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Grape berry transpiration influences ripening and must composition in cv. Tempranillo (*Vitis vinifera* L.)

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Abstract

The implications of grape berry transpiration for the ripening process and final grape composition were studied. An experiment was conducted, under controlled conditions, with fruit-bearing cuttings of *Vitis vinifera* L. cv. Tempranillo. Three doses of the antitranspirant di-1-*p*-menthene were applied directly to the bunch at the onset of veraison: 1%, 5%, and 10% (v/v) (D1, D5, and D10, respectively). A treatment with bunches sprayed with water (D0) was also included as a control. Grape and bunch transpiration, and total soluble solids (TSS) accumulation rate decreased as the dose of antitranspirant increased, thus resulting in the lengthening of the ripening period. Bunch transpiration rates were linearly correlated with the elapsed time between veraison and maturity, and with the TSS accumulation rate. The evolution of pH, malic acid and total skin anthocyanins during ripening did not show remarkable changes as a consequence of the artificially reduced bunch transpiration. However, a decoupling between TSS and anthocyanins was observed. At maturity, the bunches treated with D10 had significantly lower must acidity and higher pH and extractable anthocyanin levels, these differences being likely associated with the lengthening of the ripening period. The results show a clear implication of grape transpiration for the ripening process and final grape composition, and give new hints on the direct application of antitranspirants to the bunch as a way to regulate sugar accumulation while avoiding the concurrent delay of color development.

1 | INTRODUCTION

Fruit transpiration is a biophysical process determined by environmental parameters, such as temperature, wind speed, radiation, relative humidity, and so on, and fruit skin conductance, which is species-specific (Montanaro et al., 2012). In grapevine, the skin water permeability depends on both stomata and cuticular conductance. Since the stomata density of berry skin is very low, <1 stomata per mm^2, the main driver for grape berry transpiration is the cuticular conductance (Blanke & Leyhe, 1987). Grape berry transpiration changes throughout development, and a decline, either on a per surface area basis or on a per fresh weight basis, has been described, associated with anatomical modifications of fruit epidermis (Fahey & Rogiers, 2019; Palliotti & Cartechini, 2001; Rogiers et al., 2004). During berry development, stomata become partially or completely occluded by epicuticular wax or are transformed into lenticels, thus losing their functioning (Hardie et al., 1996). At the same time, an increase in the amount of epicuticular waxes and modifications in wax structure and

composition have been described during berry growth, which determine the reduction in the cuticular conductance (Palliotti & Cartechini, 2001; Rogiers et al., 2004).

Over the last decades, a trend toward earlier grape ripening, marked by fast berry sugar accumulation, has been reported in many regions around the world as a consequence of global warming (Martínez-Lüscher et al., 2016; Mira de Orduña, 2010; Petrie & Sadras, 2008; van Leeuwen et al., 2019). This advanced maturity has also been coupled with a lower concentration of acids and anthocyanins, high pH and the lessening of aromatic components in the must (Keller, 2010; Kuhn et al., 2014). The phenomena can result in wines with a high alcohol concentration, low acidity and an undesirable concentration of aromatics and phenolic substances (Palliotti et al., 2014). In this context, the application of antitranspirants has been proposed to maintain plant water status and delay sugar accumulation in berries. The application of film-forming antitranspirants to only the upper portion or the entire canopy, including the bunch zone, has been tested as a suitable strategy to hinder berry sugaring and to delay ripening (Brillante et al., 2016; Di Vaio et al., 2019; Gatti et al., 2016; Palliotti et al., 2013; Silvestroni et al., 2020). Such reduction in sugar accumulation was mainly associated with a significant source limitation produced as a consequence of the physical blockage of the stomata, thus limiting the CO_2 entry and leaf photosynthetic activity. Nevertheless, berry transpiration in response to antitranspirant treatment has not been assessed in these studies and it is difficult to ascertain if this kind of products has a direct effect on the fruit itself (Fahey & Rogiers, 2019).

Besides its important role in water balance, fruit transpiration also acts as one of the driving forces for sugar accumulation. During ripening, phloem becomes the primary water source for berries and constitutes the preferential pathway in post-veraison berries, although recent studies showed that xylem appears functional and connected between the vine and the berries after veraison (reviewed in Dai et al., 2010). Lang and Thorpe (1986) postulated that the key mechanism involved in the assimilate flow into the berries is a gradient in water potential from stem to bunch. Water loss through transpiration, which leads to a lower turgor within the berry and maintains the gradient of water potential between the fruit and the stem, may promote the importing of assimilates into the berry (Rebucci et al., 1997). Nevertheless, the literature on the response of fruit ripening to changes in transpiration rates still is very limited. Morandi et al. (2010) observed that high transpiration rates enhanced phloem import, with subsequent positive effects on solute accumulation and fruit growth of peach. Zhang et al. (2017) reported a lower solute accumulation rate in grape berries of cv. Concord, Merlot and Syrah with a forced reduction in transpiration with a commercial antitranspirant. Also, a linear correlation between berry transpiration and net sugar intake in the grapes has been described in cv. Sangiovese plants with manipulated grape berry transpiration (Rebucci et al., 1997). These studies are mainly focused on sugar accumulation, whereas little information is available on the impact of reduced grape transpiration on other important constituents of grapes, such as organic acids and anthocyanins. In this work, we wanted to take a step forward, and we aimed to know how berry transpiration affects grape ripening and sugar accumulation, as well as the content of other key constituents of red wine grapes, such as organic acids and anthocyanins, with important implications in the final grape and wine quality. We hypothesized that grape transpiration plays a significant role in grape development and that low transpiration rates lengthen the maturation period, which may have potential implications for the accumulation of grape constituents and final grape quality. This practical information may shed light on different alternative practices of antitranspirant application in order to achieve the desired berry composition.

2 | MATERIALS AND METHODS

2.1 | Plant material and experimental design

Vitis vinifera L. cv. Tempranillo is an important red variety native from La Rioja (Spain) and currently widely cultivated in the Iberian Peninsula and South America. It is characterized by having a large limb with five to seven lobes, a medium-large cluster, a short-medium peduncle, and a compact bunch. The berries have a circular shape, are small and uniform with a thick, bluish-black skin, whereas the pulp has no or very weak color. Tempranillo must have an intense color and low acidity, being a good base of wine blends (Chomé et al., 2003).

