Received : 17 December 2018; Accepted : 22 February 2019; Published : 6 March 2019
DOI : 10.20870/oeno-one.2019.53.1.2366

Anthocyanin content and composition in four red winegrape cultivars (*Vitis vinifera* L.) under variable irrigation

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ABSTRACT

Aim: The aim of this study was to determine and compare anthocyanin content and profile under variable irrigation regimes in four red grape cultivars (*Vitis vinifera* L.), the Greek indigenous cvs. Agiorgitiko and Xinomavro, alongside Syrah and Grenache noir.
Methods and results: Three irrigation treatments were applied in a 6-year-old vineyard comprising all four varieties in a block design, starting at bunch closure (E-L 32) through harvest of 2012 and 2013: full irrigation (FI) at 100% of crop evapotranspiration (ET_c), deficit irrigation (DI) at 50% of ET_c and non-irrigated (NI). The identification of the compounds was performed by HPLC. Results showed that, under the hot summer conditions of the Greek climate, the four cultivars had a similar response regarding vigor and yield parameters, with values increasing with water supply. Anthocyanin concentration was maximized under non-irrigated conditions in all cultivars, but anthocyanin profile and relative distribution of individual anthocyanins among irrigation treatments showed a strong cultivar effect.
Conclusion: Xinomavro seemed to favor the synthesis of more stable forms of anthocyanins under limited water supply (acylated over non-acylated and tri-oxygenated & methoxylated on the B-ring over di-oxygenated & hydroxylated) while Agiorgitiko had an opposite behavior, which might imply a need for a different irrigation strategy.
Significance and impact of the study: To the best of our knowledge, this is the first comparative report of anthocyanin composition and profile in berry skin, under contrasting water status, for the two most important red winegrapes of Greece.

KEYWORDS

grapevine, water regime, phenolic compounds, acylated anthocyanins, tri-oxygenated anthocyanins

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INTRODUCTION

Anthocyanidins of grape berry skins play a key role in wine color intensity, hue and stability. In *Vitis vinifera* L. berries, anthocyanidins occur as 3-*O*-glucosides (anthocyanins), substituted with two (di-oxygenated: cyanidin- and peonidin-3-*O*-glucosides) or three (tri-oxygenated: delphinidin-, petunidin-, and malvidin-3-*O*-glucosides) hydroxyl (-OH) and/or methoxyl (-OCH_3) groups in the side-ring (B) of the flavonoid structure. Position 6 of glucose can also be esterified (acylated anthocyanins) by acetic (acetylated derivatives), p-coumaric (p-coumaroylated derivatives) and, less commonly, caffeic (caffeoylated derivatives) acids (Squadrito *et al*., 2010).

The composition of anthocyanins (relative abundance of individual anthocyanins, ratio of di-oxygenated vs. tri-oxygenated side-ring forms, ratio of acylated vs. non-acylated derivatives, etc) is variable among grapevine varieties (Kallithraka *et al*., 2005; Monagas *et al*., 2003). It has been shown that the profile and structure (e.g. esterification) of skin anthocyanins have an important role in the anthocyanin interactions, such as self-association and co-pigmentation, thereby affecting the intensity and stability of red color in wine (Han and Xu, 2015; González-Manzano *et al*., 2008). Color intensity increases with the number of substituted groups on the B-ring (di-oxygenated forms are more red while tri-oxygenated shift to blue) and with the replacement of hydroxyl by methoxyl groups (i.e. malvidin has the darkest color). Methoxylated anthocyanins (malvidin and peonidin) are also more stable than hydroxylated ones to environmental and viticultural factors (Castellarin and Di Gaspero, 2007).

Environmental and management conditions can significantly influence grape anthocyanin content; among these, irrigation is by far the most studied. In most reports, a mild water deficit positively affects the accumulation of anthocyanins during ripening (Casassa *et al*., 2015; Zarrouk *et al*., 2012). This is attributed to multiple influences, either direct, such as enhanced expression of specific anthocyanin genes (Castellarin *et al*., 2007), or indirect, such as reduced berry size and vegetative growth (the latter improving canopy microclimate and allocation of assimilates to berries) (Keller, 2015). However, under Mediterranean climate conditions, negative effects of severe water limitations on the amount of anthocyanins have also been reported (Arrizabalaga *et al*., 2018; Teixeira *et al*., 2013). Apart from anthocyanin content, environmental (Bucchetti *et al*., 2011) and agricultural (Kyraleou *et al*., 2016a) factors can additionally affect anthocyanin composition. However, most studies involving irrigation effects on anthocyanins focus on total anthocyanin levels rather than on their compositional characteristics that can also affect intensity and stability of wine color.

