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The Exogenous Application of Abscisic Acid Induce Accumulation of Anthocyanins and Phenolic Compounds of the 'Rubi' Grape

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Abstract

The abscisic acid (ABA) is related to the formation of certain polyphenols, such as anthocyanin, improving the nutritional and commercial quality of the red grapes. In this study, we verified whether the exogenous application of *S*-ABA favors the accumulation of anthocyanin, the total phenolic compounds and the antioxidant activity of the 'Rubi' grape. The experiment was performed during the cycle 2014/2015 with the 'Rubi' vine (*Vitis vinifera* L.) grafted in the rootstock 420-A. The experimental design was composed of randomized blocks, with four treatments and six blocks, in a total of 24 experimental parcels, constituted of one plant each. We used the *S*-ABA isomer in different concentrations and times of application, 400 mg·L^{-1} *S*-ABA in the beginning of the ripening (BR); 400 mg·L^{-1} at BR + 200 mg·L^{-1} at 25 days after first application (DAFA); 400 mg·L^{-1} at BR + 400 mg·L^{-1} at 25 DAFA; besides the control (0 mg·L^{-1}). After 40 days of the first application, the samples were harvested and we verified that the *S*-ABA induced the accumulation of anthocyanins and total phenolic compounds in the 'Rubi' grape. From these results we can recommend two applications of *S*-ABA, 400 mg·L^{-1} at BR + 200 mg·L^{-1} at 25 DAFA.

Keywords

Vitis vinifera, *S*-ABA, Table Grape, Bioactives Compounds

1. Introduction

The 'Rubi' grape (*Vitis vinifera* L.) is a fine table cultivar and occurred from a

mutation from the 'Italia' grape, distinguishing only by the pink color of the berry [1]. However, in some regions of tropical climate, the colored grapes show an undesirable color in the moment of the harvest and this is mainly due to the low thermal amplitude and high rainfall. This lack of color can be supplied by the use of growth regulators, as the abscisic acid (ABA) that promotes the synthesis and accumulation of anthocyanins, which are pigments that provide color to the fruits, flowers and vegetables [2].

The fruit color has a central role in the direct consumer attraction and for this reason, is one of the most important ampelographic traces and also is one of the main characteristics to be considered in order to evaluate the quality by phenotyping of a potential genotype [3]. The proportion of pigments such as chlorophylls, carotenoids and anthocyanins determines the external color and appearance of the fruit. Some of the phytochemicals of the polyphenols class, such as anthocyanins, are considered as important markers of the commercial values of grapes [4], besides acting as antioxidants in the human organism, preventing a series of generative diseases [5].

The concentration of phenolic compounds in fruits and vegetables is regulated by genetic, environmental and physiological factors, such as temperature, light, rainfall, soil, chemical products and growth regulators. Many agronomic strategies as alteration of the environmental conditions, water availability, grafting and stimulating agents have been employed to increase the biosynthesis of phenolic compounds in fruits and vegetables [6]. The exogenous application of abscisic acid (ABA) in the beginning of the berries ripening is a way of increasing the synthesis of secondary metabolism compounds, including (poly) phenols. This growth regulator is involved in various biochemical and physiological processes, promoting the synthesis and accumulation of anthocyanins, which directly influence the color development. The expression of this pigment depends on internal factors as the ABA, which induces the transcription factor MYB1A, protein responsible for regulating gene transcription genes that compose the anthocyanins biosynthetic pathway in colored grapes [7].

The efficiency of exogenous application of ABA has been demonstrated in the synthesis and accumulation of anthocyanins (polyphenols) in the peel of many grape cultivars [8] [9] [10] [11], as well as it provides uniformity of color, improves the quality of the treated grapes [8] [12], and increase the phenolic compounds contents [11] [13]. Anthocyanin performs an important role in the color and in the sensorial characteristics of fruits and vegetables, beyond its protective effects against certain types of cancer and cardiovascular diseases [14] [15]. The accumulation of these compounds adds commercial quality to the product, as well as attracts consumers that, increasingly, search for fruits with better nutritional quality as a natural fount of antioxidants.

