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RESEARCH ARTICLE

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Effects of irrigation and shoot thinning on the size and phenolics content of developing grape berries (*Vitis vinifera* L. cv. Tempranillo)

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Abstract

Aim of study: The concentration of phenolics in the grape berries can be influenced by cultural practices such as irrigation or thinning. The main objective of the present study was to evaluate the effect of combinations of these practices on grape size and phenolics content.

Area of study: The trial was carried out in an experimental cv. Tempranillo vineyard located in Extremadura, Spain.

Material and methods: Two irrigation regimes were considered: rainfed vines (non-irrigated, *NIr*), and 100% ET_c irrigated vines *(Ir)*. For each irrigation treatment, two cropping levels were studied: low shoot-thinning *(LT)* vs high shoot-thinning *(HT)* implemented in winter and spring, respectively. Berry weight, and total phenolics, proanthocyanidin, and anthocyanin concentrations were determined at eight stages of berry development in three consecutive years (2014, 2015, and 2016).

Main results: Specific weather conditions of each year affected phenolics accumulation differently. In 2014, where maximum temperatures were low and an important rainfall occurred at Stage II, both the *NIr-LT* and *NIr-HT* treatments led to the greatest concentrations of total phenolics, proanthocyanidins, and anthocyanins. In 2015, where a little rainfall was registered at Stage II, the berries from the *NIr-HT* and *Ir-HT* treatments accumulated the greatest total phenolics and proanthocyanidin contents, but the *NIr-LT* and *NIr-HT* treatments led to the greatest accumulation of anthocyanins. Finally, in 2016, where high maximum temperatures and scarce rainfall were registered, the *Ir-LT* and *Ir-HT* treatments presented the greatest concentrations of total phenolics, proanthocyanidins, and anthocyanins.

Research highlights: A significant effect of irrigation and thinning was observed on berry size and phenolic content, as well as year × thinning interaction.

Additional keywords: berry development; berry weight; phenolic compounds; proanthocyanidins; anthocyanins.

Abbreviations used: DAA (days after anthesis); ET_0 (reference evapotranspiration); ET_c (crop evapotranspiration); *HT* (high shoot-thinning); *Ir* (irrigation at 100% ETc); *LT* (low shoot-thinning); *NIr* (no irrigation)

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Introduction

Phenolics are synthesized both during the plant normal development (Romero *et al.,* 2010; Molero de Ávila *et al.,* 2019) and in response to such situations as stress and UV radiation (Naczk & Shahidi, 2004). Proanthocyanidins and anthocyanins constitute the two most abundant classes of phenolics in grape berry skin, being responsible for the resulting taste and colour of wine, respectively. Some of the steps of their biosynthetic pathways are common, but they occur at different stages of berry development (Adams, 2006; Boss & Davies, 2009). For example, proanthocyanidins, also known as condensed tannins, mainly accumulate before veraison (Cadot *et al.,* 2006), but it is unclear whether or not their total amounts and/or composition in the skins change during ripening. On the other hand, the amounts of anthocyanins vary depending on the cultivar, ecological conditions, and cultural practices (Adams, 2006; Downey *et al.,* 2006). Their accumulation starts at veraison, being linked to berry softening, colouration, and sugar influx, and continues throughout ripening (Boss *et al.,* 1996). The greatest concentration of anthocyanins reaches around harvest (Esteban *et al.,* 2001; Kennedy *et al.,* 2002; Downey *et al.,* 2004), although, surprisingly, some studies report a decline in total anthocyanins just before harvest (Ryan & Revilla, 2003) and/or during over-ripening (Roggero *et al.,* 1986).

'Tempranillo' variety is originally from northern regions of Spain and it is one of the most important cultivars for production of red wines in Spain. It has been spread to warm regions as Extremadura (SW Spain), where high-quality wines are produced. Because of Tempranillo is a cultivar very sensitive to water deficit (Esteban *et al.,* 2001; Girona *et al.,* 2009) and Extremadura is characterized by scarce and irregular rainfalls, irrigation practice has been implemented to improve yields and grape quality (Hardie & Coinsidine, 1976).

It is known that berry size depends on cultivar, weather conditions (light and temperature especially), irrigation and crop load (Reynier, 2002; Matthews & Nuzzo, 2007). Among the viticultural and environmental parameters investigated for various grape varieties, water deficit is known to influence berry development (Hardie & Considine, 1976; Matthews & Anderson, 1988; Ojeda *et al.,* 2001). Also, proanthocyanidin and/or anthocyanin metabolism varies with irrigation practices (Esteban *et al.,* 2001; Kennedy *et al.,* 2002; Ojeda *et al.,* 2002). Roby *et al.* (2004) reported that water stress increased concentrations of tannins and anthocyanins in the skin, independently of its effect on berry size.

Crop load is usually regulated in order to achieve a higher cluster solar exposition and a greater phenolic accumulation. Crop level can be regulated in several ways. On the one hand, cluster thinning reduces the yield and leads to better fruit quality (Guidoni *et al.,* 2002). However, many researchers have found no clear effect in fruit quality after cluster thinning (Keller *et al.,* 2005). These apparent inconsistencies could be attributable to differences in the moment of thinning and in the plant variety (Dokoozlian & Hirschfelt, 1995; Guidoni *et al.,* 2002). On the other hand, shoot thinning reduces plant vegetative vigour promoting an improvement of grape quality through modifying the source/sink balance and decreasing shading over cluster zone (Mota *et al.,* 2010). Given the environmental characteristics of the Extremadura region, shoot thinning may be suitable and efficient for regulating 'Tempranillo' grape phenolic content.

In summary, irrigation and thinning practices influence the biosynthesis and accumulation of phenolics in the grape berry during its development, and ultimately on wine quality. The objective of the present work was to test the combined effect of two irrigation regimes and two crop levels on 'Tempranillo' berry weight and total phenolics, anthocyanins, and proanthocyanidins content considering three consecutive years.

Material and methods

Experimental site and plant material

The experiment was conducted during three consecutive years (2014, 2015, and 2016) in a vineyard planted on a clay loam soil with Richter 110 rootstock grafted in 2001 to *Vitis vinifera* L. cv. 'Tempranillo'. The vineyard was located in the experimental fields of the *Instituto de Investigaciones Agrarias La Orden-Valdesequera* belonging to the *Centro de Investigaciones Científicas y Tecnológicas de Extremadura* (CICYTEX), *Junta de Extremadura* (38° 51' N; 6° 40' W; 186 m a.s.l.). Vine spacing was 2.5 m between rows and 1.2 m within each row (3333 vines ha^{-1}). They were trained as bilateral cordons (Royat), and winter pruning was to leave six spurs per vine with two buds per spur.