Plant material consisted of dormant cuttings taken from Estación de Viticultura y Enología de Navarra (EVENA, Olite, Spain). Fruit-bearing cuttings were obtained as described by Mullins and Rajasekaran (1981) with some modifications (Arrizabalaga et al., 2018). First, rooting was stimulated by immersing the cuttings in indole butyric acid in a warm bed (27°C) into a cold chamber (5°C). One month later, and once the cuttings developed roots, they were transplanted into pots with a substrate composed of peat: perlite (2:1, v/v). Plants were transferred to the greenhouse, where they grew at 24°C/14°C and 50%/70% relative humidity (RH) (day/night) under natural daylight supplemented with high-pressure metal halide lamps (OSRAM®), providing a photosynthetic photon flux density of 500 μmol m^{-2} s^{-1} at inflorescence level. Plants were manually pruned and maintained with four leaves until fruit set. Then, the main shoot was allowed to grow up to about 12 leaves in all the plants, thus maintaining a constant leaf area to fruit fresh mass ratio. A single bunch per plant was allowed to grow. Irrigation was performed with water alternated with nutrient solution (Ollat et al., 1998).

At the onset of veraison (defined when three to four grapes in the bunch started to turn red), plants were divided into four homogenous groups of eight plants each. These plants were selected to have similar intergroup and intragroup variability in terms of grape bunch size. Each group was treated using a spray with a different dose of di-1-*p*-menthene ($C_{20}H_{34}$, VaporGard®, Miller Chemical & Fertilizer Co.), corresponding to 1%, 5%, and 10% of antitranspirant diluted in water (D1, D5, and D10, respectively). The di-1-*p*-menthene, also known as pinolene, is a water emulsifiable organic concentrate for use on plants and designed to reduce transpiration by forming a clear, soft, and flexible film that retards normal moisture loss (Palliotti et al., 2010). A group of plants was sprayed with water as a control treatment (D0).

The plants of the different treatments were homogeneously distributed in the greenhouse and grew until maturity under the temperature, RH and light conditions described above. Maturity was considered when grape berry total soluble solids (TSS) content was about 23°Brix.

2.2 | Ripening time

The ripening time was calculated as the number of days from the onset of veraison to maturity. Both stages were assessed for each plant individually. The onset of veraison was determined visually when three to four berries in the bunch started to turn red. To determine maturity, the TSS content of two to three berries per bunch was periodically measured.

2.3 | Grape berry and bunch transpiration

Berry and bunch transpiration were measured with a new measuring device as recently described (Morales et al., 2022). Transpiration rates were measured in detached Tempranillo grapes from five different plants during the ripening period and in detached bunches at maturity, assuming that detaching from the bunch (berries) or from the plant (bunches) does not alter fruit transpiration, as previously verified by Zhang and Keller (2015) and Morales et al. (2022). Weekly, from the beginning of the treatments, five berries per bunch were taken from five different plants of each treatment ($n = 5$). Berries were sampled from the top and middle portion of the bunch, which allocate the highest number of berries. These were weighted to determine their fresh mass (FM) before measuring transpiration. Additionally, the bunches of eight plants per treatment were sampled at maturity and weighted before transpiration measurements. The berries chamber consisted of a 50 cm^3 syringe. The bunch chamber consisted of a methacrylate box (30 × 15 × 15 cm). Both chambers were equipped with small fans inside to ensure air homogeneity. The chambers were supplied with air previously conditioned at 40% RH using a condensation trap. The rate of air flow to the chambers was 400 ml min^{-1} and 1 L min^{-1}, berry and bunch chamber, respectively. The relative humidity (RH) of the air sample taken from the outlet duct was measured with a RH sensor (Vaisala). Both chambers were placed into a plant growth chamber maintained at 24°C to ensure the stability of measurements. Readers are referred to Morales et al. (2022) for a complete and detailed description of the measuring device. After the measurements, the berries and bunches sampled were stored at −20°C for subsequent analyses.

2.4 | Evolution of total soluble solids, malic acid, pH, and skin anthocyanins in the berries

Total soluble solids (TSS), pH, and total skin anthocyanins were analyzed 0, 7, 14, 21, 28, 35, and 42 days after the beginning of treatments in five berries sampled from five different plants. Malic acid was measured on day 0, 7, 21, and 35. For that, grapes were manually separated into skin, pulp and seeds. TSS, pH, and malic acid were measured in the must extracted from the pulp: TSS using a desktop refractometer (Abbe Digital 315RS, Zuzi); malic acid with an enzymatic method (Enzytec™ L-Malic Acid, Boehringer Mannheim/R-Biopharm); and pH with a pH meter (Crison Instruments). In order to calculate the sugar content per berry, total sugars per berry mass were estimated from TSS according to the International Organization of Vine (OIV, 2016). The skins were lyophilized and, subsequently, ground with a batch ball mill (Retsch MM400). Total skin anthocyanins was measured according to the method described in Arrizabalaga et al. (2018). Briefly, 50 mg of sample were taken and 2 ml of methanol–HCL (0.1%) was added. The samples were stirred for 60 min and centrifuged at 4100*g* for 10 min at 4°C. The supernatant was taken and diluted in methanol–HCL (0.1%) to subsequently measure the absorbance in a mini-1240 UV spectrophotometer (Shimadzu) at 536 nm. The calibration curve used was prepared with malvidin-3-*O*-glucoside (Sigma-Aldrich).

2.5 | Technological and phenolic maturity

At maturity, 15 berries per plant ($n = 8$) were taken and pooled, then extracted and centrifuged for 10 min at 4100*g*. In the supernatant, TSS and pH were measured as described previously. Total acidity was determined by titrating 2 ml of extracts against NaOH 0.01 N according to the OIV (2016) and expressed as g tartaric acid per L of must. Additionally, 15 other berries per plant ($n = 8$) were ground in a batch ball mill. The homogenate was divided into two centrifuge tubes in equal and known volumes. Samples were macerated for 4 h at 4°C with either a HCl solution (pH 1) for total anthocyanin determination, or a tartaric acid solution (pH 3.2) for the analysis of extractable anthocyanins, color intensity, tonality index, and total polyphenol index (TPI). After maceration, samples were centrifuged at 4100*g* for 10 min at 4°C. The supernatant was quickly separated to avoid re-suspension. Total and extractable anthocyanins were determined using the SO_2 bleaching method (Ribéreau-Gayon & Stonestreet, 1965). Both data were used to calculate the cellular extractability of anthocyanins (AE; Nadal, 2010). For the determination of TPI, 0.5 ml of the extract macerated at pH 3.2 were diluted 100 times and absorbance was measured at 280 nm (Ribéreau-Gayon & Stonestreet, 1965). The seed maturity (SM) index was calculated from TPI and extractable anthocyanin values (Nadal, 2010). Color intensity and tonality were analyzed according to Glories (1984) and Glories and Augustin (1993) in the supernatant of the sample macerated at pH 3.2. Color intensity was determined by the sum of the measured absorbances at 420, 520, and 620 nm. Tonality index was determined by the ratio between the measured absorbances at 420 and 520 nm.