Grapevine (*Vitis vinifera* L.) cultivation under Mediterranean climatic conditions is facing new challenges (van Leeuwen and Darriet, 2016). Climatic models predict significant rises in annual mean temperatures accompanied by water scarcity, leading to increases in evaporation rates (Tolika *et al*., 2008); thereby, irrigation of traditionally non-irrigated vineyards is becoming a common practice in southern Europe (Costa *et al*., 2016). While the response of berry anthocyanins to water conditions has been extensively studied in a range of winegrape varieties, little is known about indigenous Greek winegrape varieties which could be considered as a future alternative for varietal selection and breeding programs under climate change scenarios.

Agiorgitiko and Xinomavro (*Vitis vinifera* L.) are important Greek red grape varieties, representing a major fraction of Greece's wine production. Xinomavro is cultivated almost exclusively in northern Greece producing Protected Denomination of Origin (P.D.O. Naoussa, Amyndeon, Rapsani and Goumenissa) red wines. Grapes and wines of Xinomavro are poor in anthocyanins despite being rich in tannins (Kallithraka *et al*., 2006), making Xinomavro vinification a challenging task for winemakers. The cultivar Agiorgitiko originates from the area of Nemea in Peloponnesus (southern Greece) giving Protected Denomination of Origin (P.D.O. Nemea), deeply colored, red wines, with lower tannin concentration than Xinomavro (Kallithraka *et al*., 2011). However, Agiorgitiko wine color intensity tends to decline during fermentation, possibly due to the low levels of tannins that can bind with anthocyanins at the early stages of fermentation (Ribéreau-Gayon *et al*., 2000).

In Greece, vineyard area under irrigation has been steadily increasing during the last three decades. Moreover, since temperature and rainfall patterns are predicted to change,

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increasing the risk of drought in Greece's viticultural regions (Koufos *et al*., 2018), supplemental irrigation will greatly affect the ability of existing varieties to ripen fruit. The aim of this study was to determine and compare anthocyanin content and profile under variable irrigation regimes in four red grape cultivars (*Vitis vinifera* L.), namely Agiorgitiko and Xinomavro, alongside the well studied French cvs. Syrah and Grenache noir, under the typical semiarid conditions of Greece.

MATERIALS AND METHODS

1. Vineyard site and experimental design

A field trial in Thessaloniki, northern Greece (37° 79' N, 22° 61' E, 280 m) was carried out for two consecutive years (2012 and 2013) in a 6-year-old vineyard, planted with four red grape varieties (*Vitis vinifera* L. cvs. Xinomavro, Agiorgitiko, Syrah and Grenache noir), onto 1103 Paulsen (*V. rupestris* × *V. berlandieri*) at 4000 vines per ha (1.0 m × 2.5 m), in three replicated vineyard blocks. The vineyard was located on a loamy-clay soil (30% sand, 25% silt and 45% clay). Vines were trained on a vertical trellis with three fixed wires and spur-pruned on a bilateral cordon system to a standard 16 nodes per vine.

Starting at bunch closure (E-L 32) through harvest, three water regimes were applied: irrigation at 100% of crop evapotranspiration ET_c (FI), irrigation at 50% of ET_c (DI) and non-irrigated (NI). Each treatment was replicated three times in randomized blocks, with eight consecutive plants for each replication. The water quantities were applied by a drip irrigation system equipped with 4 L h^{-1} drip emitters positioned at 50 cm intervals along the pipe, on either side of the trunk. ET_c was estimated from the potential evapotranspiration data (calculated by the Penman-Monteith method) obtained from an on-site automatic weather station (iMETOS, Pessl Instruments GmbH, Weiz, Austria). Total water supply was 413 mm for FI and 129 mm for DI treatments in 2012 and 385 mm for FI and 120 mm for DI in 2013.

