07 | Nd |
| Cyanidin-3-G (**39**) | Nd | 0.23 ± 0.07 | Nd |
| Petunidin-3-G (**40**) | Nd | 0.33 ± 0.10 | Nd |
| Malvidin-*p*-cou (**41**) | Nd | 1.41 ± 1.16 | Nd |
| Cyanidin-*p*-cou (**42**) | Nd | 0.48 ± 0.35 | Nd |
| Peonidin-*p*-cou (**43**) | Nd | 0.41 ± 0.16 | Nd |
| **Terpenes** (ng $mg^{-1}$ fr. wt) | | | |
| Cymene (**48**) | Nd | 41.34 ± 4.15 *a* | 16.12 ± 3.57 *b* |
| D-Limonene (**49**) | Nd | 55.41 ± 4.88 *a* | 36.36 ± 9.49 *a* |
| Eucalyptol (**50**) | Nd | 26.36 ± 3.43 *a* | 19.46 ± 4.64 *a* |
| γ-Terpinene (**51**) | Nd | 425.90 ± 39.07 *a* | 248.22 ± 62.13 *a* |
| Terpinolene (**52**) | Nd | 9526.01 ± 827.34 *a* | 6981.09 ± 1711.06 *a* |
| α-Farnesene (**53**) | Nd | 44.76 ± 10.49 *a* | 12.13 ± 0.60 *b* |
| β-Farnesene (**54**) | Nd | 147.96 ± 34.76 *a* | 19.31 ± 5.29 *b* |
| Bergamotene (**55**) | Nd | 27.20 ± 5.72 *a* | 3.84 ± 0.39 *b* |
| Nerolidol (**56**) | Nd | 3903.48 ± 586.12 *a* | 5278.33 ± 2297.05 *a* |
| Farnesol (**57**) | Nd | 4229.93 ± 391.64 *a* | 3721.05 ± 961.36 *a* |
| α-Tocopherol (**58**) | 643.58 ± 84.62 *a* | 372.64 ± 71.60 *b* | 213.49 ± 33.33 *b* |
| **Membrane sterols** (ng $mg^{-1}$ fr. wt) | | | |
| Stigmasterol (**59**) | 30.73 ± 0.92 *a* | 14.45 ± 1.59 *b* | 9.46 ± 0.59 *c* |
| γ-Sitosterol (**60**) | 372.76 ± 24.17 *a* | 185.61 ± 18.04 *b* | 146.69 ± 4.99 *b* |

anthocyanins, except for the glycosylated malvidin (**36**) and peonidin (**37**), the acetylated and *p*-coumarylated malvidin (**44** and **41**), cyanidin (**45** and **42**) and petunidin (**46** and **47**). In this sense, $GA_3$ (**2**) seemed to impair the biosynthesis of anthocyanins in berries, in correspondence with the high level of L-tyrosine (**18**) detected (Table 2 and Supplementary Table S5). Similar results were obtained in apple skins, where applications with $GA_3$ (**2**) delayed ripening and anthocyanin accumulation (Awad and De Jager, 2002). It has been confirmed, in *Arabidopsis* plants, that gibberellins act as negative regulators of the expression of the transcription factors *AtMYB75* and *AtMYB90*, which bind to the promoter region of *AtUFGT* (UDP-glucose: flavonoid 3-*O*-glucosyltransferase), a key gene for anthocyanins synthesis and its accumulation (Loreti et al., 2008). A similar mechanism may be happening here. On the other hand, $GA_3$ (**2**) treatment highly enhanced the proportion of *p*-coumarylated anthocyanins in berries, while reducing the percentage of acetylated ones (Supplementary Table S11). $GA_3$ (**2**) treatment highly increased the percentage of malvidins as well, reducing the proportion of delphinidins, cyanidins and petunidins as compared to the control (Supplementary Fig. S1), while no differences in peonidin percentages were observed (Supplementary Fig. S1).

After ABA (**1**) treatment, only the content of petunidin-3-glucoside (**40**) was reduced, while no significant differences were observed for the other anthocyanins in berries. There is extensive literature on the effect of abiotic stress, such as water stress, on anthocyanin biosynthesis and accumulation in grapevine berries (Kennedy et al., 2002; Koundouras et al., 2006; Ojeda et al., 2002). In the present work, no differences in anthocyanin levels were found between control and ABA (**1**) applications; however, ABA (**1**) anticipated ripening, and color development was achieved 7 days earlier than control. Therefore, it can be suggested that ABA (**1**) is involved in abiotic stress alleviation by synthesizing anthocyanins (antioxidant compounds). On the other hand, in grapevine plant root tissue, no anthocyanins were detected.

### 2.4.3. Terpenes

Terpenes are secondary metabolites with antioxidant properties playing a protective role against abiotic (Alonso et al., 2015; Gil et al., 2012; Kolb et al., 2001; Leicach et al., 2010) and biotic stresses (Escoriaza et al., 2013; Leitner et al., 2008; Pontin et al., 2015). However, there is scarce information regarding the effect of ABA (**1**) and $GA_3$ (**2**) on terpene contents in different grapevine tissues. In this sense, Alonso et al. (2015) found that ABA (**1**) applications to grapevine plants increased the content of mono- and sesquiterpenes in leaves.