Two irrigation treatments were tested. The first was a rainfed control, in which the vines were not irrigated *(NIr)*. In the second treatment, irrigation *(Ir)* started when the stem potential reached 0.6 MPa (Williams & Baeza, 2007), as measured at midday using a pressure chamber (Model Soil Moisture Crop, Santa Barbara, CA, USA), and it was maintained over the course of grape development at amounts needed to replace 100% of crop evapotranspiration (ET_c). This last parameter was determined with a weighing lysimeter located in the experimental vineyard (Picón-Toro *et al.,* 2012). Irrigation started on June 9th 2014, May 13th 2015, and June 15th 2016. The total amount of water applied in the irrigation treatments, as well as the values of the reference evapotranspiration (ET_0), ET_c, and rainfall were determined from fruit set to veraison and from veraison to harvest.

For the two irrigation regimes, crop load was controlled by shoot thinning. High crop load vines were subjected to a "low shoot-thinning" *(LT)* treatment, with 12 shoots vine^{-1} (vines were pruned in winter to six spurs with two buds each). Some vines were subjected to a low crop load treatment, adjusting them to 6 shoots vine^{-1} to constitute the "high shoot-thinning" *(HT)* treatment. This pruning adjustment was done on April 23rd 2014, April 24th 2015, and May 3rd 2016, corresponding to phenological stage 12 (Eichhorn & Lorenz, 1977). Therefore, four treatments were applied as the result of the combination of irrigation and shoot thinning practices: *NIr-LT* (non-irrigation and low thinning), *NIr-HT* (non-irrigation and high thinning), *Ir-LT* (irrigation and low thinning), and *Ir-HT* (irrigation and high thinning).

The experiment had a split-plot design, with four sub-plots corresponding to each treatment. The sub-plots were divided into four replicate blocks, comprising six rows of 18 vines each one. The surrounding perimeter was taken as vine guards, with neither the two outermost rows of each sub-plot nor the two outermost vines of each row being used for sample collection.

A selection criterion was set for grape sampling in order to minimize berry heterogeneity. During berry development and the first stages of ripening, samples were selected on an equatorial diameter basis (Table 1). On the other hand, during the later ripening stages (Table 1), the berries were classified in accordance with their soluble solids content as determined by their density in different NaCl solutions (Carbonell-Bejerano *et al.,* 2012). Altogether, berries were collected at eight stages of development corresponding to different days after anthesis (DAA) (May 11th 2014 and 2015, and May 23rd 2016): (1) pre-veraison corresponding to berries 4-6 mm in diameter (10 DAA); (2) pre-veraison corresponding to berries 7-8 mm in diameter (18 DAA); (3) pre-veraison corresponding to berries 10-11 mm in diameter (30 DAA); (4) onset-veraison corresponding to berries 11-12 mm in diameter (45 DAA); (5) mid-veraison corresponding to berries 12-13 mm in diameter when grapes are coloured at 50 % (65 DAA); (6) end-veraison corresponding to berries completely coloured (approximately 20 ^{o}Brix, density 120-140 g L^{-1} NaCl) (79, 86 and 73 DAA in 2014, 2015 and 2016 seasons, respectively); (7) first harvest when the berries of at least one of the treatments had reached to commercial ripening (23-24.5 ^{o}Brix, density 150-170 g L^{-1} NaCl) (93, 93 and 80 DAA in 2014, 2015 and 2016 seasons, respectively); and (8) second harvest when the berries of the rest of the treatments had reached to commercial ripening (23-24.5 ^{o}Brix, density 150-170 g L^{-1} NaCl) (100, 100 and 87 DAA in 2014, 2015 and 2016 seasons, respectively). Berries reaching firstly to commercial ripening were from *HT* treatment in 2014 and 2015, and from *Ir* treatment in 2016.

Berries were carefully picked from random plants on each date. They were picked from the central part of south-oriented clusters early in the morning, and then transported to the laboratory in an ice cooler at 4 °C. The berries were then cut at the base of the pedicel, rinsed, and dried. Finally, they were weighed (model M-Prove AY-412 balance, SARTORIUS) to determine average berry weight, and frozen at -40 °C for later phenolics extraction.

Extraction of phenolic compounds

The phenolic extracts were obtained using entire grapes following the procedure described by Singleton & Rossi (1965) with some modifications. Samples were homogenized in extraction solvent (methanol 80%) in a 1:4 ratio (w:v). After 30 min stirring, the liquid extract

Table 1. Sampling criteria for berry collection during 'Tempranillo' grape development.

Sampling	Days after anthesis (DAA)			Date			Phenological phase	Selection criterion			
	2014	2015	2016	2014	2015	2016		Equatorial diameter (≈ mm)	Berry density 2014	2015	2016
-		-		11 May	11 May	23 May	Anthesis	-		-	
1		10		22 May	20 May	2 Jun	Pre-veraison	4-6		-	
2		18		29 May	28 May	10 Jun		7-8		-	
3		30		10 Jun	9 Jun	22 Jun		10-11		-	
4		45		25 Jun	24 Jun	7 Jul	Onset-veraison	11-12		-	
5		65		15 Jul	14 Jul	27 Jul	Mid-veraison	12-13		-	
6	79	86	73	29 Jul	4 Aug	4 Aug	End-veraison	-	120-140 g L^{-1} (20 ^{o}Brix)		
7	93	93	80	12 Aug	11 Aug	11 Aug	First harvest	-	150-170 g L^{-1} (24 ^{o}Brix)		
									HT	*HT*	*Ir*
8	100	100	87	19 Aug	18 Aug	18 Aug	Second harvest	-	150-170 g L^{-1} (24 ^{o}Brix)		
									LT	*LT*	*NIr*

was separated from the solid residues by centrifugation at 3000 rpm for 15 min (Centrifuge Eppendorf 5810R, Hamburg, Germany). The extraction procedure was repeated thrice, the final extract resulting in a mixture of the three supernatants. This was filtered through a 0.45 µm nylon membrane and stored in a freezer until assay for total phenolics, anthocyanin, and proanthocyanidin contents. One extraction was performed for each experimental block.

Determination of phenolic compounds

Total phenolics content

The extracts total phenolics contents were determined by the Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965) with some modifications. Briefly, 1 mL of diluted extract was oxidized with 5 mL of Folin-Ciocalteu reagent (10%) and neutralized with 4 mL of Na_2CO_3 solution (7.5%). After mixing and keeping the samples at 50 °C (water bath) for 5 min, their absorbances were read at 760 nm against a buffer blank in a UV/VIS spectrophotometer (Biomate 6UV-Vis, THERMO SCIENTIFIC). A calibration curve was constructed using gallic acid standard solutions (0–100 mg L^{-1}). The total phenolics content was expressed as mg of gallic acid equivalent (GAE) per g fresh weight. All extracts were assayed in triplicate and the results were expressed as means ± standard errors.