In this way, the aim of this study was the effects of the (*S*)-cis-abscisic acid (*S*-ABA) exogenous application in the anthocyanins content, phenolic compounds and in the antioxidant capacity of the 'Rubi' grape.

2. Material and Methods

2.1. Location and Plant Material

The experiment was performed during the cycle 2014/2015 of the 'Rubi' vine (*Vitis vinifera* L.) grafted in the rootstock '420-A' spaced out 4 × 2 m, in the sixth production year, supported in the pergola system, located in São Miguel Arcanjo, state of São Paulo (23˚31'S, 47˚35'W and altitude 660 m), Brazil. The soil of the region was classified as Dystrophic Red Latosol [16] and the climate region, according to Köppen classification, as *Cwa*, with average annual rainfall of 1.396 mm, temperature average of 20.4˚C and 70.6% relative humidity.

The grapevines were pruned at August 2014, leaving six buds for each productive branch and subsequently, 2% hydrogen cyanamide was applied aiming the induction and standardizes prouting. In the beginning of the gems sprouting, the vegetative branches were eliminated and 2 to 3 sprouts with cluster were kept by branch. The other cultural treatments, as shoot and branch thinning, branch training, fertilization, weed, pests and diseases control were performed according to the techniques employed in the cultivation region.

2.2. Experimental Design and Treatments

The experimental design was composed of randomized blocks, with four treatments and six blocks, in a total of 24 experimental parcels, constituted of one plant each. The effect of the isomer *S*-ABA supplied by Valent BioScience Corporation (Libertyville, IL, USA) (100 g·L^{-1} of active ingredient) was evaluated in different concentrations, 400 mg·L^{-1} *S*-ABA in the beginning of the ripening (BR); 400 mg·L^{-1} at BR + 200 mg·L^{-1} at 25 days after first application (DAFA); 400 mg·L^{-1} at BR + 400 mg·L^{-1} at 25 DAFA; beyond the control (no *S*-ABA application), in a total of 4 treatments. It was added to all treatments 0.3 mL of BreakThru® (Evonik Industries, Germany) non-ionic surfactant. It was considered as BR for the first application softening berries and color change, which presented soluble solids values of 9˚ Brix and acidity of 0.9% of tartaric acid. The clusters were sprayed in the morning using costal sprayer (40 kg f^{-1} pressure) with JA1 hollow cone nozzle tips to provide complete and uniform coverage, employing 900 L·ha^{-1}.

2.3. Harvest and Characteristics Evaluated

The harvest was performed 40 days after the first *S*-ABA application, were harvested five representative clusters in each experimental parcel and sampling 10 berries per cluster for the analyses of the anthocyanins content, total phenolic compounds, total flavonoids, total chlorophyll, total carotenoids and total antioxidant activity. The berries were cut in half to remove the seeds and the peel and pulp were pulverized in liquid nitrogen and stored at −80˚C until the moment of the analyses.

Chlorophyll, anthocyanins and carotenoids were determined according to Sims and Gamon [17]. The extraction was performed in cold acetone/Tris-HCl

buffer solution (80:20 vol:vol, pH 7.8), in ambient protected from light. The readings were made in spectrophotometer BEL Photonics®, SP 2000 UV/vis. The absorbance was converted in mg 100 g^{-1} of fresh weight, through the following equations:

$$\text{Anthocyanin} = 0.08173A_{537} - 0.00697A_{647} - 0.002228A_{663} \quad (1)$$

$$\text{Chlorophyll } a = 0.01373A_{663} - 0.000897A_{537} - 0.003046A_{647} \quad (2)$$

$$\text{Chlorophyll } b = 0.02405A_{647} - 0.004305A_{537} - 0.005507A_{663} \quad (3)$$

$$\text{Carotenoids} = \left[A_{470} - \left(17.1 \times \left(\text{Chl } a + \text{Chl } b\right) - 9.479 \times \text{Anthocyanin}\right)\right] / 11926 \quad (4)$$

The total chlorophyll content was obtained from the sum of Equation (2) and Equation (3).