Supplementary Table S12 shows the mono-, sesqui-, di- and triterpenes content in leaves of grapevine plants. ABA (**1**) and $GA_3$

**Table 2**
Primary and secondary metabolite levels assessed in berries of *Vitis vinifera* L. cv. Malbec measured at the stage of full veraison (70 DAA in control plants, 63 DAA in ABA (**1**) and 75 DAA in $GA_3$ (**2**) treated plants). Values are means ± SE, n = 5. Different letters indicate significant differences ($p < 0.05$). Nd: not detected. fr. wt: fresh weight. G: glycosylated. acet: acetylated. p-cou: p-coumarilated.

| Metabolite | Berry | | |
|---|---|---|---|
| | Control | ABA | $GA_3$ |
| **Primary metabolites** | | | |
| **Monosaccharides** (mg $g^{-1}$ fr. wt) | | | |
| Glucose (**6**) | 75.43 ± 1.69 *a* | 54.61 ± 0.72 *b* | 61.72 ± 7.23 *ab* |
| Fructose (**7**) | 73.40 ± 1.59 *a* | 55.76 ± 0.86 *b* | 62.09 ± 6.43 *ab* |
| **Disaccharide** (mg $g^{-1}$ fr. wt) | | | |
| Sucrose (**4**) | 8.82 ± 0.89 *a* | 4.36 ± 0.21 *b* | 8.63 ± 0.70 *a* |
| **Organic acid** (mg $g^{-1}$ fr. wt) | | | |
| Malic acid (**11**) | 8.80 ± 0.77 *b* | 13.84 ± 0.20 *a* | 11.26 ± 1.31 *ab* |
| **Amino acids** (ng $mg^{-1}$ fr. wt) | | | |
| L-Proline (**3**) | 142.82 ± 3.66 *b* | 62.21 ± 31.95 *b* | 406.71 ± 107.87 *a* |
| L-Phenylalanine (**16**) | 11.01 ± 0.99 *a* | 10.64 ± 3.15 *a* | 19.22 ± 8.22 *a* |
| L-Tyrosine (**18**) | 1.75 ± 1.05 *b* | 4.74 ± 3.41 *b* | 19.89 ± 7.97 *a* |
| L-Glutamic acid (**19**) | 39.19 ± 9.58 *b* | 33.83 ± 14.77 *b* | 78.71 ± 11.12 *a* |
| L-Asparagine (**20**) | 1.15 ± 0.44 *b* | 2.16 ± 1.18 *ab* | 4.30 ± 1.05 *a* |
| L-Aspartic acid (**21**) | 165.25 ± 45.64 *b* | 96.01 ± 28.49 *b* | 338.23 ± 51.88 *a* |
| L-Glutamine (**22**) | 1.47 ± 1.18 *a* | 3.81 ± 2.90 *a* | 7.38 ± 3.43 *a* |
| **Secondary metabolites** | | | |
| **Anthocyanins** (μg $mg^{-1}$ fr. wt) | | | |
| Malvidin-3-G (**36**) | 2077.48 ± 118.28 *a* | 1627.24 ± 238.51 *a* | 1872.72 ± 148.49 *a* |
| Delphinidin-3-G (**38**) | 861.67 ± 19.58 *a* | 791.31 ± 54.95 *a* | 336.46 ± 8.47 *b* |
| Cyanidin-3-G (**39**) | 94.34 ± 11.32 *a* | 104.65 ± 13.75 *a* | 36.25 ± 1.34 *b* |
| Petunidin-3-G (**40**) | 716.78 ± 22.98 *a* | 614.90 ± 26.99 *b* | 358.47 ± 2.60 *c* |
| Cyanidin-*p*-cou (**42**) | 6.95 ± 1.58 *b* | 7.79 ± 5.81 *b* | 26.57 ± 7.65 *a* |
| Peonidin-*p*-cou (**43**) | 181.85 ± 22.70 *a* | 129.98 ± 15.54 *ab* | 110.42 ± 3.32 *b* |
| Cyanidin-3-acet (**45**) | 15.94 ± 1.12 *a* | 19.97 ± 2.22 *a* | Nd |
| Petunidin-3-acet (**46**) | 169.03 ± 9.99 *a* | 146.45 ± 16.23 *a* | 48.44 ± 4.18 *b* |
| Delphinidin-3-acet (**72**) | 164.52 ± 8.64 *a* | 156.65 ± 22.18 *a* | 31.92 ± 4.89 *b* |
| Peonidin-3-acet (**73**) | 17.97 ± 2.68 *ab* | 21.49 ± 1.30 *a* | 11.13 ± 1.06 *b* |
| Delphinidin-*p*-cou (**74**) | 115.68 ± 13.83 *a* | 89.11 ± 9.06 *a* | 43.67 ± 4.99 *b* |
| **Terpenes** (ng $mg^{-1}$ fr. wt) | | | |
| γ-Terpinene (**51**) | Nd | 1.02 ± 0.29 *a* | 0.73 ± 0.21 *a* |
| Terpinolene (**52**) | Nd | 27.00 ± 6.23 *a* | 23.38 ± 4.40 *a* |
| Nerolidol (**56**) | Nd | 27.12 ± 2.08 *a* | 22.28 ± 4.29 *a* |
| Pinene (**61**) | Nd | 3.27 ± 0.87 *a* | 3.40 ± 1.10 *a* |

