POSSIBLE ROLES OF BOTH ABSCISIC ACID AND INDOL-ACETIC ACID IN CONTROLLING GRAPE BERRY RIPENING PROCESS

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Abstract

Aims: The objective was to better understand the mechanisms involved in grape ripening that brings about important changes in the physiology and chemistry of the fruit. So we focused on the involvement of two growth regulators: abscisic acid (ABA) and indol-acetic acid (IAA) in controlling grape berry ripening process

Methods and results: We described the evolution of the two plant growth regulators during the development of cv. Merlot grapes (*Vitis vinifera* L.). In order to better understand the role of ABA and IAA in the ripening control, these two growth regulators were applied on the grapes at the onset of veraison. The hormonal profile was established on treated berries and different physiological parameters were assayed to evaluate the effects of both applications. The partitioning of both plant growth regulators in non-treated berries showed that ABA and IAA accumulated at the onset of ripening. Moreover, it appeared that endogenous ABA decreased progressively in the flesh while accumulated in the skin from the beginning of the colour change to maturity. The hormonal treatments modified the hormonal profile and several physiological parameters: sugar, acidity, colour, and *Botrytis* sensibility.

Conclusion: These findings suggest that both treatments have modified the ripening process. Exogenous ABA has induced advancement in grape ripening, while IAA application has delayed this process. These observations support the view that the grape ripening process may be influenced by the hormonal status.

Significance and impact of study: This study gives new information about the ripening control of the non-climacteric fruits. In grape berries, it provides evidence of a possible co-involvement of ABA and IAA in controlling ripening process.

Key words: abscisic acid, indol-acetic acid, hormonal balance, grape berry ripening, *Vitis vinifera* L.

Résumé

Objectif : L'objectif est de mieux appréhender les mécanismes impliqués dans la maturation de la baie de raisin, qui provoquent de profonds changements physiologiques et chimiques du fruit. Ainsi, nous nous sommes intéressés à l'implication de deux régulateurs de croissance : l'acide abscissique (ABA) et l'acide indole acétique (AIA) dans le contrôle du processus de maturation des baies.

Méthodes and résultats : L'évolution de ces deux régulateurs de croissance a été suivi au cours du développement de baies de cépage Merlot (*Vitis vinifera* L.). Pour mieux comprendre le rôle de l'ABA et de l'AIA dans le processus de maturation, ces deux régulateurs de croissances ont été appliqués sur les baies au moment du déclenchement de la véraison. Le profil hormonal a été établi sur les baies traitées et différents paramètres physiologiques ont été testés afin d'évaluer les effets de ces deux applications. La répartition des deux régulateurs de croissance dans les baies non traitées montre que l'ABA et l'AIA s'accumulent au début de la véraison. De plus, il apparaît que les teneurs en ABA endogène diminuent progressivement dans la pulpe alors qu'elles s'accumulent dans la pellicule du début véraison à maturité. Les traitements hormonaux modifient le profil hormonal et différents paramètres physiologiques : les teneurs en sucre, l'acidité, la couleur, et la sensibilité vis-à-vis de *Botrytis*.

Conclusion : Ces résultats suggèrent que les deux traitements ont modifié le processus de maturation. L'ABA exogène a induit un avancement dans la maturation des baies, alors que l'application d'AIA a retardé ce processus. Ces observations laissent supposer que le processus de maturation du raisin est influencé par le statut hormonal.

Signification et impact de l'étude : Cette étude apporte de nouvelles informations sur le contrôle de la maturation des fruits non-climactériques. Dans les baies de raisin, il semble évident qu'il existe une co-implication de l'ABA et de l'AIA dans le contrôle du processus de maturation.

Mots clés : acide abscissique, acide indole acétique, équilibre hormonal, maturation de la baie de raisin, *Vitis vinifera* L.