2. Vine parameters

Stem water potential (Ψ_{stem}) measurements were conducted with the use of a pressure chamber, according to Choné *et al*. (2001). In each measurement set, four mature leaves per plot of the inside part of the canopy were enclosed in plastic bags and covered with aluminum foil for at least 90 min before measurement, to allow equilibration of Ψ. Measurement of Ψ_{stem} was performed at midday (12h30 to 13h30), on a weekly basis. Only the central four vines of each plot were used for measurements. Cluster temperature (°C) was determined simultaneously to Ψ_{stem} on eight clusters of the same vines per plot, using an HI 99551 infrared thermometer (Hanna Instruments, Keysborough, Australia). Vine vigor was assessed by a non-destructive estimation of leaf area per vine according to the method of Lopes and Pinto (2000) on four vines per plot. On the same vines, measurements of pruning weight per vine (kg $vine^{-1}$) were obtained during the dormant period.

3. Berry analysis

Four samplings took place after veraison in 2012 (DOY 204, 220, 235, 247 for Syrah and DOY 208, 225, 240, 252 for the others) and in 2013 (DOY 195, 209, 224, 240 for Syrah and DOY 201, 215, 229, 246 for the others). Samples of 200 berries were collected randomly from the central four vines of each plot. Berries were pressed and the must was analyzed for total soluble solids (°Brix) by refractometry for titratable acidity (g L^{-1} tartaric acid). A subsample of 100 berries was counted and weighed to determine mean berry mass per plot and stored at -30°C for subsequent analysis of anthocyanins. At the end of the storage period, the berry samples were slowly de-frozen at 5°C, the skins were carefully removed from the pulp by hand and, after measurement of fresh skin weight, the skins were freeze-dried and grounded to a fine powder.

Anthocyanins were extracted from 1 g of dried skin with acidified methanol (1 mL L^{-1} in 0.012 mol HCl L^{-1}) for three different time duration steps (4, 18 and 24 h). After centrifugation, the three supernatants were combined in one sample. Monomeric anthocyanins were determined by HPLC consisting of a Jasco AS-1555 Intelligent Sampler, a Jasco PU 2089 Plus Quaternary Gradient Pump, a Jasco MD-910 Multiwavelength Detector and a Jasco LC-Net II/ADC (Jasco Corporation, Tokyo, Japan). A Restek Pinnacle II C18 column (250 mm × 4.6 mm, 4 μm; Restek Corporation, Bellefonte, PA, USA) was employed as described by Kyraleou *et al*. (2016b). The following compounds were identified: delphinidin-3-*O*-monoglucoside, cyanidin-3-*O*-monoglucoside, petunidin-3-*O*-monoglucoside, peonidin-3-*O*-monoglucoside, malvidin-3-*O*-

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TABLE 1. Monthly mean (Tavg), maximum (Tmax) and minimum (Tmin) temperatures (oC) and rainfall (mm) at the experimental site

	2012				2013			
	T_{avg}	T_{max}	T_{min}	Rainfall	T_{avg}	T_{max}	T_{min}	Rainfall
April	14.9	19.9	11.0	52.4	16.1	21.7	11.6	10.4
May	20.1	25.5	15.7	67.4	22.3	27.8	17.7	3.0
June	26.2	31.6	21.4	3.8	24.7	30.1	20.4	21.6
July	29.3	34.7	24.6	0.8	26.9	32.1	22.4	30.2
August	27.6	33.2	22.8	32.6	27.7	32.9	23.3	14.2
September	23.0	28.4	18.9	38.2	22.9	27.8	19.7	20.8

Irrigation treatments: FI, 100% of ET_c; DI, 50% of ET_c; and NI, non-irrigated.
Significant differences between years and among irrigation treatments are indicated by different letters (Tukey's test, $p<0.05$). * represents significance of the year × irrigation treatment (Y × I) interaction at $p<0.05$; *ns*, not significant.