**Table 3**
Primary and secondary metabolite levels assessed in roots of *Vitis vinifera* L. cv. Malbec measured at the stage of full veraison (70 DAA in control plants, 63 DAA in ABA (**1**) and 75 DAA in $GA_3$ (**2**) treated plants). Values are means ± SE, n = 5. Different letters indicate significant differences ($p < 0.05$). Nd: not detected. fr. wt: fresh weight.

| Metabolite | Root | | |
|---|---|---|---|
| | Control | ABA | **$GA_3$** |
| **Primary metabolites** | | | |
| **Monosaccharides** (mg $g^{-1}$ fr. wt) | | | |
| Glucose (**6**) | 10.86 ± 0.55 *a* | 6.01 ± 0.22 *b* | 4.15 ± 0.12 *c* |
| Fructose (**7**) | 4.34 ± 0.11 *a* | 1.95 ± 0.06 *c* | 2.70 ± 0.10 *b* |
| **Secondary metabolites** | | | |
| **Terpenes** (ng $mg^{-1}$ fr. wt) | | | |
| Nerolidol (**56**) | Nd | 62.83 ± 10.59 *a* | 101.58 ± 21.01 *a* |
| α-Tocopherol (**58**) | 8.03 ± 3.73 *b* | 18.93 ± 3.13 *ab* | 28.31 ± 2.75 *a* |
| **Membrane sterols** (ng $mg^{-1}$ fr. wt) | | | |
| Stigmasterol (**59**) | 30.68 ± 5.12 *b* | 67.73 ± 9.82 *a* | 69.88 ± 7.35 *a* |
| γ-Sitosterol (**60**) | 93.48 ± 20.11 *b* | 176.60 ± 24.71 *a* | 224.27 ± 35.11 *a* |
| Ergostenol (**62**) | 13.85 ± 2.77 *b* | 24.03 ± 3.74 *ab* | 30.57 ± 4.06 *a* |
| Squalene (**63**) | 3.93 ± 0.85 *b* | 329.59 ± 44.36 *a* | 504.77 ± 95.99 *a* |
| Taraxasterol (**64**) | 37.09 ± 15.28 *b* | 162.61 ± 19.00 *a* | 99.18 ± 29.69 *a* |
| Betuline (**65**) | 0.75 ± 0.08 *c* | 10.37 ± 1.64 *a* | 4.42 ± 0.73 *b* |

(**2**) applications induced the biosynthesis of mono- (cymene (**48**), D-limonene (**49**), eucalyptol (**50**), γ-terpinene (**51**) and terpinolene (**52**)) and sesquiterpenes (α-farnesene (**53**), β-farnesene (**54**), bergamotene (**55**), nerolidol (**56**) and farnesol (**57**)). No statistically significant differences in monoterpene contents were observed among the treatments with the exception of cymene (**48**), whose levels increased after ABA (**1**) applications (Table 1). However, in leaves that were ABA (**1**) treated, the content of sesquiterpenes α-farnesene (**53**), β-farnesene (**54**) and bergamotene (**55**) was higher, while the content of the diterpene α-tocopherol (**58**) and the membrane sterols (triterpenes) stigmasterol (**59**) and γ-sitosterol (**60**) decreased by ABA (**1**) and $GA_3$ (**2**) treatments (Table 1).

In berries (Table 2 and Supplementary Table S13), as for leaves, ABA (**1**) and $GA_3$ (**2**) applications induced *de novo* biosynthesis of mono- (γ-terpinene (**51**), terpinolene (**52**) and pinene (**61**)) and sesquiterpenes (nerolidol (**56**)), while no significant differences were observed among treatments. On the other hand, the concentration of stigmasterol (**59**) and ergostenol (**62**) was higher in $GA_3$ (**2**) treated berries, as was the content of ergostenol (**62**) after ABA (**1**) treatment. The triterpene γ-sitosterol (**60**) was, however, detected only in control plants.

In roots (Table 3 and Supplementary Table S14), the concentration of the sesquiterpene nerolidol (**56**), and the di- and triterpenes, α-tocopherol (**58**), stigmasterol (**59**), γ-sitosterol (**60**), ergostenol (**62**), squalene (**63**), taraxasterol (**64**) and betuline (**65**) was highly increased by ABA (**1**) and $GA_3$ (**2**). Moreover, applications with ABA (**1**) caused the highest concentration of betuline (**65**).