Total phenolic content (TPC) was determined using Folin-Ciocalteau method [18]. The absorbance was measured at 725 nm using a UV-VIS spectrophotometer (BEL Photonics®, SP 2000). TPC was expressed as mg gallic acid equivalent 100 g^{-1} of fresh weight.

Total flavonoids were determined according to the method described by Popova *et al.* [19], with adjustments [20]. The extraction was performed in 70% acidified methanol and the absorbance was measured at 425 nm in spectrophotometer (BEL Photonics®, SP 2000 UV/VIS). The total flavonoids were expressed in mg quercetin equivalent per 100 g of fresh weight.

The antioxidant activity was performed according to the methodology of Brand-Williams *et al.* [21], altered by Rossetto *et al.* [22]. The DPPH (2,2diphenyl, 1picrylhydrazyl hydrate) (Sigma Aldrich Brazil) in 80% methanol and the absorbance was measured at 517 nm and converted in percentage of the antioxidant capacity through the equation:

$$\%\text{ of DPPH reduction} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

The calibration curve was prepared with Trolo × at 5, 10, 15, 20 and 25 μg and the results were expressed in μg TEAC equivalent g^{-1}.

2.4. Statistical Analysis

The data were submitted to the variance analysis (F test) and the means were compared using Tukey test at 5% of probability. The correlation analysis was performed between the contents of anthocyanins, chlorophylls, carotenoids, phenolic compounds, flavonoids and antioxidant activity.

3. Results and Discussion

The application of *S*-ABA provided a raise in the intensity of the color in 'Rubi' grape berries (analyzed via anthocyanins) in all the treatments, when compared to control (**Table 1**). The color of the grape berries is resulting of the accumulation of phenolic compounds (e.g., anthocyanins) in the beginning of the ripening. Generally, it is found a high curvilinear relation between the anthocyanins content and parameters of color measures (e.g., L^* e h°) [12]. With two *S*-ABA

Table 1. Anthocyanins (Ant), total phenolic compounds (TPC), flavonoids (Flav) and carotenoids (Car) of 'Rubi' grape submitted to the application of abscisic acid (*S*-ABA).

	Treatments (*S*-ABA mg·L^{-1})	Ant (mg 100 g^{-1})	TPC (mg 100 g^{-1})	Flav[a]	Car (μg 100 g^{-1})
	Control (0)	0.16 ± 0.03* c	61.14 ± 5.02* c	0.84 ± 0.04* b	73.42 ± 8.26* a
	400 (BR)	0.22 ± 0.03 b	72.41 ± 4.34 b	0.89 ± 0.04 b	62.83 ± 4.21 b
	400 (BR) + 200 (25 DAFA)	0.30 ± 0.03 a	82.53 ± 4.96 a	0.98 ± 0.13 ab	74.74 ± 2.65 a
	400 (BR) + 400 (25 DAFA)	0.25 ± 0.02 b	78.99 ± 2.81 ab	1.16 ± 0.16 a	77.76 ± 6.68 a
	CV (%)	11.38	5.65	12.06	8.36

*Means followed by the same letter in the column do not show any significant difference by the Tukey's test at 5% probability. Note: BR = Beginning of Ripening; 25 DAFA = 25 days after the first application. [a]Expressed in mg quercetin equivalente per 100 g^{-1} of fresh sample.

applications (400 mg·L^{-1} at BR + 200 mg·L^{-1} at 25 DAFA) there was an induction of anthocyanins synthesis and accumulation (0.30 mg 100 g^{-1}), 46.66 % higher than control (0.16 mg 100 g^{-1}) (Table 1). The use of this regulator applied twice (400 mg·L^{-1} 7 days after the beginning of the ripening + 400 mg·L^{-1} at 15 days before the harvest) improved the 'Benitaka' grape color, besides improving the physical characteristic of the clusters, when compared to one application (400 mg·L^{-1} 7 days after the beginning of the ripening) [8].

The synthesis of anthocyanins can occur from the beginning of the ripening until the harvest, as well as during the first half of the ripening [23], which would justify the positive effect of the *S*-ABA application in two phases of the grapes ripening. Many studies have showed that the *S*-ABA exogenous increases the acnthocyanins contents in grapes [10] [11] [24] [25] and can also improve the color and quality of the fruit [8] [12], by increasing other classes of polyphenols (e.g., flavonoids and ellagic acid) [13].