TABLE 2. Irrigation and year effects on vegetative growth parameters

		Main Leaf Area (m^2/vine)	Lateral Leaf Area (m^2/vine)	Shoots/m	Pruning Wood Weight (kg)	Cane Weight (g)	T (^oC) berry
Xinomavro	**2012**	2.3	4.2 a	10.8	2.4	179	33.3 a
	2013	2.2	3.3 b	11.0	2.2	162	30.8 b
	FI	2.6 a	5.4 a	11.0	2.6 a	177	30.2 c
	DI	2.5 a	4.2 a	11.0	2.3 ab	170	32.0 b
	NI	1.6 b	1.7 b	10.5	2.0 b	164	33.9 a
	Y×I	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Agiorgitiko	**2012**	2.0	3.0 a	8.9 b	1.8	199 a	34.0 a
	2013	2.0	2.2 b	10.0 a	1.9	152 b	31.6 b
	FI	2.5 a	4.9 a	10.0 a	2.2 a	223 a	30.2 c
	DI	2.1 a	2.0 b	9.5 ab	1.9 b	170 b	32.9 b
	NI	1.4 b	1.0 c	8.8 b	1.5 c	133 c	35.3 a
	Y×I	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Syrah	**2012**	1.9	2.4 a	11.4	1.5 a	113 a	36.0 a
	2013	1.9	1.5 b	12.6	1.2 b	95.6 b	34.3 b
	FI	2.3 a	3.4 a	12.2	1.5 a	114 a	32.4 c
	DI	1.6 b	1.6 b	12.0	1.5 a	114 a	34.4 b
	NI	1.8 b	0.9 c	11.8	1.2 b	85.1 b	38.6 a
	Y×I	*ns*	*ns*	*ns*	*ns*	*	*ns*
Grenache noir	**2012**	2.0 a	2.4	12.2 a	1.4 a	109 a	34.3 a
	2013	1.6 b	2.2	9.9 b	1.3 b	90.1 b	31.4 b
	FI	2.1 a	3.8 a	11.2	1.6 a	114 a	30.2 c
	DI	1.9 a	1.8 b	10.3	1.5 a	112 a	33.5 b
	NI	1.5 b	1.3 c	10.2	1.0 b	72.4 b	34.8 a
	Y×I	*ns*	*ns*	*ns*	**	*ns*	*ns*

Irrigation treatments: FI, 100% of ET_c; DI, 50% of ET_c; and NI, non-irrigated.
Significant differences between years and among irrigation treatments are indicated by different letters (Tukey's test, $p<0.05$).
* and ** represent significance of the year × irrigation treatment (Y × I) interaction at $p<0.05$ and $p<0.01$, respectively;
ns, not significant.

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monoglucoside, malvidin-3-*O*-acetylglucoside and malvidin-3-(6-*O*-p-coumaroyl) glucoside. The concentration of anthocyanins was expressed as mg g^{-1} skin fresh weight of malvidin-3-*O*-monoglucoside equivalents. All analyses were performed in triplicate.

All grapes per plot were harvested at commercial harvest (3 September 2012 and 28 August 2013 for Syrah and 8 September 2012 and 2 September 2013 for Grenache noir, Agiorgitiko and Xinomavro), and total yield per plant (kg $vine^{-1}$) and average cluster weight (g) was estimated.

4. Statistical analysis

All values were averaged per plot and only the mean value per plot was used in the statistical analysis. Data were expressed as means of three replicates (n=3) and subjected to analysis of variance by means of ANOVA with irrigation treatments as the main factor by the use of SPSS 22 (Tukey's test $p<0.05$).

RESULTS AND DISCUSSION

1. Climatic conditions and vine water status

Climatic conditions for 2012 and 2013 presented differences in terms of total growth season rainfall but showed comparable mean temperatures (Table 1). Concerning rainfall, for the period April-September, the 2013 vintage was characterized by the lowest values of the last 25 years (1991-2015). Furthermore, mean temperatures for both 2012 and 2013 were higher by almost 3.5°C compared to the mean of the period 1991-2015. Although the mean temperature values were similar for both years, for 2012, maximum daytime temperatures were higher than in 2013. From June to September, there were 25 days with maximum temperatures above 35°C for 2012, compared to only 5 in 2013.