Among the different tissues, the highest terpene concentration during veraison was observed in leaves, followed by root and berries (Supplementary Tables S12, S13 and S14). The percentage of monoterpenes, in leaves and berries, increased with ABA (**1**) and $GA_3$ (**2**) applications, whereas no monoterpenes were detected in

any tissues of control plants. The proportion of sesquiterpenes varied with both tissue and the treatment, being more abundant in $GA_3$ (**2**) treated leaves (53%) (Supplementary Table S12); di- and triterpenes were found in both treatments and also in the control (Supplementary Tables S12, S13 and S14).

As no mono- or sesquiterpenes could be detected in the control plants, the percentage of di- and triterpenes was analyzed separately in each tissue (Supplementary Fig. S2). In leaves, $GA_3$ (**2**) treatment caused an increase in the percentage of squalene (**63**) compared to the control, while the proportion of α-tocopherol (**58**) was reduced (Supplementary Fig. S2a). In berries, the percentage of squalene (**63**) was increased by ABA (**1**) and $GA_3$ (**2**) (Supplementary Fig. S2b); furthermore, both PGR applications caused an increment in the percentage of α-tocopherol (**58**), stigmasterol (**59**) and ergostenol (**62**), but not of γ-sitosterol (**60**) (Supplementary Fig. S2b). In roots, the squalene (**63**) content was highly influenced by the PGR as also found in berries (Supplementary Fig. S2c). In roots, the percentage of α-tocopherol (**58**), stigmasterol (**59**), γ-sitosterol (**60**) and ergostenol (**62**) increased in control plants (Supplementary Fig. S2c), while in $GA_3$ (**2**) treated roots the percentage of taraxasterol (**64**) decreased.

From these results, ABA (**1**) seems to have a crucial role as a signal molecule, mediating terpene synthesis in all assessed organs. In this regard, Gil et al. (2012) found that high ABA (**1**) levels elicited by UV-B radiation induced enhancement of mono- and sesquiterpene contents in leaves. Concerning gibberellins, Hong et al. (2012) demonstrated that the expression of *TPS21* and *TPS11*, two members of sesquiterpene synthases of *Arabidopsis*, were up-regulated in presence of $GA_3$ (**2**) and jasmonate. They showed that MYC2, a basic helix-loop-helix transcription factor, directly binds to promoters of *TPS21* and *TPS11*, and activates their expression. Furthermore, DELLAs proteins directly interact with MYC2. Thus, the action of gibberellin or jasmonate induced degradation of DELLAs, increasing sesquiterpene biosynthesis. In this sense, applications with $GA_3$ (**2**) on grapevine plants may induce a similar mechanism, resulting in high levels of mono- and sesquiterpenes in both leaf and berry tissues and only nerolidol (**56**) as sesquiterpenes in root.

Tocopherols are antioxidant compounds which prevent photooxidative deterioration of unsaturated fatty acids, lipids and lipoproteins in the cell membrane of plants by ROS detoxification (Fahrenholtz et al., 1974; Neely et al., 1988). Membrane sterols regulate fluidity and permeability of membranes to allow coping with abiotic stress (Berli et al., 2010; Gil et al., 2012). Vögeli and Chapell (1988) found that a fungal elicitor activated the enzyme sesquiterpene cyclase and suppressed the activity of squalene synthase, the first step in synthesis of sterols. Therefore, it can be hypothesized that this mechanism could occur in leaves of plants treated with ABA (**1**) and $GA_3$ (**2**), where low levels of α-tocopherol (**58**), stigmasterol (**59**), γ-sitosterol (**60**), ergostenol (**62**), and high levels of sesquiterpenes were observed compared to the control. However, in berries, no significant differences between treatments in content of α-tocopherol (**58**) and sterols were detected. In roots, α-tocopherol (**58**) and sterol levels were higher in ABA (**1**) and $GA_3$ (**2**) treatments compared to the control, whereas sesquiterpene concentrations were lower in berries and roots compared to leaves. According to this data, it is possible that ABA (**1**) and $GA_3$ (**2**) activate oxidative stress protection instead of membrane stability, and this effect is organ dependent. Altogether, the results here suggest that ABA (**1**) and $GA_3$ (**2**) act as signal molecules in abiotic stress alleviation in grapevine plants cv. Malbec.

## 3. Conclusions

To our knowledge, this is the first report investigating the effect of ABA (**1**) and $GA_3$ (**2**) on primary and secondary metabolite levels in leaves, berries and roots of grapevine plants at veraison. Both plant growth regulators elicited a response in all assessed tissues. ABA (**1**) seemed to play a major role in leaf protection, since this phytohormone elicited synthesis of a large amount of compounds against osmotic (L-proline (**3**)), oxidative (acidic AAs, anthocyanins, terpenes) and pathogen related (terpenes) stresses. On the other hand, some compounds synthesized after $GA_3$ (**2**) application, such as L-proline (**3**) and acidic AAs in berries, could be correlated to the high endogenous ABA (**1**) content of that tissue. However, ABA (**1**) and $GA_3$ (**2**) seem to affect independently the synthesis of polyphenols and terpenes. In the case of terpenes, both phytohormones induced synthesis of sesquiterpenes in leaves, possibly at the expense of membrane sterols. Thus ABA (**1**) and $GA_3$ (**2**) may stimulate antioxidant damage protection instead of membrane stability. In conclusion, ABA (**1**) and $GA_3$ (**2**) applications to grapevine plants cv. Malbec affect the synthesis of primary and secondary metabolites in leaves, berries and roots. These treatments could thus alert and prepare plants to cope with biotic and abiotic stresses.