INTRODUCTION

Fruit ripening has direct implications to a significant component of human diets, nutrition and agriculture. This ripening process is a complex developmental program in which senescing tissues undergo programmed changes in firmness, texture, coloration, flavour and susceptibility to microbial infection (Coombe and McCarthy, 2000; Brownleader *et al.*, 1999; Brummell *et al.*, 2004). Fruit growth is controlled by numerous hormones. It is well known that ethylene plays an important role in climacteric fruit ripening. In contrast with the great deal of information regarding the regulation of ripening in climacteric fruit, much less is known about non-climacteric ones, such as grapes. At present, no single growth regulator appears to play a positive role analogous to that played by ethylene in the ripening of climacteric fruits. The influence of exogenous ethylene exposure of table grapes, usually used to affect positively the quality of commercial fruits during storage, has been reviewed and Palou *et al.* (2003) have concluded that ethylene treatment did not improve the postharvest life of grapes. This indicated that ethylene played minor function in harvested grapes. It has been suggested that regulators such as abscisic acid (ABA), auxins and polyamines, can be important in fruit development and ripening (Davies *et al.*, 1997; Ponappa and Miller, 1996).

The role of ABA in stomatal closure, adaptation to stress, seed dormancy and photosynthesis is well known (Zeevaart and Creelman, 1988). This plant growth regulator is also associated with grape maturation: endogenous ABA levels increase during ripening, with a maximal level at the end of the colour change period, called « veraison » by viticulturists (Antonin *et al.*, 2003; Broquedis, 1987; During *et al.*, 1978; Gagné *et al.*, 2006) and control the expression of some genes encoding for phenol compounds (Jeong *et al.*, 2004) or cell wall hydrolases (Lohani *et al.*, 2004).

Auxin is thought to be implicated in the acid growth theory by inducing proton efflux and hyperpolarization of the plasma membrane. Its action may affect cell elongation, cell wall loosening and in the increase of growth (Fukuda and Tanaka, 2006; Rayle and Cleland, 1992). It has been observed for a long time that auxin can negatively control the ripening of some non-climacteric fruits. In banana, it has been shown that the expression of many ripening specific genes can be down-regulated by treatments with an exogenous auxin (Lohani *et al.*, 2004). During the early climacteric tomato fruit growth, it has been put forth that IAA controls the expression of some genes encoding for cell wall-modifying proteins, such as expansin, endoglucanase and xyloglucan transglycosylase (Català *et al.*, 2000). In grape, Davies *et al.* (1997) suggest that this hormone plays a negative

role in the regulation of ripening. In fact, it has been shown that treatments with a synthetic auxin are able to delay the expression of a number of ripening-related genes.

The interaction between ABA and ethylene signal pathways has been described in several studies (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000; Ross and O'Neill, 2001) but its mode of action is still unclear. Studies of ABA and auxin have revealed that these pathways impinge on each other. Many physiological studies have suggested that these two hormones functionally interact for example in roots, as regulators of growth, development and tropisms (Casimiro *et al.*, 2003). However, despite recent progress in understanding the intermediate and downstream steps in ABA and auxin signalling (Delong *et al.*, 2002; Dharmasiri and Estelle, 2004; Navarro-Avino and Bennet, 2005), the mechanisms of hormone action remain obscure. Here we report studies on a possible interaction of ABA and IAA in the involvement of berry ripening. We observed the effects of ABA and IAA treatments on the hormonal balance and ripening process. Our physiological approach sheds new light on the possible links of these classic hormones during a non-climacteric fruit ripening.

MATERIALS AND METHODS

Plant material

The experiment was conducted on berries of *Vitis vinifera* L. cv. Merlot in a Bordeaux vineyard (France). Grape clusters were collected at different stages of ripening during the year 2003: a green stage, 25 days after anthesis (DAA); different points during the colour change period: 10 %, 50 %, 80 % and 100 % of red coloured, corresponding respectively to 46, 49, 53, 56 DAA; a sample during maturation, 10 days after the end of the colour change; and maturity, corresponding to the harvest, 89 DAA. Random samples of 6 grape clusters on different plants were selected for each stage, immediately frozen in liquid nitrogen and berries were stored at -80°C until analyses, except for maturity index and infection tests.