2.6 | Statistical analysis

We applied an analysis of variance (one-way ANOVA) to determine the effect of the antitranspirant treatments on the parameters

analyzed. When the effect of the treatments was statistically significant ($p < 0.05$), the differences among doses of antitranspirant were analyzed with the post hoc test of least significant difference (LSD). Regression analysis was used to evaluate relationships between quantitative variables: total skin anthocyanins versus TSS, as well as elapsed time veraison-maturity and TSS accumulation rate versus bunch transpiration. Analysis of residuals was used to test the effects of the antitranspirant on the relationship between TSS and total skin anthocyanins. For that, the equation obtained after including all the treatments was fitted to the data, and the effect of the antitranspirant dose on residuals was tested with an analysis of variance. Phenological and grape composition parameters at maturity were also analyzed using a principal component analysis (PCA). Statistical analysis was performed using the XLSTAT 7.5.2 statistical package.

3 | RESULTS

3.1 | Grape berry and bunch transpiration in response to the application of antitranspirant

The treatments with antitranspirant, especially the highest doses (D5 and D10), significantly reduced grape berry transpiration in the first 3 weeks after the application compared with the treatment without antitranspirant, D0 (Figure 1A). In the following 2 weeks (days 28 and 35), transpiration was quite similar in all the treatments but a slight difference was observed again in week 6 (day 42), D0 showing significantly higher transpiration rates compared with the rest of the treatments. The differences in the cumulative transpiration of the berries increased throughout the ripening period. At the end of the ripening period (day 42), the treatments with antitranspirant (D1, D5, and D10) reduced significantly the cumulative berry transpiration with respect to D0 (Figure 1B). Bunch transpiration, measured at maturity, decreased significantly as the dose of antitranspirant increased (Table 1), in accordance with the results of berry transpiration. The application of antitranspirant did not produce significant changes in the bunch fresh mass at maturity. Individual berry weight showed a tendency to decrease with an increasing dose of antitranspirant (differences statistically not significant; Table 1).

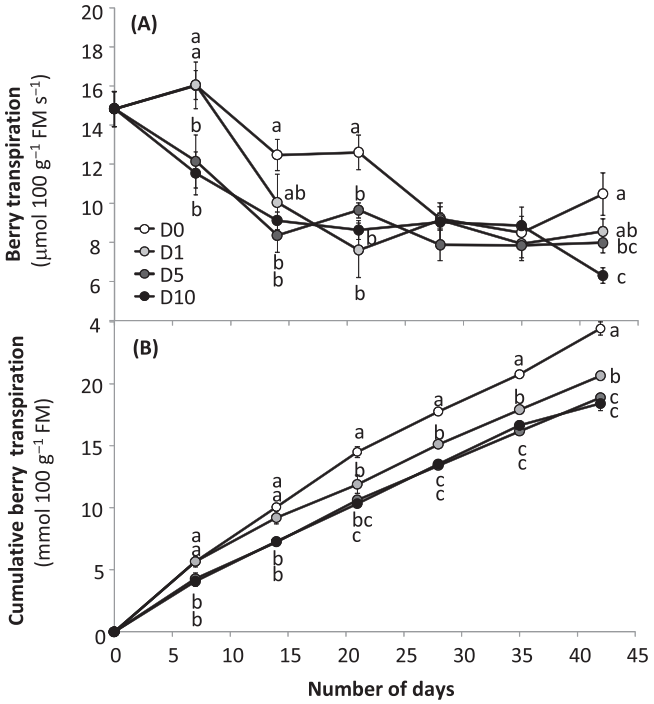

FIGURE 1 Evolution of grape berry instantaneous (A) and cumulative (B) transpiration in *Vitis vinifera* L. cv. Tempranillo bunches sprayed with 0% (v/v, D0), 1% (D1), 5% (D5), and 10% (D10) of antitranspirant. Measurements were done weekly from the beginning of the treatments (onset of veraison). Data are means ± standard errors. $n = 5$ biological replicates, each being five berries taken from one plant, total of five sampled plants. Means with letters in common within each same sampling date are not significantly different according to LSD-test ($p > 0.05$). FM, fresh mass.

3.2 | Evolution of TSS, pH, malic acid, and skin anthocyanins throughout ripening

TSS gradually increased throughout berry ripening from values around 9.4°Brix at the beginning of the treatments (onset of veraison) up to around 20°Brix 6 weeks later (Figure 2A). On day 7, the grapes sprayed with the highest dose of antitranspirant (D10) showed significantly lower TSS than those of the treatments D0 and D1. In addition, from day 21 onwards, the bunches not treated with antitranspirant (D0) tended to have higher must TSS than those sprayed with the highest doses (D5 and D10), these differences being statistically significant on day 42. Malic acid decreased throughout ripening, thus leading to an increase in pH (Figure 2B,C). No significant differences among treatments were observed for these two parameters within each sampling date, except for malic acid on day 7, when D10 showed significantly higher values than D1 and for pH at maturity, when D10 showed higher pH values than D0. The concentration of total skin anthocyanins increased gradually until maturity, reaching levels of 44–48 mg g^{-1} skin dry matter in the last sampling date, with minor differences among treatments (Figure 2D).

The pooled data supported a strong linear relationship ($R^2 = 0.89$, $P < 0.001$) between total skin anthocyanins and TSS (Figure 3). When we calculated the regressions for each antitranspirant dose, we observed a slope-dependent decoupling between these two parameters, induced by the application of the antitranspirant, especially in the highest doses (D5 and D10; Figure 3A). The values of the slopes for each antitranspirant dose were 3.7, 3.9, 4.3, and 4.0 for D0, D1, D5, and D10, respectively. The positive mean residuals for the treatments D5 and D10 compared with the negative mean residuals for D0 and D1 (Figure 3B) indicates a higher concentration of anthocyanins at the same concentration of soluble solids in those berries sprayed with high doses of antitranspirant over the ripening period.