2. Vegetative and reproductive growth

Canopy growth was higher for the indigenous cultivars Xinomavro and Agiorgitiko, compared to Syrah and Grenache noir, with particularly high values under FI regime (Table 2). Concerning years, the drier 2013 was generally associated with lower vegetative growth, in all varieties. High water supply (FI) led to a more vigorous development in all varieties, with lateral leaf area being the most responsive among measured parameters. Indeed a pronounced lateral growth was observed for the more vigorous cvs. Xinomavro and Agiorgitiko, under FI conditions. Pellegrino *et al*. (2005) reported that, for the Syrah variety, the growth of the leaf area and especially of the secondary leaf surface is more sensitive to water stress and can be used as a water shortage indicator.

The number of shoots per vine did not presumably affect canopy growth since it was similar among irrigation treatments, following a uniform pruning (except Agiorgitiko where NI plants showed lower number of shoots compared to FI). Except for two cases, there was no year × irrigation interaction for vigor parameters, showing a consistent effect of water conditions on vine vegetative growth. Irrigation-related decreases in canopy growth and extension in space were probably responsible for the increased temperature of the clusters in the NI vines, in all cultivars (Table 2).

Total yield and all its major components (cluster number - except Syrah -, cluster weight and berry weight) were reduced under NI treatment (Table 3) compared to FI, with DI presenting intermediate values. It has been previously reported that water deficit can lead to smaller berry size in Cabernet-Sauvignon (Intrigliolo *et al*., 2016), Sangiovese (Merli *et al*., 2015) and Tempranillo (Zarrouk *et al*., 2012) in Mediterranean-type climates. Productivity was lower in the two French cvs. during the hotter 2013 compared to 2012 but this was not observed for Xinomavro and Agiorgitiko. Skin-to-berry weight ratio was modified by irrigation only in Xinomavro and Grenache noir, with higher values for the berries of the non-irrigated vines. According to Roby and Matthews (2004), water deficit can lead to higher proportion of skins per berry, which in turn affects the chemical composition of must during fermentation in red vinification (Shellie, 2011). Must soluble solids responded positively to water limitation in all varieties, while for titratable acidity there was an opposite trend, except for Xinomavro and Grenache noir (Table 3). Except for one case, there was no year × irrigation interaction for yield and must composition parameters, showing a consistent effect of water conditions on vine reproductive development.

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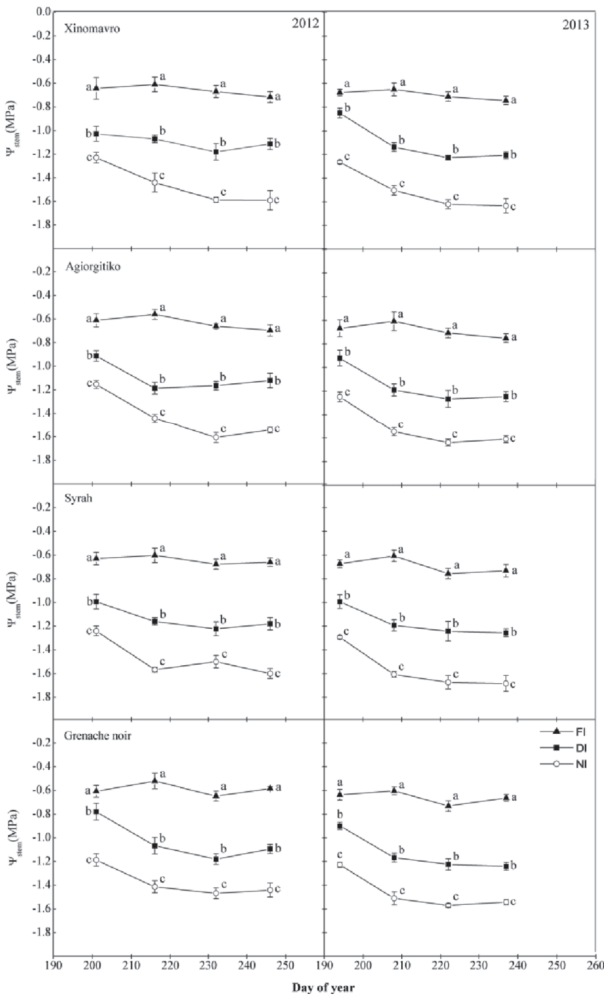


FIGURE 1. Midday stem water potential (Ψ_{stem}) seasonal pattern, in 2012 and 2013
Irrigation treatments: FI, 100% of ETc; DI, 50% of ETc; and NI, non-irrigated. Bars indicate ±S.E. of the mean value. Significant differences among treatments within samplings and years are indicated by different letters (Tukey's test, $p<0.05$).