Proanthocyanidin content

The proanthocyanidin content of the grape extracts was determined by vanillin-HCl assay as described by Broadhurst & Jones (1978). Briefly, 0.5 mL of diluted extract was mixed with 3 mL of vanillin solution in methanol (4%) and 1.5 mL of HCl. The reaction mixture was incubated for 15 min at room temperature. Absorbance was measured at 500 nm against a buffer blank without vanillin in a UV/VIS spectrophotometer. A calibration curve was prepared using catechin standard solutions (0–335 mg L^{-1}). The proanthocyanidin content was expressed as mg of catechin equivalents per g fresh weight. All extracts were assayed in triplicate and the results were expressed as means ± standard errors.

Anthocyanin content

The monomeric anthocyanin content of the grape extracts was measured using a modified pH differential method (Boyles & Wrolstad, 1993). Diluted extract was mixed thoroughly with 0.025 M potassium chloride buffer of pH 1 in a 1:2 ratio (v:v). Other amount of diluted extract was similarly mixed with a sodium acetate buffer of pH 4.5, stirred, and left to stand for 15 min for the reaction to take place and stabilize. The absorbances at 510 nm (wavelength of maximum absorbance) and 700 nm were measured with a UV/VIS spectrophotometer against buffer blanks at pH 1.0 and pH 4.5. The absorbance readings were converted to total mg of malvidin 3-glucoside (Mv-3-glu). The anthocyanin content was calculated as follows:

$$\text{Total monomeric anthocyanins (mg/100 g)} = \\ = \Delta A \times MW \times 1000/ (\varepsilon \times C)$$

$$\Delta A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}$$

In this formula, A is the absorbance, MW (463.3 g mol^{-1}) is the molecular weight of Mv-3-glu, ε (28,000 M^{-1} cm^{-1}) is the molar absorptivity of Mv-3-glu, and C is the concentration of the grape extract in mg mL^{-1}. The anthocyanin content was expressed as mg of Mv-3-glu equivalents (Mv-3-glu E) per 100 g fresh weight. All extracts were assayed in triplicate and the results were expressed as means ± standard errors.

Statistical analysis

Results are expressed as means and standard errors calculated over all replicates. All the data were subjected to an ANOVA using the SPSS 23.0 software package, and Tukey's test was used to establish the significance of differences between means at a $p<0.05$ level. Student's t-test is also used in this work. The data were analysed using irrigation and crop load as the main factors, and including the irrigation × crop load, irrigation × year, and crop load × year interactions.

Results

The results obtained in the present study are shown in four figures showing the weather conditions (Fig. 1), differences between irrigation treatments and thinning treatments on berry weight and total phenolics (Fig. 2), differences between irrigation treatments and thinning treatments on proanthocyanidins and anthocyanins (Fig. 3) and differences between combined treatments on berry weight, total phenolics, proanthocyanidins and anthocyanins at harvest (Fig. 4).

Weather conditions

The meteorological parameters of temperature (°C) and rainfall (mm) were measured by a weather station located at the site of the experimental vineyard.

During the experimental period (from May to August), maximum temperatures were in the ranges of 23-33 °C, 28-35 °C, and 24-37 °C in 2014, 2015, and 2016,

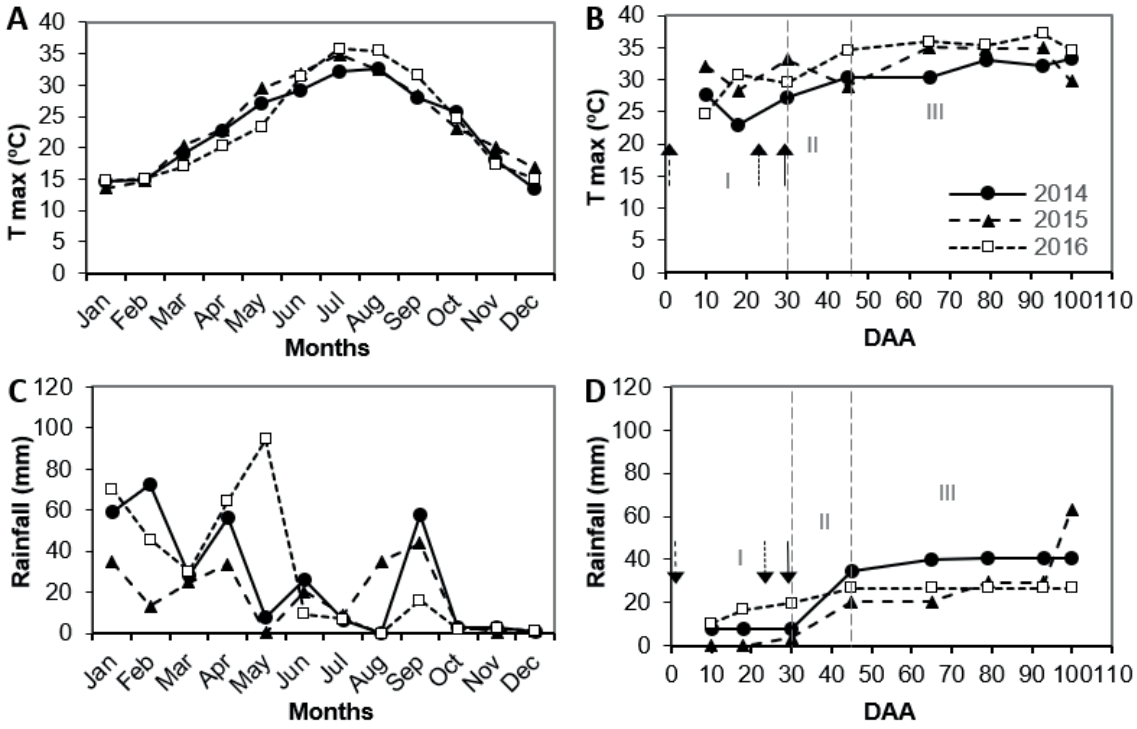

Figure 1. Temperature and rainfall recorded at the experimental vineyard during the 2014, 2015, and 2016 seasons. A: Monthly maximum temperature (°C). B: Maximum temperature during the berry development period (°C). C: Monthly total rainfall (mm). D: Accumulated rainfall during the berry development period (mm). I: Fast growth phase. II: Lag phase. III: Ripening phase. DAA: Days after anthesis. Arrows indicate the onset of the irrigation treatment.

respectively (Fig. 1B). There were extremely high temperatures in the 2016 summer. The major precipitation events were recorded before anthesis (in May) in all the years studied, especially in 2016 (Fig. 1C). During grape development (Fig. 1D), major rainfall events occurred during Stage II in 2014 and 2015 (26.34 and 19.8 mm, respectively). Also, rainfall of 34.05 mm was measured between first and second harvest in 2015 (between 93 and 100 DAA). In 2016, there was light rainfall spread out over Stages I and II (16.25 mm). The accumulated rainfall values at the end of the study periods were 41, 63, and 27 mm in 2014, 2015, and 2016, respectively.