TABLE 1 Bunch and berry fresh mass (FM) and bunch transpiration at maturity in *Vitis vinifera* L. cv Tempranillo treated with 0% (v/v, D0), 1% (D1), 5% (D5), and 10% (D10) of antitranspirant. Data are means ± standard errors ($n = 8$ plants). Means without letters or with letters in common are not statistically different according to LSD-test ($p > 0.05$). FM, fresh mass.

Treatment	Bunch FM (g)	Berry FM (g)	Bunch transpiration (μmol H_2O 100 g^{-1} bunch FM s^{-1})
D0	81.5 ± 10.7	1.43 ± 0.42	6.26 ± 0.64 ab
D1	72.8 ± 10.6	1.22 ± 0.42	6.77 ± 0.49 a
D5	72.1 ± 12.5	1.26 ± 0.50	5.26 ± 0.50 bc
D10	95.8 ± 24.2	1.11 ± 0.31	4.27 ± 0.40 c

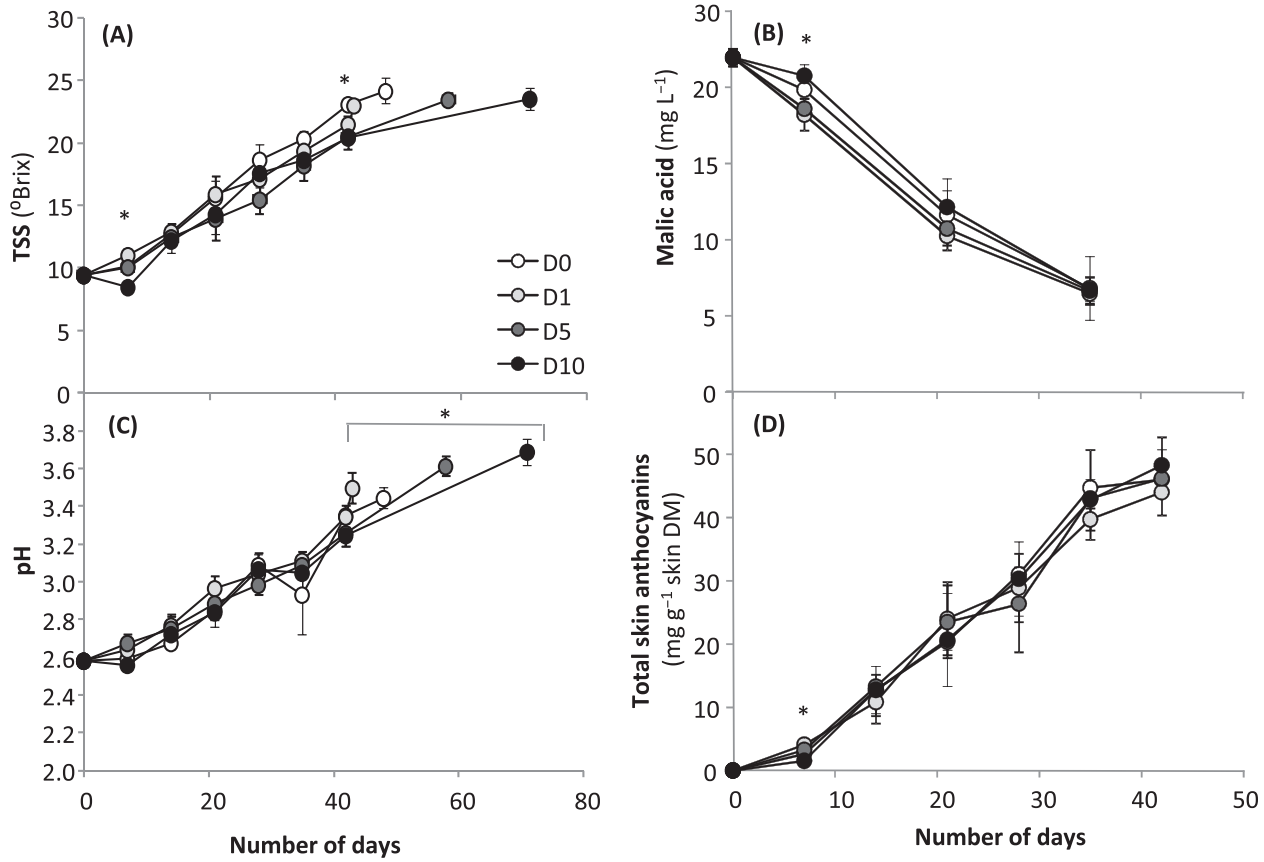

FIGURE 2 Evolution of total soluble solids (TSS, A), pH (B), malic acid (C), and total skin anthocyanins (D) in bunches of *Vitis vinifera* L. cv Tempranillo treated with 0% (v/v, D0), 1% (D1), 5% (D5), and 10% (D10) of antitranspirant. Measurements were done weekly from the beginning of the treatments (onset of veraison) for TSS, pH, and total skin anthocyanins (malic acid measured only at day 0, 7, 21, and 35). Charts A and C also include data at maturity (last point of each treatment). Data are means ± standard errors ($n = 5$ biological replicates, each being five berries taken from one plant, total of five sampled plants). * indicates statistical differences among doses either within each sampling date or stage (maturity in the case of TSS and pH) ($p < 0.05$). DM, dry mass.

3.3 | Effect of antitranspirant on technological and phenolic maturity

The levels of TSS were similar among treatments at maturity due to the fact that bunches were harvested when each one reached a fixed °Brix value (around 23°Brix) (Table 2). Must acidity significantly decreased as the antitranspirant rate increased, which was associated with a significant increase in pH (Table 2). The concentration of total and extractable anthocyanins increased with increasing the dose of antitranspirant (significant differences for extractable anthocyanins), without significant changes in the percentage of extractability of these compounds (Table 2). The levels of color intensity tended to increase in those berries sprayed with the highest doses of antitranspirant (D5 and D10), although the differences were not statistically significant. The application of antitranspirant did not have a significant effect on the tonality and TPI of the must. The seed maturity index significantly decreased as the antitranspirant dose increased.