Independently on the concentration, treatments with two *S*-ABA applications were efficient in the induction of total phenolic compounds (TPC) and flavonoids in 'Rubi' grape (Table 1). For total flavonoids, there was a high concentration in the grapes treated with 400 mg·L^{-1} BR + 400 mg·L^{-1} 25 DAFA distinguishing from the control (Table 1). The variations in the flavonoid content can be attributed to genetic and climatic factors, management of the vineyard, degree of ripeness, among others [26]. In this study, all treatments received the same management and the same growing conditions, thus, the grapes ripening

degree promoted the synthesis and accumulation of flavonoids, which is evidenced by the intense pigmentation of the berries, provided after *S*-ABA application.

With two applications of 400 mg·L^{-1} of *S*-ABA, the grapes showed 1.16 mg 100 g^{-1} of flavonoids, 27.58% superior when compared to the control (0.84 mg 100 g^{-1}). The anthocyanins and flavonoids are products of the phenylalanine ammonia lyase (PAL - EC 4.3.1.5) and of the cinnamate 4-hydroxylase (C4H - EC 1.14.13.11) [27] activities. In this way, the color of the 'Rubi' grape using 400 mg·L^{-1} BR + 200 mg·L^{-1} at 25 DAFA, may be a consequence of the induction of the enzymes activities.

There was a significant effect of the treatments in the total chlorophyll levels and total carotenoids of 'Rubi' grapes (**Table 2**). Grapes that were not treated with *S*-ABA showed the highest content (104.22 μg 100 g^{-1}), not differing statistically from the grapes treated with two applications of *S*-ABA (400 mg·L^{-1} BR + 200 mg·L^{-1} at 25 DAFA), which showed a content of 102.16 μg 100 g^{-1} (**Table 2**). The result obtained with the grapes that received application of *S*-ABA showed chlorophyll content similar to the control, what can be explained by the analyses in the whole berries (peel and pulp), because the 'Rubi' grape shows white pulp.

Grapes treated with two *S*-ABA application showed the highest of total carotenoids content (74.74 and 77.76 μg 100 g^{-1}), distinguishing from the ones that received only one application (62.83 μg 100 g^{-1}), however, they did not differ from the control treatment (73.42 μg 100 g^{-1}) (**Table 1**). Some studies that quantify the chlorophyll and carotenoids in the berries of colored grapes are scarce, mainly the ones submitted to the application of abscisic acid. According to Rustioni *et al.* [28] these compounds perform an important role in the grapes pigmentation, but especially in white grapes, when the anthocyanins are not present. In the grapes, as well as in its derivatives, the content and profile of the carotenoids influence in the fruit bouquet and are considered determining factors for the aroma, beyond its importance to the human health (pro-vitamin A and antioxidant activity) [29].

Even though the analyzed compounds with antioxidant potential have been affected by the *S*-ABA application, there was no variation in the total antioxidant

Table 2. Total chlorophyll and total antioxidant activity of the 'Rubi' grape, submitted to the application of abscisic acid (*S*-ABA).

Treatments (*S*-ABA mg·L^{-1})	Chlorophyll total (μg 100 g^{-1})	Antioxidant activity^{aNS**}
Control (0)	104.22 ± 7.34* a	341.35 ± 28.93*
400 (BR)	90.71 ± 7.38 b	322.96 ± 18.19
400 (BR) + 200 (25 DAFA)	91.28 ± 8.22 b	341.16 ± 24.34
400 (BR) + 400 (25 DAFA)	102.16 ± 13.19 ab	346.96 ± 29.03
CV (%)	7.94	7.62

*Means followed by the same letter in the column do not show any significant difference by the Tukey's test at 5% probability. Note: BR = Beginning of Ripening; 25 DAFA = 25 days after the first application. [a]Expressed in μg Trolox equivalent g^{-1} of fresh sample. [NS]**Non-significant.

activity measured via DPPH (Table 2). The antioxidant capacity in grapes treated with abscisic acid is variable in grapes cultivars. Sandhu *et al.* [13] studied the effects of the ABA exogenous in grapes 'Alachua' (table grape) and 'Noble' (wine grape) and verified that the ABA increased the antioxidant capacity of the 'Noble' grape in two times (8 and 10 days after the first and second application, respectively), while that for 'Alachua' no differences were found in no sampling period. In the juice of the 'Isabel' grape, Yamamoto *et al.* [10] found no correlation between the content of phenolic compounds and antioxidant activity.