Berry development and ripening

Berry weight evolution followed a double sigmoidal pattern with two periods of growth (Stage I and Stage III) (Figs. 2A-F). Lag phase (Stage II) only was observed under *NIr* treatments in 2014 and 2015, while berry weight experienced a light increase in the rest of the cases during this stage (Table S1 [suppl]). The greatest weight gain took place in Stage III from the onset to the end of veraison (Table S1 [suppl]), Figs. 2A-F). In 2014 and 2015, berry weight did not vary at the end of maturation, but it fell significantly in 2016 (Table S1 [suppl]), Figs. 2A-F), probably due to the water loss induced by the high temperatures registered during this year.

In 2014, the *Ir-LT* treatment led to the lowest berry weight, and the greatest values corresponded to the *NIr-HT* treatment, reaching 2.32 g fruit^{-1} at second harvest (100 DAA, Table S1 [suppl]). In 2015, the *Ir-LT* and *Ir-HT* treatments gave the greatest berry weights (Table S1 [suppl]). Finally, in 2016, the greatest weights corresponded to the *Ir-HT* treatment, producing berries weighing 1.24 g fruit^{-1} at second harvest (87 DAA, Table S1 [suppl]).

Although *Ir* treatments would normally contribute to increased berry size as it happened in 2015 and 2016 (Figs. 2B and C), the grapes sampled in 2014 presented significantly lower weights than the grapes from the *NIr* treatments (Fig. 2A). Thinning also significantly modified berry weight, with greater weights under HT treatments in most of the stages analysed during the growth period in all three years (Figs. 2D-F).

In 2014 and 2015, the HT treatments, which firstly reached to full ripeness (93 DAA), led to greater weights than the *LT* treatments (Table S1 [suppl]), highlighted boxes in solid square). The *LT* treatments delayed the ripening (100 DAA) and promoted lower berry weights than the *HT* treatments under the *NIr* conditions, but there were no significant differences under the *Ir* conditions

Table 2. Significant effects of irrigation, shoot thinning, irrigation × shoot thinning, year, irrigation × year and shoot thinning × year on berry weight, total phenolics, proanthocyanidins and anthocyanins concentration at harvest. Statistical significances are based on Student's t-test: ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; n.s., $p>0.05$.

	Significance of effects			
	Berry weight	**Total phenolics**	**Proanthocyanidins**	**Anthocyanins**
Irrigation	***	*	ns	***
Thinning	***	***	***	**
Irrig. × Thin.	***	ns	ns	ns
Year	***	***	***	***
Irrig. × Year	***	***	***	***
Thin. × Year	ns	ns	*	**

(Table S1 [suppl], highlighted boxes in striped square). In 2016, the first harvest was for the *Ir* treatments at 80 DAA, and the second harvest was at 87 DAA. In both cases, the *Ir* grapes had greater weights than those of the *NIr* treatments (Table S1 [suppl]).

With regard to fully ripened grapes, while *Ir-LT* and *Ir-HT* had the greatest berry weights in 2015 and 2016 (Table S1 [suppl], Figs. 4B and 4C), the greatest weights corresponded to *NIr-HT* in 2014 (Fig. 4A). There were significant effects of irrigation and thinning on berry weight, as well as of the interaction between irrigation and thinning (Table 2).

The effect of the year on berry weight was checked at harvest (Table 2). The greatest weights were attained in 2014 (Fig. 4A), and the lowest in 2016 (Fig. 4C). The differences in berry weight between years could be related to seasonal factors. In 2014, the maximum temperatures were lower (Fig. 1B) and the rainfall higher (Fig. 1D) than in the other two years. These conditions could induce greater berry weights in this season. Conversely, the higher maximum temperatures (Fig. 1B) and sparse rainfall (Fig. 1D) during grape development in 2016 could have led to smaller berries at harvest. Irrigation × year interaction significantly modified berry weight, but no significant differences were found on berry weight due to thinning × year interaction (Table 2).

Total phenols

The evolution of the total phenolics concentration during berry development presented the same trend in the three years regardless the treatment (Table S1 [suppl], Figs. 2G-L). There was a sharp decrease in Stage I, but no significant changes during Stage II (Table S1 [suppl], Figs. 2G-L). Stage III was characterized by a steady fall in total phenolics until the end of veraison, followed by a stabilization around harvest (Table S1 [suppl], Figs. 2G-L).

In 2014 and 2015, the *NIr-LT* treatments generally led to the greatest total phenolics concentrations until the end of veraison (Table S1 [suppl]). However, the greatest total phenolics levels at 100 DAA corresponded to *NIr-HT* with 5.55 and 3.77 mg g^{-1} fresh weight in 2014 and 2015, respectively (Table S1 [suppl]). There were no significant differences in this parameter between the combined treatments during the ripening period in 2016 (Table S1 [suppl]).

The *Ir* treatments reduced the total phenolics concentrations in 2015 and 2016 (Figs. 2H-I), and in 2014 at the end of the period studied (Fig. 2G). However, at the beginning of fruit development in the 2014 season, grapes under *Ir* treatments accumulated higher phenolic content (Fig. 2G). These differences could be attributable to the effect of irrigation practice could be masked by the heavy rainfall recorded at early developmental stages in 2014.

The effect of thinning on total phenolics accumulation varied during grape development. The *HT* treatments usually led to lower levels of total phenolics during berry development. However, at the end of ripening (100 DAA), those values were very similar (2016) (Fig. 2L) or significantly greater under *HT* treatments (2014 and 2015) (Figs. 2J and 2K). The high temperatures recorded at the end of ripening in 2014 and 2015 (Fig. 1B) could have stimulated greater synthesis of phenolic compounds in the *HT* vines. Furthermore, phenolic pigments accumulation at final stages of ripening could be a consequence of higher exposure to solar radiation in *HT* vines.

In 2015, the grapes that firstly reached to 24 ºBrix were those corresponding to the *HT* treatment (first harvest, Table 1). At the time of this first harvest (93 DAA, Table 1), their phenolics concentrations were significantly greater than in the grapes of the *LT* group (Table S1 [suppl], highlighted boxes in solid square). But at the time of second harvest (100 DAA, Table 1), there were no significant differences between the two treatments (Table S1 [suppl]). In 2014 and 2016, no significant differences were found on phenolics concentrations under *HT* and *LT* treatments, neither at the first (93 and 80 DAA in 2014 and 2016, respectively) nor at the second harvest (100 and 87 DAA in 2014 and 2016, respectively).