## 4. Experimental

### 4.1. Plant material and experimental conditions

Cuttings of *Vitis vinifera* L. cv. Malbec were obtained from one-year-old cane-pruned cv. Malbec shoots collected from an experimental vineyard at INTA-Mendoza (Mendoza, Argentina). Cuttings were treated as explained in Murcia et al. (2016), then used for the experiments.

The assay was set in a random design with three treatments and five replicates per treatment. The samples were taken at veraison, (100% colored berries, onset of ripening), using individual plants as experimental units. Treatments consisted of application of ABA (**1**), $GA_3$ (**2**) and $H_2O$ (control) solutions with a weekly frequency from fruit set (10 days after anthesis, DAA) until full veraison, which occurred at 70 DAA in control plants, 63 DAA in ABA (**1**) treated plants, and 75 DAA in $GA_3$ (**2**) treated plants, respectively. Solutions were sprayed with a hand-held sprayer onto the whole plant (leaves and bunches) until runoff, during late afternoon to minimize ABA (**1**) photodegradation. Treatment doses were: 250 μg $mL^{-1}$ ABA (**1**) (±—S-*cis*, *trans* abscisic acid, PROTONE SL, Valent BioSciences, Libertyville, IL, USA), 500 μg $mL^{-1}$ $GA_3$ (**2**) (GIBERELINA KA, S. Ando & Cía. SA, Buenos Aires, Argentina) and control ($H_2O$). All solutions were supplemented with 0.05% (v/v) Triton X-100 as surfactant. All samples were taken the day after the last hormone application. At the end of the study, the plants were dissected, keeping berries, leaves and roots at −80 °C for further analysis.

### 4.2. Soluble sugars, alditols and organic acids determinations

Measurements of soluble sugars, alditols and organic acids were carried out by gas chromatography-flame ionization detection (GC-FID) according to Bartolozzi et al. (1997) with some modifications. Briefly, 3 g (fresh weight, fr.wt) of deseeded berries, 3 g (fr. wt) of fully expanded leaves, and 3 g (fr. wt) of powdered root tissue per biological replicate were ground to a fine powder using a mortar and pestle. The powder was transferred to a 50 mL Falcon tube containing 0.05 M imidazole:EtOH (20 mL pH 7, 50:50, v/v) and β-phenyl-glucopyranoside (1 mL, 2.5 g/100 mL), as internal standard. The mixture was shaken 18 h at room temperature and then centrifuged 10 min at 7000 *g*. The supernatant was transferred to a new 50 mL tube, the pellet was re-extracted with imidazole solution (20 mL) and the supernatant was added to the previous one.

Aliquots of berry extract (2 mL) and of leaves and root extracts (4 mL) were dried by an air stream, with the reconstituted residues solubilized with pyridine (500 μL), hexamethyldisilazane (250 μL) and trimethylchlorosilane (50 μL), and heated at 50 °C for 1 h. Trimethylsilyl derivatives were injected into a Varian CP 3800 GC equipped with a splitter injector, a flame ionization detector and a HP-1 J &W Scientific capillary column (60 m length, 0.25 mm inner diameter, and 0.25 mm film thickness) (Chrompack, Middelburg, The Netherlands). The temperature of the injector and detector was 350 °C. The column temperature was held at 140 °C for 1 min, then increased at 6 °C min 1–230 °C, at 8 °C min 1–270 °C, at 12 °C min 1–330 °C and at 20 °C min 1–350 °C and finally held at 350 °C for 6 min. The retention times of standards of the main sugars, alditols and organic acids present in the samples were used for identification.

For each analyte, a calibration line was built by internal standard method using the following ratios: analyte concentration (aC)/internal standard concentration (ISC) and analyte area (aA)/internal standard area (ISA). β-phenyl-glucopyranoside was used as internal standard. Analytes quantification was obtained applying the following formula aC = Ka * ISC * (aA/ISA), where ka is a coefficient obtained from each calibration curve.

### 4.3. Polyphenol extraction

Anthocyanins and low molecular weight polyphenols (LMWPPs) were extracted from grape skins, leaf and root samples using established methods as a reference with some modifications (De Nisco et al., 2013; Nicoué et al., 2007; Xu et al., 2011). Phenolics were extracted from homogenized fresh grape skin (0.5 g) and from powdered leaf and root material (1 g) by using MeOH:HCl (5 mL, 99:1, v/v) solution. The extraction was performed by sonication during 30 min at 25 °C. The procedure was repeated two times for leaf and root samples, with the obtained supernatants combined. The mixture was centrifuged for 10 min at 1300 *g*, and the extracts were made up to 10 mL with extraction solvent. Finally, an aliquot (1 mL) of each extract was evaporated to dryness, with each residue reconstituted in the initial mobile phase, filtered through a 0.45 μm filter and then analyzed by high performance liquid chromatography-multiple wavelength detector (HPLC-MWD). This procedure was followed for direct determination of anthocyanins in grape skin and LMW-PPs in leaf and root samples. For the quantification of LMW-PPs in skins and anthocyanins in leaves and roots, additional sample preparations were necessary.