Experimental treatments

The grapes were treated at the onset of their coloration (veraison) by spraying all clusters of 10 plants with aqueous solutions ($2.10-4$ mol.L^{-1}) of the plant growth regulators ABA ([14375-45-2], Sigma-Aldrich France) and IAA ([87-51-4], Sigma-Aldrich France). Each cluster was sprayed with 10 ml of the solution or sprayed with water (control) containing 0.1 % Tween80 as a wetting agent.

Growth regulator analysis

Free ABA was determined following the method described by Antonin *et al.* (2003) and IAA was

determined according to the method of Abbas *et al.* (1999). The two hormones were extracted from 15 whole grape berries or from isolated skin and pulp, obtained by peeling 20 freezed berries. The extraction was first carried with methanol 80 % (v/v), followed by purification with polyvinyl poly-pyrrolidone and a final extraction with diethyl-ether. The plant growth regulators were determined with a HPLC in combination with UV spectrophotometry for ABA (λ: 254 nm) and with fluorescence spectrometry for IAA at 280 and 360 nm excitation and emission wavelengths, respectively. Quantification was performed by including regularly a mix of ABA and IAA standards in each HPLC sequence. The assays were validated independently with purified hormone extracts by mass spectrometry.

Determination of ripening physiological markers

Chlorophyll levels. Pericarp chlorophyll contents were extracted and determined by spectrophotometry according to Fougère-Rifot *et al.* (1995).

Soluble contents. Berry samples (200) were wrapped in double-layer cheesecloth and crushed with a small hand press. The resulting juice was centrifuged at 3,000 g for two minutes to remove debris. Aliquots of the supernatant were retained for immediate analysis of titritable acidity by titration with NaOH (Guymon and Ough, 1962), and soluble solids by refractometry (Amerine and Ough, 1980).

Polyamine levels. Polyamines were extracted according to the method described by Rifai *et al.* (2005) and analyzed via HPLC in combination with fluorescence spectrophotometry according to the method of Smith and Davies (1985), adapted to our plant material by Geny *et al.* (1997).
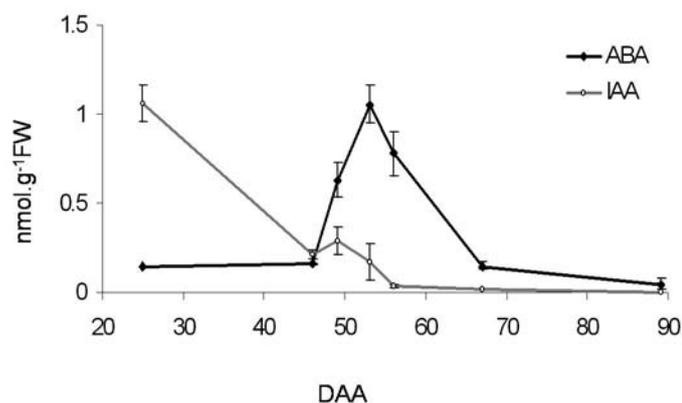
Botrytis susceptibility. *Botrytis* susceptibility was determined by an inoculation of *Botrytis cinerea* (Pers.) spores (2.10^5 spores.L^{-1}) on the surface of isolated ripe berries, initially disinfected. Berries were incubated in a high relative humidity at 20 °C and visually infected berries were recorded every 5 days during a 35-d period.

Statistics

Analytical data are the mean ± SD of 4 experiments, each experiment consisting of 15 frozen berries for hormones and chlorophyll determination and 50 fresh berries for the soluble contents and B*otrytis* susceptibility determination. Statistic analyses were used to evaluate treatment effects by LSD Fisher test ($\alpha = 0.05$).