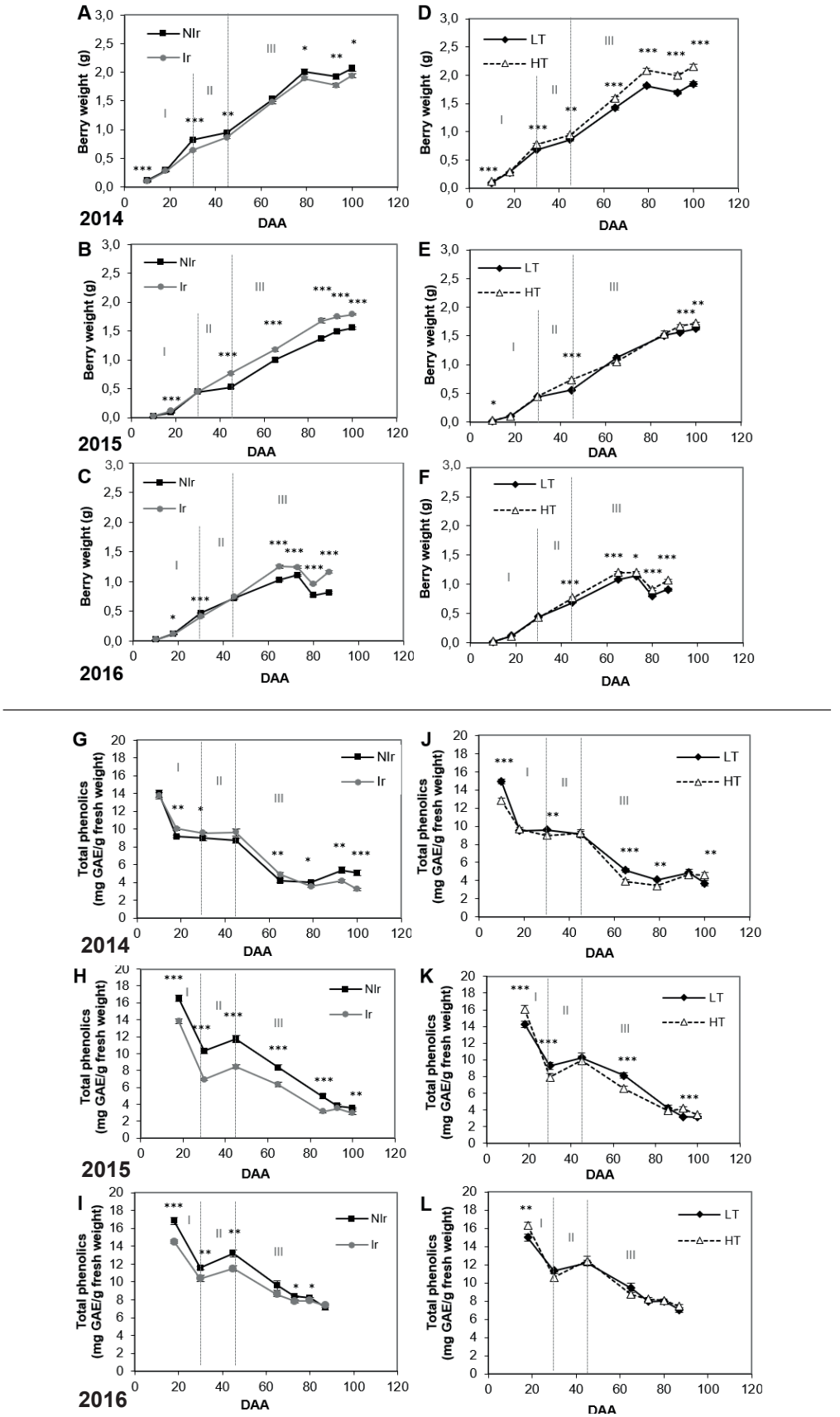

Figure 2. Differences between irrigation treatments (*NIr:* no irrigation; *Ir:* irrigation at 100% ETc) and shoot thinning treatments (*LT:* low shoot-thinning; *HT:* high shoot-thinning) on 'Tempranillo' berry weight (A, B, C, D, E, F) and total phenolics (G, H, I, J, K, L) as a function of days after anthesis (DAA) in 2014 (first row), 2015 (second row), and 2016 (third row). I: Fast growth phase. II: Lag phase. III: Ripening phase. Vertical bars indicate standard errors (n=150 for berry weight and n=18 for total phenolics). Statistical significances are based on Student's t-test: ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; n.s., $p>0.05$.