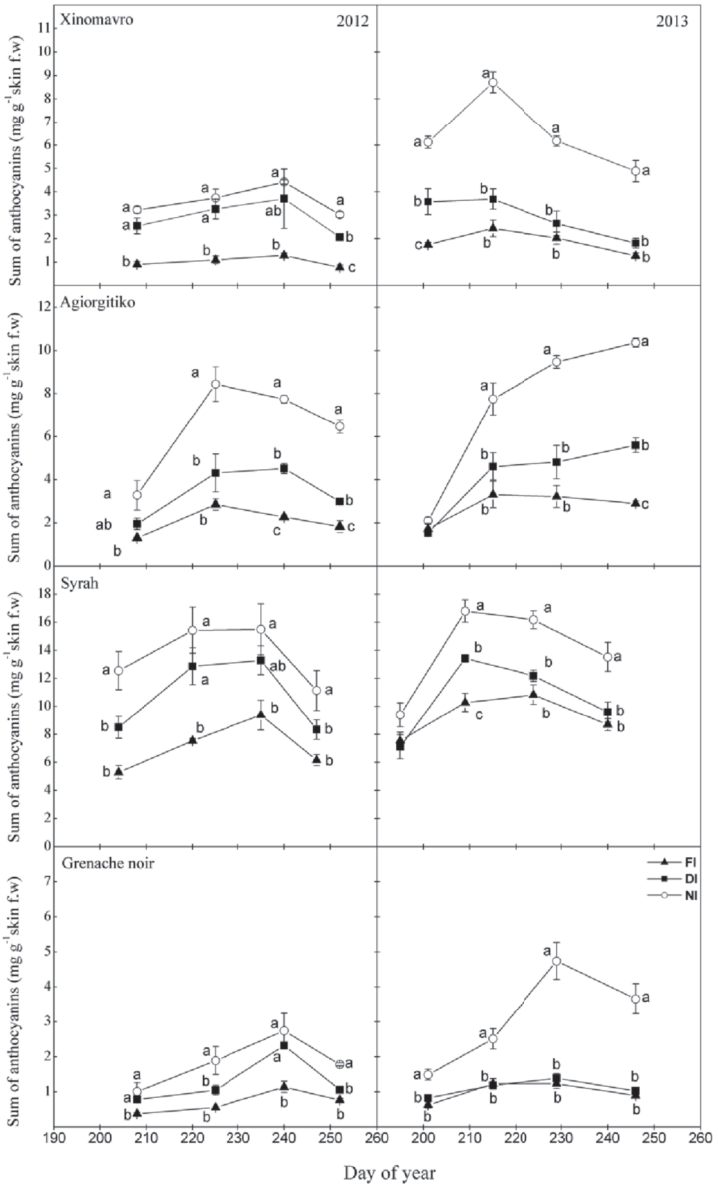


FIGURE 2. Sum of anthocyanin seasonal pattern, in 2012 and 2013
Sum of anthocyanins: delphinidin-3-*O*-glucoside (Dp), cyanidin-3-*O*-glucoside (Cy), petunidin-3-*O*-glucoside (Pt), peonidin-3-*O*-glucoside (Pn), malvidin-3-*O*-glucoside (Mv), malvidin-3-*O*-acetylglucoside (MvA) and malvidin-3-(6-*O*-p-coumaroyl) glucoside (MvC).
Irrigation treatments: FI, 100% of ET_c; DI, 50% of ET_c; and NI, non-irrigated. Bars indicate ±S.E. of the mean value. Significant differences among treatments within samplings and years are indicated by different letters (Tukey's test, $p<0.05$).