RESULTS

ABA and IAA levels during ripening

The evolution of free ABA contents in grape berries (figure 1) revealed that it accumulated during the colour change period with a maximal concentration appearing at 80 % red coloured (59 DAA). In the pulp and skin, ABA profiles appeared similar than whole berry (data not shown) with both cases an ABA accumulation during veraison. Nevertheless, we noted a modification in the repartition of this hormone, expressed per berry, in both pericarp tissues (skin and pulp), as shown in figure 2: at the early stages of development, free ABA was essentially present in the pulp tissue, and from the beginning of the colour change process to maturity, ABA levels decreased progressively in the flesh and increased in the skin. At maturity, this hormone was largely present in the skin. This repartition has been also observed in other cultivars and during two consecutive seasons.

Figure 1 - Evolution of free ABA and IAA in Merlot berries during development.

Fifty days after anthesis (DAA) corresponds to veraison and 95 DAA to the date of harvest.
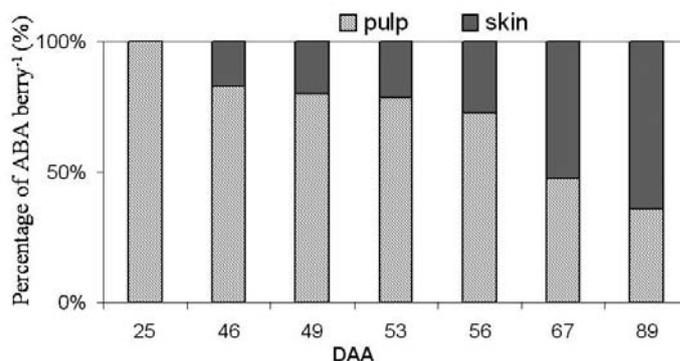Data are expressed as the means ±SDs of 4 different determinations.

Figure 2 - Partitioning of ABA in Merlot grape berries between pulp and skin tissues during fruit development, from the green stage (25 DAA) to harvest (89 DAA).

ABA contents were conduced on isolated pulp and skins of control berries and were expressed on a per berry basis. Data are expressed as the means ±SDs of 4 replicates.

Indol-acetic acid was present in large quantities during the early stages of berry development and then decreased until fruit maturity (figure 1). Moreover, IAA content showed a rapid and brief increase at 50 % coloration, which corresponded to the enhancing of the second growth phase. Then the levels decreased and stayed quite low until maturity. The same evolution has been observed in Cabernet-Sauvignon and Sauvignon blanc cultivars (data not shown). This peak in IAA levels appeared to be concomitant with the beginning of ABA accumulation in grape berries.

Effect of treatments on some parameters related to ripening

The ABA-treated berries had significantly less chlorophyll compared to the control, while the IAA treatment significantly delayed chlorophyll loss in the grape pericarp (table 1). Both treatments were carried out at the onset of skin coloration, when 1 % of the berries in the same grape cluster were beginning to colour. Our results indicated that both treatments modified the time of chlorophyll pigment degradation and in parallel modified the onset of anthocyanin pigment accumulation. Moreover, ABA-treated berries showed a more rapid red pigmentation than control and IAA-treated fruits showed an opposite effect (data not shown).

At maturity, the juice of ABA-treated berries showed a higher concentration of soluble solids than the control, while IAA-treated berries showed opposite results (table 2). Both treatments did not modify significantly the berry weigh at the harvest. ABA application did not significantly affect the titratable acidity whereas IAA-treated berries showed a lower acidity. Polyamines contents are also affected followed treatments. At harvest,

the levels were significantly lower in ABA-treated berries than control and significantly higher in IAA-treated ones. Considering the values presented here, ABA-treated berries were more ripe than control whereas the IAA-treated ones were significantly less ripe that the other two treatments.

The modification of the senescence process was also confirmed by *in vivo Botrytis cinerea* sensitivity. The number of fresh isolated berries, collected 89 DAA, was noted regularly after an inoculation by a solution of fungal spores (figure 3). The number of infected berries increased progressively during the 35d after inoculation. Treatments modified berry sensitivity during fruit maturation: berry infection development was faster in berries treated with ABA and inversely, slower in IAA-treated berries.