### 4.4. Purification of the anthocyanin fraction from leaf and root samples

For determination of anthocyanins in leaf, additional preparation was required due to their low concentration. Extracts (3 mL aliquots) were individually evaporated to dryness and dissolved in 0.1% $HCO_2H$ in $H_2O$ (3 mL). Anthocyanins were concentrated and separated from non-pigments by solid-phase extraction (SPE). Briefly, the re-dissolved extract (500 mg) was loaded onto a SPE cartridge Sep-pak® $C_{18}$ (Waters, Milford, Massachusetts, USA), that had been pre-conditioned with 0.1% $HCO_2H$ in MeOH and 0.1% $HCO_2H$ in $H_2O$, respectively. Anthocyanins were adsorbed onto the column while sugars, acids, and other water-soluble compounds were removed by eluting with 0.1% $HCO_2H$ in $H_2O$. Anthocyanins were eluted through the cartridge with 0.1% $HCO_2H$ in MeOH (3 mL). The acidified MeOH fractions were evaporated to dryness, with the residues reconstituted in initial mobile phase of anthocyanins and analyzed by HPLC-MWD.

For LMW-PPs determination, extract aliquots (6 mL) were evaporated to dryness and dissolved in $H_2O$ (5 mL). LMW-PPs were extracted according to a previously reported method (Fontana and Bottini, 2014), with some modifications. Briefly, the re-suspended sample was extracted with MeCN:$HCO_2H$ (2.5 mL, 97.5:2.5, v/v). For phase separation NaCl (1.5 g) and $MgSO_4$ (4 g) were added, shaken 1 min and centrifuged for 10 min at 1300 *g*. Then, an aliquot (1 mL) of the upper MeCN phase was transferred to a 2 mL clean tube containing $MgSO_4$, PSA (Primary-Secondary Amine) and $C_{18}$, vortexed and centrifuged. Finally, an aliquot of each extract was evaporated to dryness, with the residues reconstituted in the initial mobile phase and analyzed by high performance liquid chromatography-multiple wavelength detector (HPLC-MWD).

### 4.5. HPLC-MWD analysis

#### 4.5.1. Low molecular weight polyphenols (LMWPPs)

HPLC separations/quantifications were carried out with a Dionex Ultimate 3000 HPLC-MWD system (Dionex Softron GmbH, Thermo Fisher Scientific Inc., Germering, Germany) and a reversed-phase Kinetex $C_{18}$ column (3.0 mm × 100 mm, 2.6 μm) Phenomenex (Torrance, CA, USA). Ultrapure $H_2O$ with 0.1% $HCO_2H$ (A) and MeCN (B) were used as mobile phases. Analytes were separated using the following gradient: 0–2.7 min, 5% B; 2.7–11 min, 30% B; 11–14 min, 95% B; 14–15.5 min, 95% B; 15.5–17 min, 5% B: 17–20, 5% B. The mobile phase flow was 0.8 mL $min^{-1}$. The column temperature was 35 °C and the injection volume 5 μL. LMWPPs present in samples were quantified by using an external calibration with pure authentic standards to achieve unambiguous identification of analytes. Standards of caftaric acid (**26**) (≥97%), caffeic acid (**27**) (99%), *p*-coumaric acid (**28**) (99%), (−)-epigallocatechin gallate (**29**) (≥95%), (−)-gallocatechin gallate (**30**) (≥99%), quercetin-3-glucoside (**31**) (≥90%), kaempferol-3-glucoside (**32**) (≥99%), syringic acid (**33**) (≥95%), (−)-epicatechin (**34**) (≥95%), (−)-gallocatechin (**35**) (≥98%), gallic acid (**66**) (99%), (+)-catechin (**67**) (≥99%) were purchased from Sigma-Aldrich. The standard of 2-(4-hydroxyphenyl) ethanol (OH-tyrosol (**5**)) (≥99.5%) was obtained from Fluka (Buchs, Switzerland). Linear ranges between 2 and 1000 μg $mL^{-1}$ with coefficient of determination ($r^2$) higher than 0.998 were obtained for all the studied LMW-PPs.