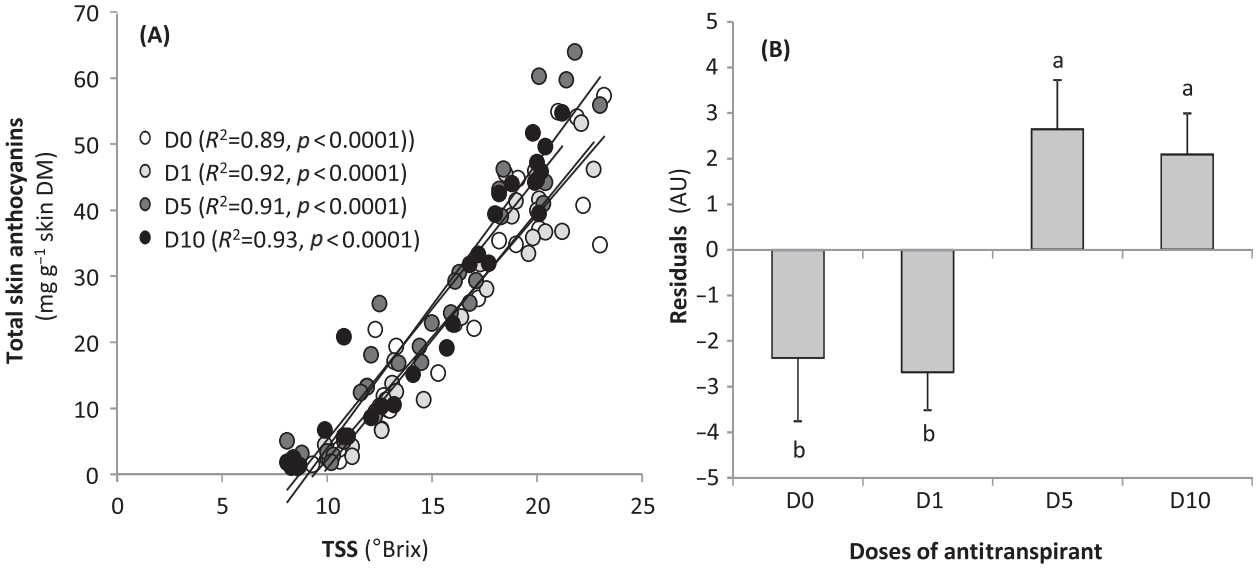

FIGURE 3 (A) Relationships between total soluble solids (TSS) and total skin anthocyanins and (B) residuals of the regressions in bunches of *Vitis vinifera* L. cv Tempranillo treated with 0% (v/v, D0), 1% (D1), 5% (D5), and 10% (D10) of antitranspirant. In (A), the lines represent the fitted regressions, considering only those values within the linear phase where TSS and anthocyanins increase in parallel (Sadras et al., 2012). In (B), bars are means ± standard errors ($n = 5$ biological replicates, each being five berries taken from one plant, total of five sampled plants). Means with letters in common are not significantly different according to LSD-test ($p > 0.05$). DM, dry mass.

TABLE 2 Grape technological and phenolic maturity of *Vitis vinifera* L. cv Tempranillo treated with 0% (v/v, D0), 1% (D1), 5% (D5), and 10% (D10) of antitranspirant. Data are means ± standard errors ($n = 8$ biological replicates, each being 15 berries from one plant, total of 8 sampled plants). Means without letters or with letters in common are not statistically different according to LSD-test ($p > 0.05$). AE, anthocyanin extractability; SM, seed maturity; TPI, total polyphenol index

Treatment	°Brix	Titratable acidity (g L^{-1})	pH	Total anthocyanins (mg L^{-1})	Extractable anthocyanins (mg L^{-1})	AE (%)	SM (%)	Color intensity	Tonality	TPI
D0	24.1 ± 2.9	7.80 ± 1.98 a	3.44 ± 0.15 b	338.0 ± 42.0	213.0 ± 12.1 b	32.9 ± 4.5	70.6 ± 1.8 a	3.10 ± 0.20	0.47 ± 0.02	29.0 ± 6.2
D1	23.0 ± 1.1	7.63 ± 2.48 a	3.50 ± 0.23 ab	314.2 ± 26.8	208.4 ± 15.0 b	32.7 ± 2.2	69.0 ± 2.9 ab	3.08 ± 0.30	0.45 ± 0.01	28.1 ± 7.9
D5	23.4 ± 1.4	6.14 ± 1.06 ab	3.61 ± 0.15 ab	391.9 ± 48.5	250.0 ± 15.7 ab	30.2 ± 6.2	63.6 ± 2.0 bc	3.26 ± 0.27	0.47 ± 0.02	27.1 ± 4.6
D10	23.5 ± 2.6	5.61 ± 1.58 b	3.70 ± 0.19 a	398.9 ± 55.7	271.0 ± 16.9 a	28.5 ± 4.2	61.9 ± 1.8 c	3.75 ± 0.39	0.49 ± 0.01	28.4 ± 2.7

3.4 | Relationship between bunch transpiration and ripening

The bunches treated with antitranspirant took longer to reach maturity than those not treated (D0) (Figure 4A). The elapsed time between veraison and maturity increased in 10 and 23 days in those bunches sprayed with the highest doses of antitranspirant (D5 and D10, respectively) compared with D0. The accumulation rate of both TSS and sugars per berry significantly decreased in the treatments D5 and D10, up to around 26% compared with D0 and D1 (Figures 4B and S1, respectively). A negative, linear correlation was found between the bunch transpiration rates measured at maturity and the elapsed time between veraison and maturity (Figure 4C). Bunch transpiration was also positively correlated with TSS accumulation rate (Figure 4D).

3.5 | Principal component analysis

A principal component analysis (PCA) of descriptive data regarding bunch transpiration, phenology, and technological and phenolic maturity was conducted to better visualize the impact of the antitranspirant doses, as well as to identify the variables involved in the bunch response to these treatments. Figure 5A shows the score plot, where samples of each treatment are grouped in the plot of the first and second principal component: PC1 versus PC2. PC1 accounted for about 39% of the total variance, whereas PC2 covered 25%. The treatments D0 and D10, corresponding to the most extreme treatments, were clearly separated in the score plot. The loading plot (Figure 5B) reveals that the distinction between D0 and D10 was mainly associated with a longer ripening period (veraison-maturity), higher pH and lower acidity values in the must of the berries treated with D10 compared with