Effects of hormonal treatments on ABA and IAA contents

The previous results indicated a modification in different parameters related to grape ripening. The hormonal profile was performed to show if the treatments had also modified it. Figure 4 showed that in control berries, treated with water and Tween, the evolution of ABA profile was similar than in non treated berries presented in figure 1. In contrast, both hormonal treatments modified the time course of ABA content during the colour change period (figure 4). Application of ABA induced a significant increase in ABA levels 5 day post-treatments. ABA levels were higher until 80 % colour change, corresponding to the maximal concentration, then when berries are full red until maturity, the levels were the same that in control berries. It was found that IAA treatment also resulted in an advancement of ABA accumulation. The maximal concentration was

Table 1 - Effect of hormonal treatments, made at the onset of skin coloration (42 DAA), on chlorophyll contents (µg.berry^{-1}) some days after the application.

The collection on day 0 was made just before the applications. Means in a column followed by a different letter are significantly different according to Fisher test, $\alpha = 0.05$.

	Days after treatments		
	0	4	8
Control	10,00	5.09 [b]	4.07 [b]
ABA-treated	10,00	1.81 [c]	0.56 [c]
IAA-treated	10,00	6.94 [a]	7.22 [a]

Table 2 - Effect of ABA and IAA treatments on the soluble contents, titritable acidity and polyamines contents in mature grape berries.

Means in a column followed by a different letter are significantly different according to Fisher test, $\alpha = 0.05$.

	Berry weight (g)	Soluble solids (g.L^{-1})	Titritable acidity (g tartric acid.L^{-1})	Polyamines (nmol.g^{-1} FW)
Control	104.9 [a]	223.3 [b]	3.7 [a]	85.9 [b]
ABA-treated	99.6 [a]	228.0 [a]	3.7 [a]	50.0 [c]
IAA-treated	101.6 [a]	215.0 [c]	3.4 [b]	110.2 [a]

observed 7 days before control level. In contrast, no significant effects of these two treatments were observed on IAA contents (data not shown).

DISCUSSION

The primary objective of the present investigation was to describe the evolution of two plant growth regulators, ABA and IAA, in berries of *Vitis vinifera* L. cv. Merlot during ripening. ABA accumulates during the colour change period, in agreement with other works (Antonin *et al.*, 2003; DaPeng *et al.* 1997; Gagné *et al.*, 2006), suggesting a central role of ABA in triggering veraison and ripening. This accumulation is clearly related to the change of skin coloration. In addition of the modifications of ABA contents in the berries, it has been shown by many studies that the change in berry colour and the modifications in the chlorophyll loss were related to the increase in ABA concentration (Gagné *et al.*, 2006; Wang *et al.*, 2005). Our results suggested then that hormonal changes due to the modifications of ABA statues could influence and induced the onset of ripening process.

An interesting observation was the specific repartitioning of this hormone in the pericarp tissues. It appears that the amount of ABA increases progressively in the skins from the onset of the coloration of grapes to maturity and at the opposite, decreases in pulp tissue. This observation could suggest a migration of ABA in skins during ripening or a new-synthesis of ABA in the skins. This movement of ABA in the pericarp is probably responsible for the physiological changes that occur during grape ripening, particularly phenolic compound accumulation, biosynthesis and changes in cell wall structure. In skins, sugar and ABA concentrations are predominant determinants for anthocyanin biosynthesis in black cultivars. Sugar and potassium ions accumulate in skin cells, along with phenolic compounds (Coombe and McCarthy, 2000). Furthermore, Hiratsuka *et al.* (2001) have shown that ABA enhanced skin pigmentation and was also involved in the activation of some anthocyanin biosynthesis enzymes. We suggest that the evolution of these compounds is under ABA regulation and that this regulation might be related to its migration in the skins. These conclusions can explain the abundance of ABA in coloured varieties skins but white cultivars show the same repartition of this hormone. In this sense, ABA must likely participate in a multitude of other physiological changes because of its central signalling cascade role in plant tissue (Himmelbach *et al.*, 2003). During grape ripening, the source of synthesis of ABA is doubtful. Antonin *et al.* (2003) suggested that, during ripening, the majority of ABA is transported from leaves to berries where it accumulates. In contrast, Okamoto *et al.* (2004) reported that ABA levels were not always dependent on leaf ABA levels during ripening. In addition, it has been reported that the source of ABA in the pericarp can be the pericarp itself (Cawthon and Morris, 1982). Therefore, a possible explanation may be that the skin synthesizes ABA during ripening, influencing differences in the hormone repartition between the pulp and skin tissues.