#### 4.5.2. Anthocyanins

For HPLC-MWD analysis of anthocyanins, separations were carried out in a reversed-phase Kinetex $C_{18}$ column (3.0 mm × 100 mm, 2.6 μm) Phenomenex (Torrance, CA, USA). The mobile phase consisted of ultrapure $H_2O$:$HCO_2H$:MeCN (87:10:3, v/v/v; eluent A) and ultrapure $H_2O$:$HCO_2H$:MeCN (40:10:50, v/v/v; eluent B) using the following gradient: 0 min, 10% B; 0–6 min, 25% B; 6–10 min, 31% B; 10–11min, 40% B; 11–14 min, 50% B; 14–15 min, 100% B; 15–17 min, 10% B; 17–21 min, 10% B. The mobile phase flow was 1 mL $min^{-1}$, column temperature 25 °C, and injection volume 5 μL. Quantifications were carried out by area measurements at 520 nm, and the anthocyanin content was expressed as malvidin-3-glucoside (**36**), using an external standard calibration curve (1–250 μg $mL^{-1}$, $R^2$ = 0.9984). The confirmation of the anthocyanins compounds detected with HPLC-MWD was confirmed by comparison with the elution profile and identification of analytes achieved in our previous work using UPLC-MS (Antoniolli et al., 2015).

#### 4.5.3. Free amino acids determination and quantification

For the determination and quantification of free AAs, berry skin (50 mg fr. wt.), fully expanded leaf (50 mg fr. wt.) and root (50 mg fr. wt.) were individually ground to a fine powder using a mortar and pestle, and then macerated with 0.1 M HCl (1 mL). The suspension was transferred to an Eppendorf tube, added with methionine sulfone (5 μL, 1000 ppm) as internal standard. The tubes were

shaken 10 min and centrifuged for 3 min at 19,500 *g*. After that, the supernatant was purified by solid-phase extraction (SPE), using an Extract Clean SCX (GRACE, Deerfield, Illinois, USA) 100 mg/1.5 mL column pre-conditioned with 0.1 M HCl (1 mL) and milliQ $H_2O$ (3 mL). The AAs were eluted with 8M $NH_4OH$:MeOH (250 μL, 1:1, v/v), and the fraction was recollected into a 1.5 mL glass vial. Once obtained and purified the free AAs, they were derivatized with pyridine (10 μL) and ethyl chloroformate (20 μL). Then, $CHCl_3$ (90 μL) and 50 mM $NaHCO_3$ (90 μL) were added. Afterwards 55 μL, from the bottom phase, was transferred to another glass vial. Finally, extracts (2 μL) were injected into a Clarus 500 gas chromatograph equipped with Clarus 500 single-quadrupole mass spectrometer detector (GC-MS) (PerkinElmer, Shelton, CT, USA). The column used was a Perkin-Elmer Elite-5MS, crosslinked methyl silicone capillary column (30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness). The injector temperature was set at 240 °C and the injections were carried out in the splitless mode. He (purity 99.9%) was used as a carrier gas at a flow rate of 1.0 mL $min^{-1}$. The oven temperature program was set as follows: initial temperature at 70 °C for 1 min, followed by an increase of 10 °C $min^{-1}$ to 280 °C, and held for 10 min. The ionization potential was 70 eV and a range of 50–360 amu (atomic mass units) was scanned. Compounds were identified by comparison of retention times and mass spectra with a set of authentic standards to obtain unambiguous identification (L-proline (**3**), L-valine (**13**), L-leucine (**14**), L-isoleucine (**15**), L-phenylalanine (**16**), L-tryptophan (**17**), L-tyrosine (**18**), L-glutamic acid (**19**), L-asparagine (**20**), L-aspartic acid (**21**), L-glutamine (**22**), L-alanine (**23**), L-methionine (**24**), L-threonine (**25**), L-glycine (**68**), GABA (**69**), L-histidine (**70**), L-lysine (**71**)), obtained from Fluka (Sigma-Aldrich, Steinheim, Switzerland), and peak areas were referred to the standard methionine sulfone for quantification.

#### 4.5.4. Terpene determinations and quantification

Samples of fully expanded leaves and grape skins (100 mg fr. wt) were ground to a fine powder using mortar and pestle, and then macerated with $CH_2Cl_2$ (1.5 mL) and MeOH:$HCO_2H$ (1 mL, 99:1, v/v). On the other hand, samples of root (1 g fr. wt) were macerated with $CH_2Cl_2$ (2 mL) and MeOH:$H_2O$:$HCO_2H$ (1.5 mL, 80:19:1, v:v:v). The suspension was transferred to glass tubes, and the extraction was carried out overnight in darkness at 4 °C. The mixture was shaken and centrifuged 5 min at 19,500 *g*. From each $CH_2Cl_2$ phase, an aliquot (100 μL) was put into a GC insert with *n*-hexadecane (100 ng) as internal standard, and 2 μL were injected into the GC-MS. The column was the same used in AAs determinations, but in this case the flow rate of carrier gas was 0.7 mL $min^{-1}$. For determination and quantification of monoterpenes, sesquiterpenes, diterpenes and triterpenes, the oven temperature program was set as described in Pontin et al. (2015). The ionization potential was 70 eV and a range of 40–500 amu was scanned. Compounds were identified by comparison of GC retention times and full mass spectra of the corresponding standards previously injected (D-limonene (**49**) 99%, terpinolene (**52**) 85%, nerolidol (**56**) 96%, α-pinene (**61**) 99% and squalene (**63**) 98%), obtained from Fluka (Sigma-Aldrich, Steinheim, Switzerland). For the compounds that standards were not available, unambiguously identification was performed by using their fragmentation pattern and comparison with data of the NIST library. Peak areas were referred to the standard *n*-hexadecane for quantification.