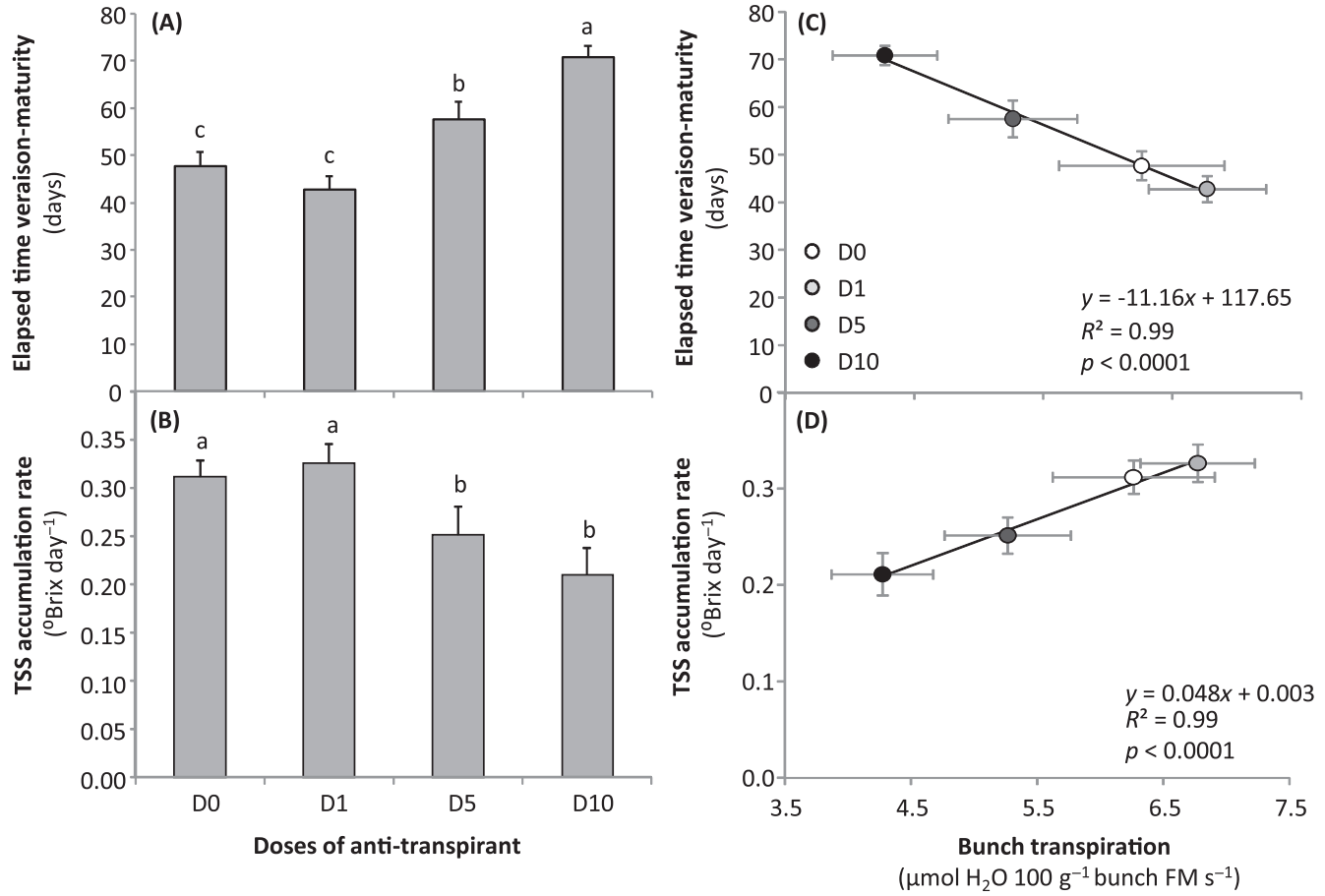

FIGURE 4 (A) Elapsed time between veraison and maturity, (B) accumulation rate of total soluble solids (TSS), and (C and D) their respective relationships with bunch transpiration rates in bunches of *Vitis vinifera* L. cv Tempranillo treated with 0% (v/v, D0), 1% (D1), 5% (D5), and 10% (D10) of antitranspirant. Data are means ± standard errors ($n = 6$–8 plants). Means with letters in common are not significantly different according to LSD-test ($p > 0.05$). In (C and D), the lines represent the fitted regressions. FM, fresh mass.

D0. This loading plot also reveals the negative correlation between bunch transpiration and the length of the ripening period (−0.504, $P < 0.05$), both variables presenting the strongest association to PC2, but with positive and negative values, respectively.

4 | DISCUSSION

Berry transpiration is a process conditioned by a series of environmental factors, such as ambient temperature and relative humidity, which determine the vapor pressure deficit (VPD). Cultivar-specific factors, such as berry surface area and skin conductance, also play a role in berry transpiration (Zhang et al., 2017; Zhang & Keller, 2015). In the present study, berry transpiration was higher at the onset of veraison and then decreased until maturity (Figure 1). Fahey and Rogiers (2019) also observed a decrease of bunch transpiration, expressed per berry surface, as the berries of cv. Müller-Thurgau, Ehrenfelser, and Riesling reached maturity. Similarly, Palliotti and Cartechini (2001) reported a reduction in transpiration, expressed on a FM basis, in Cabernet Sauvignon berries throughout development. The reduction in berry transpiration has been related to changes in the permeability of berry skin to water. Berries have a very low surface stomatal density, <1 per mm^2, depending on the variety. Furthermore, stomata are partly or completely occluded by a thick layer of epicuticular wax after fruit set (Blanke & Leyhe, 1987). Therefore, cuticular conductance is the main driver for berry transpiration, and it has been reported to peak at veraison stage, then declining over the ripening period (Zhang & Keller, 2015). This progressive reduction in cuticular conductance is associated with chemical and structural changes in epicuticular waxes, such as crystallization, even if the cuticular wax per surface area does not increase during berry ripening (Leide et al., 2007; Rogiers et al., 2004).

The treatment with antitranspirant significantly reduced the transpiration rates of both berries and bunches, this effect being more evident during the first weeks after application. The decreasing influence of antitranspirant from day 28 may be due to expansion of the berries causing gaps in the coat; however, the differences in the cumulative berry transpiration among antitranspirant doses increased throughout the experiment. The results are in agreement with Fahey and Rogiers (2019), who have recently reported that the direct application of di-1-*p*-menthene to the bunch reduced water loss by reducing bunch transpiration.