For the first time, the presence of IAA is observed during grape ripening. IAA contents showed a brief augmentation at the onset of ripening. The IAA peak is concomitant with the onset of ABA accumulation, suggesting two modes of action largely correlated to control the enhancing of the ripening process. Our results suggested that auxins are also implicated in the control of the grape ripening and it is likely that both IAA and ABA play important roles in ripening metabolism. ABA and IAA can act concomitantly to control the ripening process; ABA enhancing anthocyanins and sugar accumulations and IAA modifying cell-wall structure to
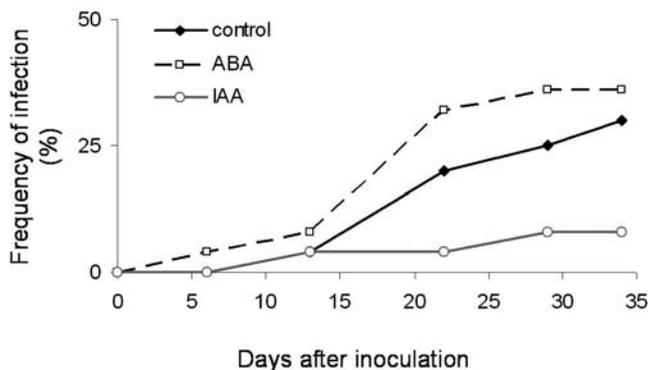

Figure 3 - Sensitivity of Merlot ripe berries sprayed with ABA and IAA to *Botrytis cinerea* infection.
The number of berries infected was scored regularly for 35 days. Each experiment was carried with a total of 50 ripe berries inoculated with a solution of *Botrytis* spores.
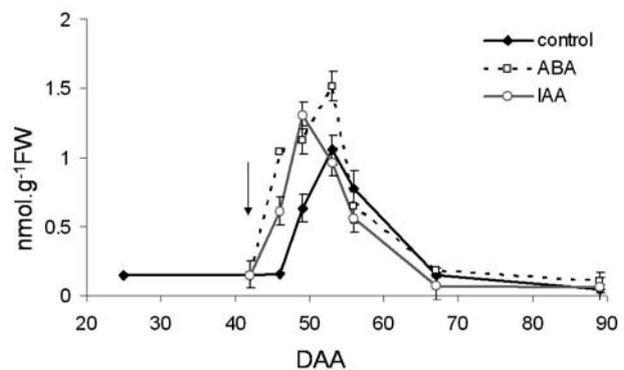

Figure 4 - Evolution of ABA in berries sprayed with ABA and IAA at veraison.
The treatment time is indicated by the arrow. Data are expressed as the means ±SDs of 4 replicates.

permit the accumulation of water and solutes in the vacuoles (Fukuta and Tanaka, 2006; Navaro-Avino and Bennet, 2005) IAA may allow cell wall elongation in relation with sugar and water flux, thus enhancing the second growth period.