3. Anthocyanin content and composition

As shown in Figure 2, the accumulation of total anthocyanins [sum of delphinidin-3-*O*-glucoside (Dp), cyanidin-3-*O*-glucoside (Cy), petunidin-3-*O*-glucoside (Pt), peonidin-3-*O*-glucoside (Pn), malvidin-3-*O*-glucoside (Mv), malvidin-3-*O*-acetylglucoside (MvA) and malvidin-3-(6-*O*-p-coumaroyl) glucoside (MvC)] followed a different pattern during ripening among the four varieties. An upward trend until harvest was exhibited only for Agiorgitiko NI skins in 2013. The concentration of total anthocyanins in FI skins (except Syrah) showed little variation during the season. In the majority of cases though, anthocyanins increased during ripening and then showed a slight decrease 10-30 days before harvest, in agreement with the findings of previous studies (Kyraleou *et al*., 2016a; Lasanta *et al*., 2014). The decrease of anthocyanin accumulation was more intense in NI treatments and could be associated with the higher temperatures of the cluster zone due to the lower

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TABLE 3. Irrigation and year effects on yield components at harvest

		Yield (kg)	Clusters/ shoot	Cluster weight (g)	Berry weight (g)	Skin/berry weight ratio	TSS (°Brix)	Titratable acidity (g/L)
Xinomavro	**2012**	5.2	1.6	240	1.7	7.3	20.4	7.9 a
	2013	4.7	1.5	238	1.7	7.7	20.7	6.3 b
	FI	5.9 a	1.8 a	292 a	1.9 a	6.4 b	19.4 c	7.3
	DI	5.1 a	1.5 ab	253 b	1.8 a	7.2 b	20.5 b	7.1
	NI	3.8 b	1.4 b	173 c	1.5 b	9.0 a	21.8 a	7.0
	Y×I	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Agiorgitiko	**2012**	4.0	1.6 a	247	2.0	8.2	20.5 b	5.2 a
	2013	3.9	1.2 b	251	1.9	9.4	21.0 a	4.8 b
	FI	4.9 a	1.7 a	281 a	2.0 a	8.2	19.8 c	5.6 a
	DI	4.3 b	1.3 b	268 a	2.1 a	8.7	20.7 b	4.8 b
	NI	2.8 c	1.2 b	197 b	1.7 b	9.4	21.7 a	4.5 b
	Y×I	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Syrah	**2012**	5.9 a	2.0	221 a	1.4	9.2 b	20.8 b	6.3 a
	2013	5.0 b	2.1	183 b	1.3	10.2 a	22.5 a	5.2 b
	FI	5.7 a	1.9	233 a	1.44 a	9.9	21.1 b	6.0 a
	DI	5.7 a	2.2	197 b	1.37 b	9.7	21.6 ab	5.8 ab
	NI	4.9 b	1.9	175 b	1.19 c	9.5	22.3 a	5.5 b
	Y×I	*ns*	*	*ns*	*ns*	*ns*	*ns*	*ns*
Grenache noir	**2012**	8.1 a	2.1 a	324	1.6 a	10.0 a	21.3	4.6
	2013	7.1 b	1.7 b	315	1.3 b	8.0 b	21.2	4.6
	FI	9.7 a	1.7 a	377 a	1.8 a	8.3 b	20.6 b	4.6
	DI	8.3 b	1.3 b	367 a	1.4 b	8.9 ab	21.1 ab	4.6
	NI	4.7c	1.2 b	214 b	1.1 c	9.7 a	22.1 a	4.5
	Y×I	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

Irrigation treatments: FI, 100% of ET_c; DI, 50% of ET_c; and NI, non-irrigated.
Significant differences between years and among irrigation treatments are indicated by different letters (Tukey's test, $p<0.05$).
* represents significance of the year × irrigation treatment (Y × I) interaction at $p<0.05$; *ns*, not significant.

vine leaf area at the end of the ripening period. It has been previously reported that high temperatures during berry development can cause degradation of anthocyanins and inhibition of their biosynthetic pathway (Mori *et al*., 2007). A vintage effect was observed for the concentration of total anthocyanins, with higher values determined in grapes from 2013, compared to the same treatments in 2012 (mostly for NI grapes). Among varieties, cvs. Xinomavro and Grenache noir had lower total anthocyanin content when compared to Agiorgitiko, while Syrah had the highest anthocyanin content in both years.