In our study, the antioxidant activity measured via DPPH can be attributed mainly to the phenolic compounds, however, other molecules as carotenoids and chlorophylls may have influenced the response, due to the properties of scavenger reactive oxygen species (ROS) [30] [31]. Other analytic methods have been used to determine the antioxidant activity in grapes (ABTS, DPPH, FRAP, ORAC, among others), increasing the difficult in the data comparison. Other studies showed the difficulty in obtaining similar data for the total antioxidant activity, making it difficult to compare the obtained data [32] [33], which can be attributed to the method utilized, or by the expression of results, or even by the analyzed material (peel, pulp, seeds or full berries).

The anthocyanins showed a positive and high correlation with the concentration of total phenolic compounds (r = 0.7852**) (Table 3), evidencing that the increase of anthocyanins is related to the increase of total phenolic compounds of the 'Rubi' grape. These compounds are directly or indirectly involved with the grapes color and red grape juice color, beyond important characteristics of the fruit quality [10].

There was a significant correlation between the total phenolic compounds and flavonoids, evidencing that the increase of the flavonoids contribute to the TPC. Nevertheless, this was already expected, since the flavonoids, as well as the anthocyanins, belong to the polyphenols class. We found that the total chlorophyll and carotenoids contributing to the increase of the flavonoids content (Table 3). According to Han *et al.* [34], the carotenoids have been described for maintaining the cell wall integrity and the efficiency of this pigment as an antioxidant

Table 3. Correlation between total chlorophyll (Cl *tot*), anthocyanins (Ant), carotenoids (Car), flavonoids (Flav), total phenolic compounds (TPC) and antioxidant activity (AA) of the 'Rubi' grape, submitted to the application of abscisic acid (*S*-ABA).

	Ant	Car	Flav	TPC	AA
Cl *tot*	−0.2326^{ns}	−0.0687^{ns}	0.5214**	0.0344^{ns}	0.1005^{ns}
Ant		−0.0687^{ns}	0.2715^{ns}	0.7852**	0.1159^{ns}
Car			0.5214**	−0.0465^{ns}	0.2170^{ns}
Flav				0.4465*	0.1005^{ns}
TPC					0.0344^{ns}

ns: non-significant; *Significant 5%; **Significant 1%, by Tukey's test (P < 0.05).

against injuries promoted by ROS depends on the synergistic interaction with flavonoids and with other carotenoids. In this way, the positive correlation found between carotenoids and flavonoids can be attributes to the synergic action and antioxidant of both phytochemicals.

It is known that well-colored grapes are more acceptable in the market of fresh fruits, thus growers should consider the application costs and the grape price to evaluate the benefits of the *S*-ABA application to increase profits. Thus, from the data, we noticed that *S*-ABA promotes the synthesis and accumulation of anthocyanins, phenolic compounds and total flavonoids of the 'Rubi' grape, mainly when applied twice, adding commercial value to this grape cultivar, since the consumers are attracted by fruits with antioxidant potential. It is also important to state that none of *S*-ABA treatments changed the berry taste, what indicates that the plant growth regulator has no influence on ripening evolution.

4. Conclusion

The application of *S*-ABA in two phases in the clusters development (beginning of ripening and after 25 days) is efficient to improve the berries color, as well as for increase of anthocyanins and phenolic compounds content of the 'Rubi' grape. The dose of 400 $mg \cdot L^{-1}$ BR + 200 $mg \cdot L^{-1}$ DAFA is enough to increase the anthocyanins, which are highly correlated to the berries color, increasing the antioxidants content of the fruit.

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