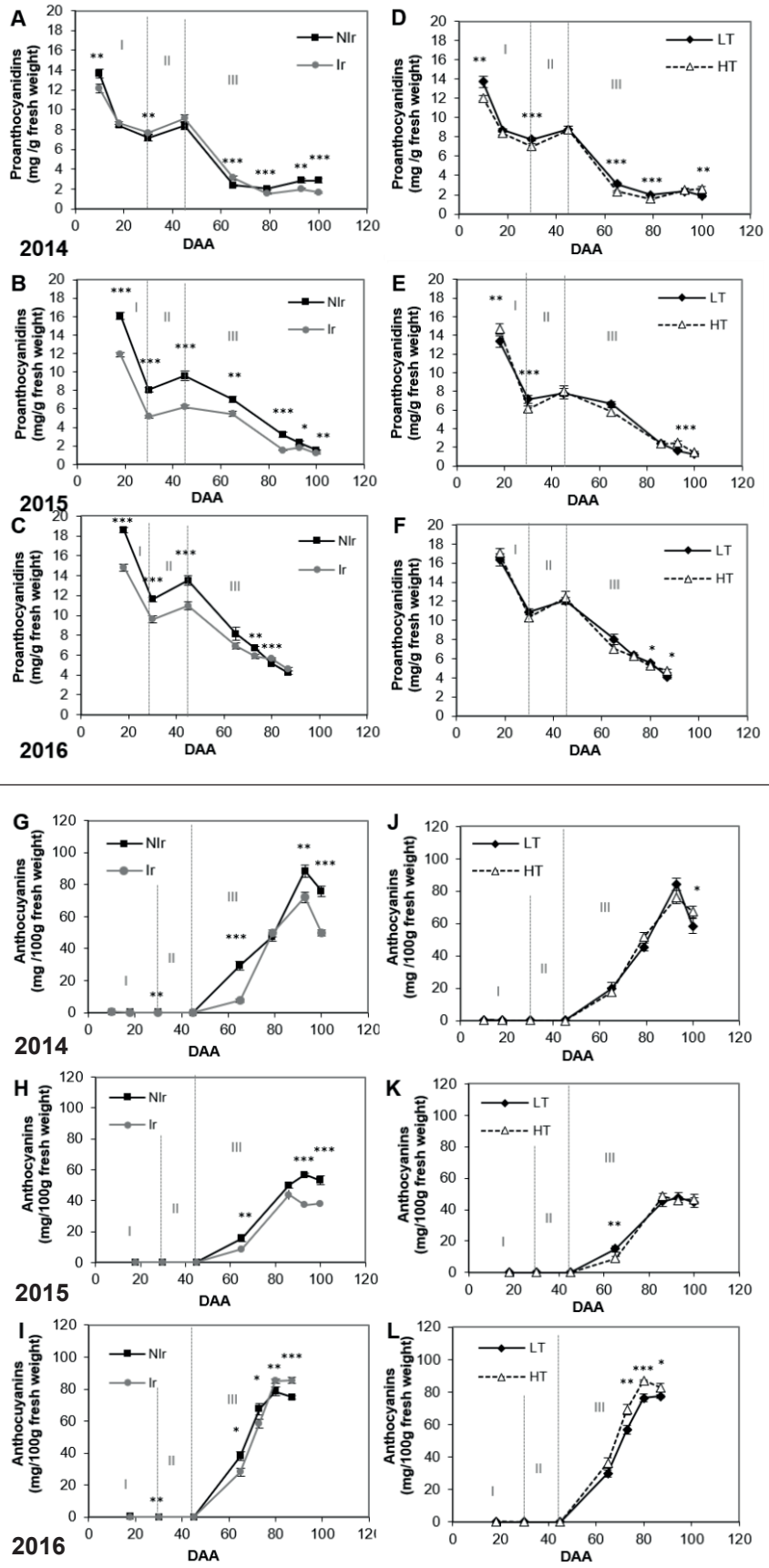

Figure 3. Differences between irrigation treatments (*NIr:* no irrigation; *Ir:* irrigation 100% ETc) and shoot thinning treatments (*LT:* low shoot-thinning; *HT:* high shoot-thinning) on 'Tempranillo' proanthocyanidins (A, B, C, D, E, F) and anthocyanins (G, H, I, J, K, L) as a function of days after anthesis (DAA) in 2014 (first row), 2015 (second row), and 2016 (third row). I: Fast growth phase. II: Lag phase. III: Ripening phase. Vertical bars indicate standard errors (n=18). Statistical significances are based on Student's t-test: ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; n.s., $p>0.05$.

At harvest, *NIr-HT* led to the greatest phenolics content in 2014 and 2015 (Figs. 4D and 4E). However, in 2016, *NIr-HT* phenolic values were similar to those of the Ir treatments (Fig. 4F). An effect of irrigation and thinning on the phenolics concentration was observable, but the interaction of these treatments was not significative (Table 2).

Season had a significant effect on phenolics content at harvest (Table 2). The greatest total phenolics concentration was recorded in the 2016 season (Fig. 4F). Table 2 indicates a significative effect of irrigation × year interaction on total phenolics, but thinning × year interaction was not significative.

Proanthocyanidins

The proanthocyanidins concentrations showed an overall decreasing trend as berry development progressed. This was especially marked during Stage I and at the beginning of Stage III, whereas, during Stage II, these concentrations remained unchanged or increased slightly, depending on the treatment (Figs. 3A-F).

In the 2014 and 2015 seasons, the *NIr-LT* treatment resulted in the greatest proanthocyanidin concentrations from fruit set until the end of veraison, but the greatest values at 100 DAA were reached with the *NIr-HT* treatment (Table S1 [suppl]). In 2016, the highest proanthocyanidin concentration was detected in grapes under *NIr* treatments until the end of veraison, but under *Ir* treatments at harvest (Table S1 [suppl]).

The effect of the treatment on proanthocyanidin concentrations was practically the same as that described for total phenolics. The *Ir* treatments generally induced lower proanthocyanidin levels during berry development in all three years studied (Figs. 3A-C). The *HT* treatments influenced proanthocyanidin concentrations differently during berry development, inducing lower levels until the end of veraison, and higher levels at harvest in all three years studied (Figs. 3D-F). This latter increase in proanthocyanidins under the *HT* treatments may have been the result of stimulation by the high temperatures reached at the end of ripening (Fig. 1B).

In the 2014 and 2015 seasons, at first harvest, the fully ripened samples *(HT)* presented greater proanthocyanidin concentrations than the yet to ripen samples *(LT)* (Table S1 [suppl], highlighted boxes in solid square), and this was still the case at second harvest (Table S1 [suppl]). In 2016, however, there were no significant differences between treatments even though they reached ripeness on different dates (Table S1 [suppl]).

At harvest, in 2014 the greatest proanthocyanidin concentration was obtained under *NIr-HT* (Fig. 4G), in 2015 under *NIr-HT* and *Ir-HT* (Fig. 4H), and in 2016 under *Ir-LT* and *Ir-HT* (Fig. 4I). The only effect observed on proanthocyanidin content at harvest was that of the thinning practice (Table 2).

In our study, there were significant differences between years in the proanthocyanidin content at harvest (Table 2), probably due to that favourable weather conditions of certain years could stimulate their synthesis and accumulation. So, the greatest values were measured in 2016 (Fig. 4I), probably due to the high temperatures recorded during the grape growth period (Fig. 1B). The low values observed in 2014 could also be attributable to the lower maximum temperatures and the higher rainfalls (Figs. 1B and 1D, Fig. 4G). Interactions between irrigation × year and thinning × year can be observed on proanthocyanidins concentration (Table 2).

Anthocyanins

Anthocyanins began to be synthesized from 45 DAA onwards in the three years regardless the treatment (Figs. 3G-L). They were rapidly accumulated until the end of veraison, but this accumulation ceased at harvest (Figs. 3G-L).

At second harvest, the greatest anthocyanin levels corresponded to the *NIr-LT* and *NIr-HT* treatments in 2014 and 2015, and to the *Ir-HT* treatment in 2016, although in that year the highest anthocyanin concentration at the beginning of ripening corresponded to the *NIr-HT* treatment (Table S1 [suppl]).

Under the *Ir* treatments, there was less accumulation of anthocyanins during ripening (Figs. 3G-I) except for the harvest of 2016 (Fig. 3I). *HT* exerted a significant positive effect on anthocyanin content during ripening in 2016 (Fig. 3L), but no significant differences were observed in either 2014 or 2015 (Figs. 3J and 3K).

In the 2014 and 2015 seasons, at first harvest, the fully ripened samples *(HT)* and the yet to ripen samples *(LT)* presented no significant differences on anthocyanin concentrations, and this was still the case at second harvest (Table S1 [suppl]). In 2016 also, there were no significant differences between treatments even though the *Ir* samples reached harvest before the *NIr* samples (Table S1 [suppl]).

At harvest, in both 2014 and 2015 anthocyanin accumulation was favoured by the *NIr-LT* and *NIr-HT* treatments (Figs. 4J and 4K), but by the *Ir-LT* and *Ir-HT* treatments in 2016 (Fig. 4L). While effects of both irrigation and thinning were observed at harvest, the interaction between the two was not significant (Table 2).