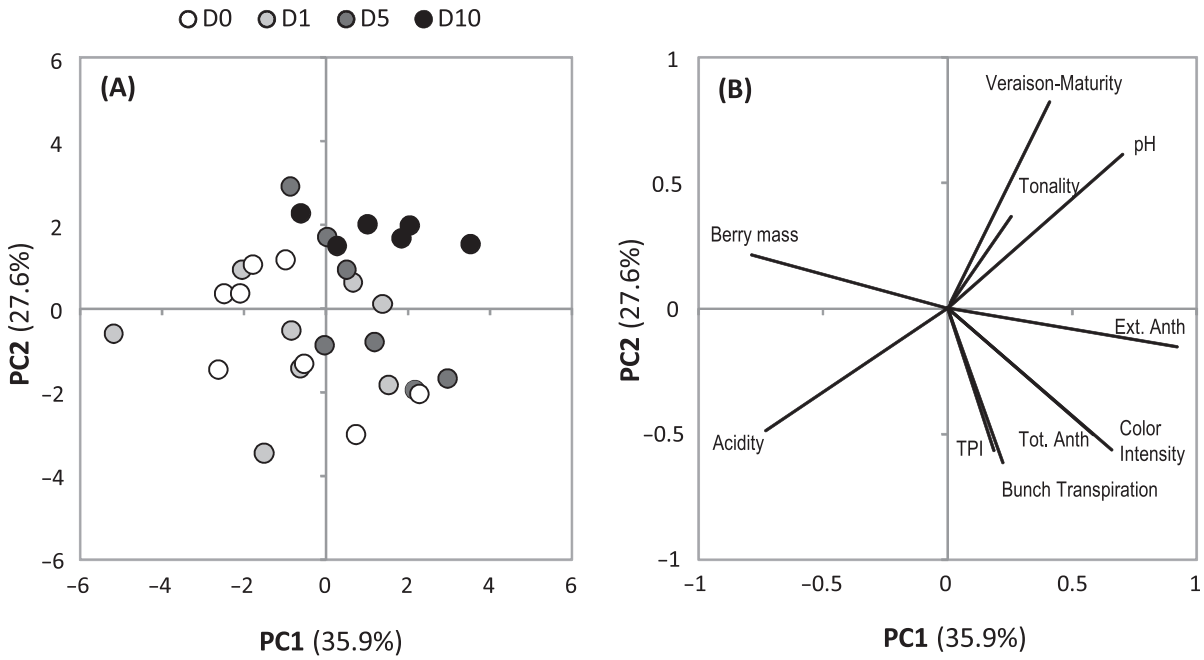

FIGURE 5 Principal component analysis of phenological development and grape technological and phenolic maturity: Score (A) and loading plot (B). Ext. Anth, extractable anthocyanins; Tot. Anth., total anthocyanins; TPI, total polyphenol index.

The impact of the application of antitranspirants to the canopy on the final grape composition has been mainly attributed to a reduction in leaf photosynthesis (Brillante et al., 2016; Di Vaio et al., 2019; Gatti et al., 2016; Palliotti et al., 2010, 2013; Silvestroni et al., 2020). However, few studies have focused on the direct effect of antitranspirant on the fruit itself, thus ruling out source limitation due to a reduced leaf C assimilation. In the present study, the application of antitranspirant to the bunch, particularly in high doses (D5, D10), significantly reduced the concentration of TSS in the berries on day 7 and 42 after the application, as well as the rates of TSS accumulation (Figure 2). Since grape maturity was considered when TSS was around 23°Brix, such slowed-down sugar accumulation led to an increase in the duration of the ripening period, thus delaying maturity in the bunches sprayed with antitranspirant. These results indicate that berry transpiration plays an important role in the accumulation of TSS in the grape and in the ripening rate. The positive correlation between bunch transpiration with the TSS accumulation rate, as well as the negative correlation between bunch transpiration and the length of the ripening period, also observed in the PCA (Figures 4 and 5), reinforces the idea of the relationship between grape berry transpiration and its capacity to accumulate solutes and to ripen. Our results are consistent with those reported by Rebucci et al. (1997) and Zhang et al. (2017), who observed a lower berry sugar accumulation in berries of Pinot Noir, Sangiovese, Concord, and Syrah cultivars with an artificially reduced transpiration. The accumulation of TSS throughout ripening results from two processes: the accumulation of sugars on the one hand and the variation in berry volume on the other. In the present study, berry size (berry FM) also showed a clear tendency to decrease as the dose of antitranspirant increased. The antitranspirant likely increased the water potential of the berry and decreased the necessary gradient required for efficient phloem unloading, thus reducing the rate of sugar accumulation and berry growth. In this line, Morandi et al. (2010) observed a lower phloem flow in peach fruits with artificially reduced transpiration during the cell expansion state, by enclosing fruits in plastic bags, which resulted in a reduced fruit growth. In addition, the accumulation rate of sugars per berry was reduced as the antitranspirant dose increased (Figure S1) despite the concurrent reduction in size. This supports the idea that a reduction in berry transpiration leads to a dynamic in sugar accumulation that is not simply the reflection of dilution/concentration effects associated with changes in berry size. Grape ripening is associated with a surplus of phloem-import water (Keller, 2015). Therefore, it is likely that berry transpiration functions as a means of discharging excess phloem-imported water, thus leading to lower turgor within the berry and maintaining the gradient of water potential between the fruit and the stem. Such gradient promotes the importing of assimilates into the berry and normal ripening (Rebucci et al., 1997; Zhang et al., 2017). Alternatively, a significant increase in berry water content has been previously reported after the application of di-1-*p*-menthene to the canopy, including the bunch (Fahey & Rogiers, 2019). Although berry water content was not measured in this study, we cannot rule out that a dilution effect may have contributed to the slowed-down accumulation of sugars in the grape in the treatments with antitranspirant. In this line, Rebucci et al. (1997) concluded that treatments applied to affect sugar accumulation via modifications of berry transpiration affect, by such a change, the berry water status itself, thus linking the two processes.

The application of di-1-*p*-menthene had a significant effect on reducing must acidity at maturity, which was concomitant with a significant increase in pH (Table 2). Gatti et al. (2016) also reported a decrease