#### 4.5.5. ABA (1) determination

For ABA (**1**) quantification, fully expanded leaves (200 mg fr. wt) and berries (400 mg fr. wt) at full veraison were used for extractions. Plant material was washed gently with running $H_2O$ prior to ABA (**1**) extraction. Measurements were carried out by GC-MS according to Berli et al. (2010), with modifications for berries-skin extraction. Each sample was homogenized in a mortar with liquid $N_2$ and extracted with MeOH:twice-distilled $H_2O$:AcOH (2 mL, 80:19:1 v/v) at 4 °C, in the case of berries. After 12 h, hexadeuterated ([$^2H_6$])-ABA, 60 ng (a gift from Professor R.P. Pharis, University of Calgary, Canada) dissolved in MeOH (2 mL) was added for ABA (**1**) quantification and allowed 1 h equilibration of the isotopes. Then, the sample was centrifuged for 10 min at 11,200 *g*, the supernatant evaporated in a rotavapor under vacuum at 35 °C. The aqueous residue was adjusted to pH 3.0 with glacial AcOH and partitioned four times with equal volumes of EtOAc saturated with 1% glacial AcOH. After solvent evaporation under vacuum at 35 °C, the residue was dissolved in $H_2O$ (1 mL) at pH 3.0 (1% AcOH) and passed through a Sep-Pak C18 reversed phase cartridge (Waters Associates, Milford, MA, USA). This elution was performed at a flow rate of 0.2 mL $min^{-1}$ using the following gradient: 1 mL each of twice-distilled $H_2O$ pH 3.0, hexane, and MeOH: 1% AcOH in $H_2O$ (80:20, v/v). The entire eluant was collected, and after solvent evaporation in vacuum at 35 °C, the residue was dissolved in $H_2O$ (1 mL) at pH 3.0. This solution was transferred to Oasis WAX (weak anion exchanger, 60 mg of material) cartridges (Waters Associates) in a gradient of MeOH:$NH_4OH$ (1 mL, 95:5, v/v), MeOH:$HCO_2H$ (1 mL, 95:5, v/v) and MeOH:$HCO_2H$ (1 mL, 98:2, v/v). The acidic elute (which contains the ABA (**1**)) was evaporated at 35 °C under vacuum and then converted to the methyl-ester (Me) derivatives with MeOH (3 μL) plus fresh ethereal $CH_2N_2$ (5 μL, 30 min at room temperature). After solvents had been eliminated under a gentle flow of $N_2$ at room temperature, the samples were dissolved in *n*-hexane (50 μL), and 1 μL was injected split–splitless into a HP-5 cross-linked methyl silicone capillary column (30 m length, 0.25 mm inner diameter, 0.25 mm film thickness) fitted in a capillary gas chromatograph-electron impact mass spectrometer (GC-MS) (Clarus 500, Perkin Elmer, Shelton, CT, USA). The GC column was eluted with He (1 mL $min^{-1}$). The GC temperature program was 100 °C–260 °C at 20 °C $min^{-1}$, then 10 min at 260 °C. The mass spectrometer was operated with electron impact ionization energy of 70 eV. The injector temperature was 230 °C, ion source temperature was 260 °C and the interface temperature was 280 °C. After performing selected ion monitoring (SIM) the amount of ABA (**1**) was calculated by comparison of the peak areas of the major ions for the Me derivative of the deuterated internal standard [$^2H_6$]-ABA (194/166) relative to its non-labelled counterpart (190/162).

#### 4.5.6. Gene expression

Samples of leaves (100 mg fr.wt) and berries (400 mg fr. wt), respectively, were used for RNA extraction according to Reid et al. (2006). cDNA synthesis and experiments of qRT-PCR were performed as described by Murcia et al. (2016). Elongation factor 1-α (*VvEF 1-α*) was used for normalization in all experiments. The primers were designed using the software Beacon Designer version 7.70 (Premier Biosoft International, Palo Alto, CA, USA) over the corresponding EST available at the NCBI GenBank database. The ratio (relative transcription amount) was obtained according to Equation (1) published in Pfaffl (2001). The primer sequences were as follows: *VvEF-1α* F: 5-GCAGCCAAGAAGAAGTGAAG-3; R: 5-CCAAGGAAGAAGGCAGAAAAC-3; *VvNCED1* F: 5-AATGCTACTGACGCTTTCT-3; R: 5-CAATCACCACCGACTTCATC-3.

#### 4.5.7. Statistical analysis

One-way ANOVA and Fisher's multiple comparison of means were used to discriminate between the averages by the minimum difference, with a significant level of $p < 0.05$. Principal component analysis (PCA) was applied to a data set of metabolites belonging to each group added for each biological replicate. The results were presented as a two-dimensional graphical display of the data

(*Biplots*). The analysis was carried out with InfoStat software (http://sites.google.com/site/fgstatistics), and the number of replicates for the reported data are specified in each figure.

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## Appendix ASupplementary data

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