There were significant differences by year in anthocyanin concentrations at harvest (Table 2). The greatest values were measured in 2016 (Figs. 4J-L), possibly as a result of the weather conditions in that year, as noted above for the total phenolics and the proanthocyanidins. Interactions between irrigation × year and thinning × year were significative on anthocyanin content (Table 2).

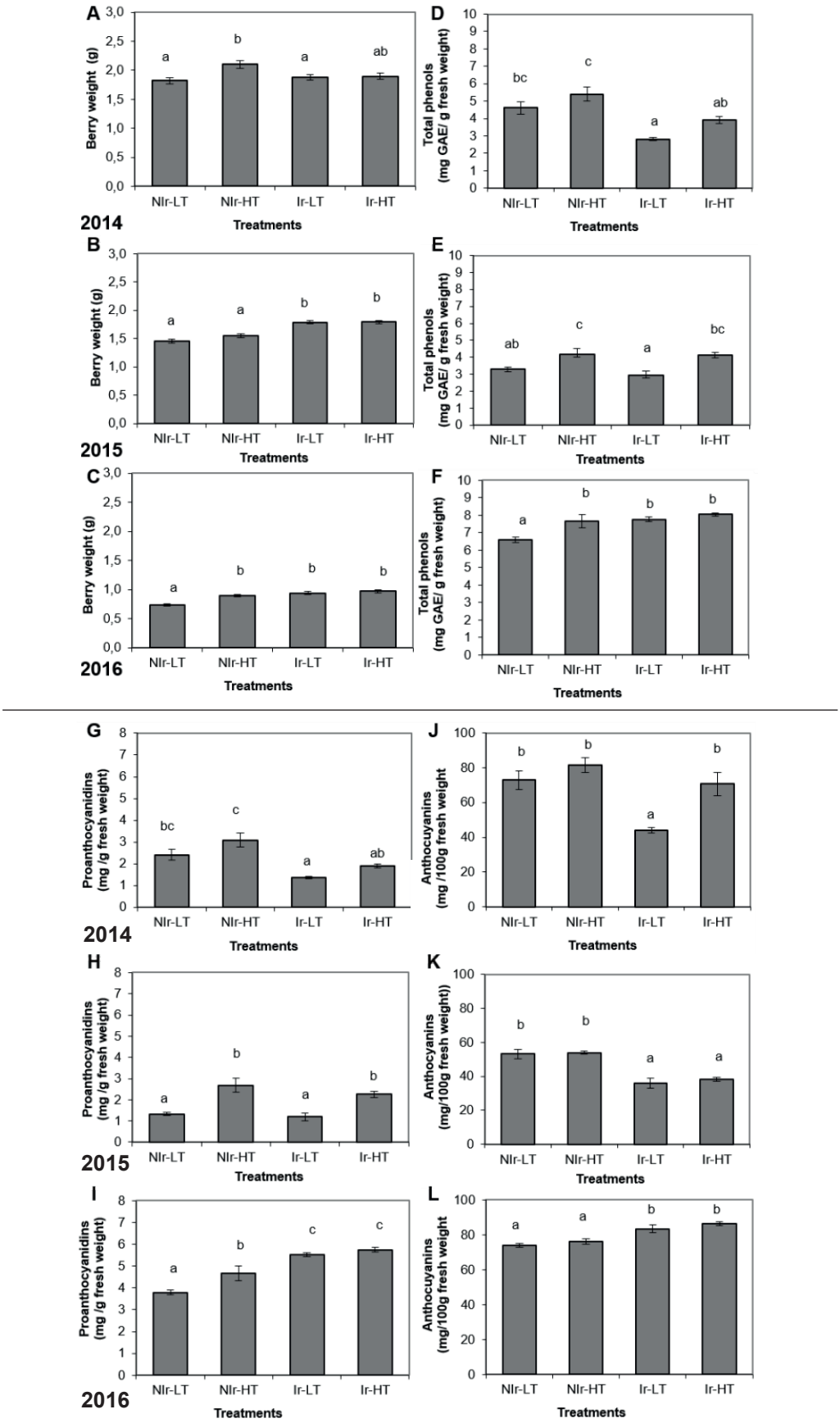

Figure 4. Differences between treatments (*NIr-LT:* non-irrigated and low shoot-thinning; *NIr-HT*: non-irrigated and high shoot-thinning; *Ir-LT:* irrigated 100% ETc and low shoot-thinning; *Ir-HT:* irrigated 100% ETc and high shoot-thinning) on 'Tempranillo' berry weight (A, B, C), total phenolics (D, E, F), proanthocyanidins (G, H, I), and anthocyanins (J, K, L) at harvest in 2014 (first row), 2015 (second row), and 2016 (third row). Vertical bars indicate standard errors (n=75 for berry weight and n=9 for phenolic compounds). The letters indicate significant differences between treatments at the $p<0.05$ level based on an ANOVA and Tukey's HSD test.

Discussion

Effects of cultural practices on berry development

In our study, berry growth followed a double sigmoid pattern in concordance with results described in varieties such as Cabernet Sauvignon (Basile *et al.,* 2011), Shiraz (Ollé *et al.,* 2011), and Tempranillo (Girona *et al.,* 2009; Valdés *et al.,* 2009; Intrigliolo & Castel, 2011).

Irrigation generally led to greater berry weights, in accordance with most other published data (Esteban *et al.,* 2001; Girona *et al.,* 2009; Valdés *et al.,* 2009; Intrigliolo & Castel, 2011). However, we found a reverse pattern in the 2014 season. A possible explanation for this discrepancy could be that 2014 was characterised by a heavy rainfall (26.34 mm) during stage II. This important rainfall could mask the effect of irrigation practice due to the high water accumulation in soil.

Concerning thinning practices, our findings were that high shoot-thinning produced greater berry weight, in agreement with previous published data (Reynolds *et al.,* 1994a,b; Intrigliolo & Castel, 2011). Similar results have been found with cluster thinning treatments (Diago *et al.,* 2010).

Our results also showed that the effects of the different combinations of irrigation and thinning treatments on berry weight varied according to the year. In 2015 and 2016, the *Ir-HT* treatment led to the greatest weights during ripening. While this is in concordance with the results of Intrigliolo & Castel (2011), Keller *et al.* (2008) found no effect of the interaction between irrigation and crop load treatments on berry weight in any year studied. On the other hand, although Valdés *et al.* (2009) reported irrigation effect on berry weight, they did not detect neither significative effects of crop level nor interaction between irrigation and crop load on berry weight.

Effects of cultural practices on total phenols during grape development

In the present study, we found a sharp decrease of total phenols at Stage I but no changes at Stage II, as has been reported by other workers during berry development (Crippen & Morrison, 1986). Also, we observed total phenolics amounts declined at the beginning of ripening, followed by a stabilization around harvest, in concordance with other studies (Romero *et al.,* 2010).