Treatments carried out at veraison were performed to monitor the ripening process and to show the importance of the hormonal balance on the control of grape ripening. ABA profile and ripening indicators were modified by both treatments. Berries show an enhancement in ABA accumulation, five days after the applications. These results suggested that hormonal balance (IAA and ABA levels on the onset of change colour) may act on ABA signalling pathways or could play a role on its accumulation in grape and/or on its synthesis in the berry. The literature tends to indicate antagonistic functions of the two growth regulators ABA and IAA, in the control of fruit development. For example, application of auxins can delay grape berry ripening (Davies *et al.*, 1997) and exogenous ABA treatment enhanced the accumulation of anthocyanins in the skin (Jeong *et al.* 2004). However, in banana fruit, it has been shown that ABA treatments decreased the firmness and modulated the activities of some softening hydrolases (Lohani *et al.*, 2004), indicating that ABA application may induce an acceleration of fruit ripening. These authors also indicate that exogenous IAA may inhibit fruit softening and delay the ripening by modelling cell wall degrading enzyme activities. In our study, IAA-treated berries showed a delay in the ripening process characterized by lower sugar contents at maturity, lower sensitivity to fungal infection and higher polyamine contents. Peppi *et al.* (2006) have shown that the application of ABA at veraison on grapes increased the anthocyanin levels in skins. These results are in accordance with our results: ABA enhances skin colour change, as we found that chlorophyll pigments decreased more rapidly in ABA-treated berries. Moreover, Canti *et al.* (in press) have also found that an application of ABA on grapes, induced an enhancement of the harvest time, confirming a positive effect of exogenous ABA in the reducing the ripening period.

This *Botrytis* susceptibility is positively correlated with the degree of grape maturity and negatively correlated with polyamine levels (Monasterio, 2004), confirming that IAA application may have delayed grape ripening. In contrast, ABA-treated berries were more sensitive to the infection which was in concordance with Audenaert *et al.* (2002). These authors have shown that exogenous application of ABA increased the susceptibility of tomato plant to fungal pathogens by negatively modulating the salicylic acid-dependent defence pathway, possibly one of the mechanisms by which ABA levels determines *Botrytis* susceptibility.

Moreover, Martinez Noel *et al.* (2001) have demonstrated a putative role of IAA, dose-dependant, in the inhibition of fungal growth, by down-regulating plant and fungal glucanase. At the date of our infection test (73 DAA), IAA levels were the higher in IAA-treated skin berries so that these elevated levels may have function as a natural defence for pathogen spread and Botrytis development. Nevertheless, our results also show that IAA treatment involves an accumulation of ABA before that control while ripening is delayed. In this case, the delay of ripening could be explained by strong contents of polyamines which are longer maintained during berry development. Polyamines were considerate by many authors as biomarkers for plant regeneration (Shoeb *et al.*, 2001) and have been reported as anti-senescence agents, with their endogenous levels being related with growth and development processes (Paschalidis *et al.*, 2001). In this study polyamines can be considered as senescent markers, suggesting that both hormonal treatments have affected ripening process. The results obtained in our study in relation to the content of polyamines in skin suggest a possible interconnection between polyamines and hormonal activity, also reported by Rastogi and Davies (1991). On the other hand, Navarro-Avino and Bennet (2005) and Fukuda and Tanaka (2006) showed that treatments with ABA or IAA could have similar effects on the expression of some genes implicated to the cell multiplication. A number of studies have shown that plant hormones and auxins in particular can affect significantly the uptake and further transport of nutrients within the plant by regulating the sink action of developing tissues. Although ABA is involved in plant growth inhibition and auxins are involved in plant growth development, Navarro-Avino and Bennett (2005) show that both IAA and ABA increase cytosolic calcium. Moreover, the list of genes regulated by both ABA and IAA is continually on the increase. Results of expression profiles in rice shoots using cDNA macroarrays showed that a group of clones regulated by ABA were also regulated by IAA (Lin *et al.*, 2003). These observations indicate the complicated interactions of signal pathways with these two hormones.

CONCLUSION

Our results show that ABA and IAA were complementary in enhancing veraison and initiating grape berry ripening. The balance between these two plant development regulators could be responsible for the different patterns of ripening observed after the different treatments, but the molecular understanding of their interaction during grape berry ripening is far from comprehensive. A specific study of certain genes involved in the ripening process in relation to hormonal treatments could provide important information about their ability to be controlled by ABA and IAA.

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