in berry titratable acidity in vines sprayed (entire canopy) with the same type of antitranspirant at pre-flowering and pre-veraison, probably associated with source limitation induced through a decrease in net photosynthesis. In our case, the treatments were applied directly to the bunch, so any effects due to leaf gas exchange reduction are ruled out. In addition, the product was applied at the onset of veraison, when the maximum accumulation of acids in the grapes has been reached. Therefore, the results suggest a higher acid degradation in the grapes treated with high doses of antitranspirant during the ripening period. Malic acid, one of the predominant acids in grapes (Iland et al., 2011), is the only high-proportion organic acid that is actively metabolized throughout ripening since the large quantity of tartrate present in the grapes is not used in primary metabolic pathways (Sweetman et al., 2009). Analyzing the impact of the antitranspirant on the evolution of malic acid over the first 35 days after veraison (Figure 2), no remarkable effects were observed. In fact, the rate of malic acid degradation was similar, around -0.44 mg L^{-1} day^{-1}, in all the treatments assayed ($P > 0.05$). These results suggest that rather than a direct effect of di-1-*p*-menthene on the processes through which malic acid is catabolized, the differences in acidity and pH at maturity may be likely a consequence of the lengthening of the ripening period produced by the antitranspirant. Bunches treated with the highest dose (D10) had, on average, up to 23 extra days to metabolize malic acid, thus reaching a lower final acidity at maturity. In addition, since organic acids, malic acid in particular, replaces sugars as a respiratory substrate, especially during the ripening phase, Gatti et al. (2016) suggested that any assimilate limitation occurring in this period might enhance this metabolic pathway. The reduction in must acidity is an undesirable feature for the Tempranillo variety, which is prone to low total acidity and high pH, especially when grown in warm areas with hot summers, which forces its acidification systematically (Ramos & Martínez de Toda, 2018).

Another remarkable response to the antitranspirant treatments was the increase in the concentration of total and extractable anthocyanins in the must at maturity, statistically significant only in the second case (Table 2). A review of the literature on the application of antitranspirants reveals contradictory results about their impact on grape anthocyanins. Some authors reported a decrease in the anthocyanin concentration after spraying the canopy with di-1-*p*-menthene-based antitranspirants (Brillante et al., 2016; Palliotti et al., 2013), or a delayed coloration of the berries after its direct application to the bunch (Zhang et al., 2017). In contrast, a significant increase in anthocyanins has been observed in other cases (Di Vaio et al., 2019; Gatti et al., 2016; Palliotti et al., 2010). In our study, although not statistically significant, there was an impact of the antitranspirant on reducing berry size, thus showing an inverse relationship between the concentration of total and extractable anthocyanins in the must with berry fresh mass. Some authors have reported that changes in berry size do not necessarily involve changes in the relative skin mass as it can be expected according to the surface area-to-volume ratio of the approximately spherical berries (Palliotti et al., 2010). However, we cannot rule out that, in the present study, the changes observed in the anthocyanin levels at maturity are associated with a concentration effect due to a higher surface/volume ratio as a consequence of the reduction in berry weight. The significant decoupling of the sugar: anthocyanin ratio induced by the treatments may also explain the differences observed at maturity (Figure 3, Table 2). Similarly, Gatti et al. (2016) reported a decoupling between sugar and anthocyanin accumulation in grapes of cv. Barbera after the application of the same antitranspirant type to the entire canopy. In that case, such decoupling was produced by an earlier onset of coloring in the treated grapes compared with the control group. The present results, however, suggest that the decoupling was associated with changes in the accumulation rates induced by the antitranspirant in these two must components as indicated by the increase in the slopes of D5 and D10 treatments with respect to D0. In our case, the limitation in grape transpiration induced by di-1-*p*-menthene exerted a differential effect on TSS and anthocyanins, sugars being more negatively affected than anthocyanins. Although the formation of anthocyanins requires a source of C, the reduced import of sugars produced by the antitranspirant did not affect the accumulation of anthocyanins to a similar extent. This source of C may have been provided, at least in part, by the increased usage of organic acids observed in the pulp of the grapes treated with antitranspirant. In post-veraison fruit, malate is liberated from the vacuole and has the potential to fulfill the role of major C source for different processes, among others, the biosynthesis of secondary compounds such as anthocyanins and flavonols (Sweetman et al., 2009).

The estimations of cellular extractability of anthocyanins (EA) and seed maturity (SM) are linked to the stage or ripeness and indicate the degree of the phenolic maturity and oenological potential of the grapes (Nadal, 2010). EA was little affected by the artificially reduced transpiration in this study and all the treatments were in the optimal range for the color extraction (around 30%) (Table 2). In contrast, the application of antitranspirant significantly decreased the SM index. High SM values (>60%) characterize seeds with not polymerized tannins that give high green astringency to the resulting wine (Nadal, 2010). The treatments with high doses of antitranspirant presented better SM values, thus indicating an advanced ripeness stage, which can be explained by their longer maturation times.

From a practical point of view, and compared with the application of antitranspirant coating to the whole canopy (Brillante et al., 2016; Palliotti et al., 2013), the present results obtained under controlled conditions provide new hints to test in the vineyard the direct application of antitranspirant coating to the bunch, as a potential practice to delay maturity while minimizing the effects on the phenolic maturity of the berries. The chemical composition of the cuticular wax of fruits can be modulated by environmental cues (Dimopoulos et al., 2020; Trivedi et al., 2019). Therefore, in a context of global warming, it would be interesting to evaluate the impact of climate change-related factors, high temperature, low relative humidity or elevated air CO_2, on grape transpiration and the potential changes in the composition of cuticular wax, which may alter grape transpiration under these conditions.

The reduction of grape transpiration induced by the direct application of di-1-*p*-menthene to the bunch reduced both the TSS accumulation rate and the length of the ripening period. In contrast, the evolution of must pH, malic acid, and skin anthocyanins over-ripening

was slightly modified, thus indicating minor direct effects of the antitranspirant coating on the metabolism of these compounds. At maturity, the highest doses of antitranspirant led to enhanced anthocyanin content and pH, and reduced total acidity in the must at maturity, which was likely related to an indirect effect of the antitranspirant through a delayed maturity. In the berries with artificially reduced transpiration, the accumulation of anthocyanin was less affected than that of TSS, thus causing a decoupling between these two parameters that also contributed to improve the anthocyanin/sugar ratio at maturity. The present study helps to better understand the involvement of berry transpiration on grape ripening and composition. Results indicate that one single application of antitranspirant at the onset of veraison may be an effective way of delaying ripening without compromising anthocyanin accumulation in the berries and ensuring a correct ripeness, but with negative effects on grape acidity.

AUTHOR CONTRIBUTIONS

Conceptualization: Fermín Morales and Inmaculada Pascual; Methodology and investigation: Fermín Morales, Inmaculada Pascual, Juan José Irigoyen, María Carmen Antolín and Nieves Goicoechea; Data curation and analysis: Fermín Morales and Inmaculada Pascual; Writing (original draft preparation): Fermín Morales and Inmaculada Pascual; Writing (review and editing); Fermín Morales, Inmaculada Pascual, Juan José Irigoyen, María Carmen Antolín and Nieves Goicoechea; Funding acquisition and project administration; Fermín Morales and Inmaculada Pascual.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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