Irrigation contributed to lower amounts of total phenols in the grape berries, in agreement with other studies on the same cultivar (Esteban *et al.,* 2001; Intrigliolo & Castel, 2008; Valdés *et al.,* 2009; Garrido *et al.,* 2014, 2016). Some authors claim that the total phenols increase is a simple consequence of reduction in berry size (Kennedy *et al.,* 2000; Roby *et al.,* 2004). However, we have to discard this possibility because our results indicated an increase in phenolics irrespective of berry weight, in agreement with some other studies (Garrido *et al.,* 2014). It could be checked at harvest in 2014 when grapes under *NIr* treatments reached higher total phenols concentration but also higher weight.

The present results showed that *HT* treatments contributed to greater amounts of total phenols at harvest. This is in concordance with studies employing cluster thinning (Valdés *et al.,* 2009; Diago *et al.,* 2010; Gamero *et al.,* 2014; Garrido *et al.,* 2016) or defoliation practices (Garrido *et al.,* 2014). There are various possible explanations for the greater phenolics content at harvest caused by thinning. For instance, thinning could promote greater nutrient availability due to an improved source-sink ratio (Pastore *et al.,* 2013). The high accumulation of total phenolics at harvest could be explained as a response of the high temperatures registered during this period (Garrido *et al.,* 2014).

In our work, the greatest amount of total phenols at harvest was reached under the combination *NIr-HT*, in agreement with previous studies (Valdés *et al.,* 2009; Intrigliolo & Castel, 2011). We also found that the year had a significant effect on total phenolics concentrations, in agreement with previous studies (Gamero *et al.,* 2014). No statistically significant interaction was observed between the two practices (irrigation and thinning) in the total phenolics content, as also reported by other studies (Ortega *et al.,* 2007; Valdés *et al.,* 2009; Gamero *et al.,* 2014).

Effects of cultural practices on proanthocyanidins during grape development

We observed a progressive decline in proanthocyanidin concentrations towards a nearly constant level at the end of ripening, as previously described in the same cultivar (Niculcea *et al.,* 2013).

Our results also demonstrated that irrigation treatments reduced proanthocyanidin concentration in whole berry from the beginning of the irrigation onwards, but not at harvest in 2016 (Fig. 3C). González & Ferrer (2008) stated that the lower tannin content under greater water availability might be a consequence of dilution because of the greater berry size. Niculcea *et al.* (2013) detected no significant differences in proanthocyanidin concentrations in the whole grape berry under different irrigation treatments. In our study, the HT treatment led to greater proanthocyanidin accumulation at harvest, it coinciding with previous studies applying cluster thinning (González & Ferrer, 2008) or defoliation (Risco, 2012).

To the best of our knowledge, this has been the first study to analyse the influence of combined *NIr* and *HT* treatments on proanthocyanidin concentrations during grape berry development. The results show that *NIr-HT*

treatments contributed to favouring these concentrations at harvest. In agreement with the authors cited above, this could be due to the additive effect of these treatments leading to a greater proanthocyanidin concentration.

Effects of cultural practices on anthocyanins during grape development

The anthocyanin accumulation throughout ripening followed by a stabilization or decline around harvest that was found in our study is in concordance with previous studies (Bindon *et al.,* 2013; Niculcea *et al.,* 2013). Some authors have explained the decline in anthocyanin concentration near harvest as an effect of environmental and viticultural conditions, since high berry temperatures and sunlight exposure could inhibit anthocyanin biosynthesis (Tarara *et al.,* 2008).

Our results indicated that the *Ir* treatment reduced the anthocyanin concentration and thus berry quality, in agreement with previous studies (Intrigliolo & Castel, 2008, 2011; Valdés *et al.,* 2009). Surprisingly, the anthocyanin concentrations in 2016 presented a reverse pattern at harvest. This could be attribute to water stress since, once water deficit surpasses a certain threshold, anthocyanin synthesis may be delayed or hindered by a decrease in photosynthesis and carbon limitation (Girona *et al.,* 2009). We also found that the *NIr* treatment affected anthocyanin concentrations positively, in concordance with previous studies (Roby *et al.,* 2004; Girona *et al.,* 2009).

In concordance with previous published data (Ortega *et al.,* 2007; Intrigliolo & Castel, 2011), we found that the *HT* treatments increase anthocyanin levels. This finding is also coherent with other studies concerning cluster thinning (González & Ferrer, 2008; Valdés *et al.,* 2009; Santesteban *et al.,* 2011; Gamero *et al.,* 2014) or defoliation (Freese, 1988) on anthocyanin concentrations during berry ripening. The effect was associated with greater exposure of the clusters to direct sunlight and to higher berry temperatures because of the reduced vegetation cover (Ginestar *et al.,* 1998; Bergqvist *et al.,* 2001).

With regard to the combination of treatments, some authors have reported that the coupling of non-irrigation and low crop load increases the quantities of anthocyanins (Valdés *et al.,* 2009; Intrigliolo & Castel, 2011). In the present study, the *NIr* treatments favoured anthocyanin accumulation at harvest in 2014 and 2015, but we found no significant differences between the *NIr-HT* and *NIr-LT* treatments. On the other hand, we found that the year had a significant effect on anthocyanin concentrations, in agreement with Gamero *et al.* (2014). We detected no statistically significant irrigation × thinning interactions affecting anthocyanin content, again in concordance with the reports of other workers (Ortega *et al.,* 2007; Valdés *et al.,* 2009; Gamero *et al.,* 2014).

In summary, although seasonal variability difficulties the study of phenolics content under natural conditions, we found that hot and dry years favoured high concentrations of these compounds. Generally, a supply of water reduced the concentrations of proanthocyanidins, anthocyanins, and total phenols during berry development. The stress induced in non-irrigated vines could stimulate the biosynthesis of phenolic compounds. In the highly shoot-thinned vines, anthocyanin synthesis was markedly stimulated throughout ripening, probably because of a higher level of exposure to sunlight. However, the only observable effect of high thinning on proanthocyanidin and total phenols concentrations was at harvest, probably due to the extreme temperatures at that time. Although the non-irrigation and high thinning treatments favoured the accumulation of phenolic compounds, the effect of their combined treatment was non significative. The effect of the year on berry weight and on phenolic compounds was checked at harvest. In particular, the quality of the berries varied depending on the specific weather conditions of each year. A significant effect of irrigation × year was observed on berry size and phenolic content, but thinning × year interaction only was detected on proanthocyanidin and anthocyanin concentration. Therefore, as irrigation practice is an important factor influencing to phenolic content of berry grape it could be studied in future works through different intensities of water deficit in order to improve the grape quality and, consequently, the wine resulting.

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