Mv was the predominant glucoside in the skins of the four varieties at harvest (Kyraleou *et al*., 2016a; Ollé *et al*., 2011), followed by its coumaroyl derivative (MvC); Mv had the highest proportion in the total pool of anthocyanins in Grenache noir skins (70%) and the lowest in Syrah (50%), while the opposite was observed for MvC (Figure 3). In both Xinomavro and Agiorgitiko, Mv represented an average of 61% of total anthocyanins. The main difference between Xinomavro and Agiorgitiko was observed in Mv derivatives, %MvA being higher in Xinomavro berries, while %MvC was higher in Agiorgitiko.

Concerning the relative contribution of non-acylated (Cy, Dp, Pt, Pn and Mv) and acylated (MvA and MvC) anthocyanins, the most abundant fraction consisted of non-acylated anthocyanins (Dp, Cy, Pt, Pn and Mv) in all varieties. Acylated anthocyanins (MvA and MvC) had the lowest percentage in Grenache noir berries (13.9%), while the highest in Syrah (37.2%). For both Xinomavro and Agiorgitiko, the levels of non-acylated and acylated anthocyanins in berries were 71.5% and 28.5% of total anthocyanins, respectively (Figure 3).

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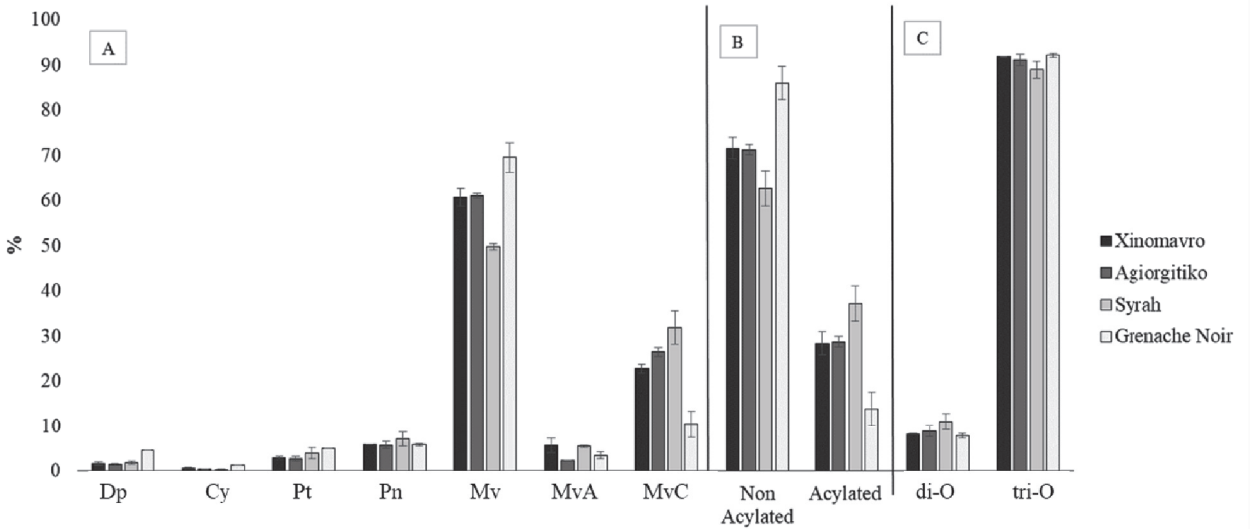


FIGURE 3. Influence of cultivar on anthocyanin composition (% total anthocyanin content) at harvest (combined years)

A. Anthocyanins: delphinidin-3-*O*-glucoside (Dp), cyanidin-3-*O*-glucoside (Cy), petunidin-3-*O*-glucoside (Pt), peonidin-3-*O*-glucoside (Pn), malvidin-3-*O*-glucoside (Mv), malvidin-3-*O*-acetylglucoside (MvA) and malvidin-3-(6-*O*-p-coumaroyl) glucoside (MvC).

B. Non-acylated anthocyanins (Sum of Dp, Cy, Pt, Pn and Mv); acylated anthocyanins (Sum of MvA and MvC).

C. di-O (Sum of Cy and Pn); tri-O (Sum of Dp, Pt, Mv, MvA and MvC).

Bars indicate ±S.E. of the mean value.

In our study, the percentage of blue tri-oxygenated derivatives was higher than the red di-oxygenated derivatives in all cultivars and it ranged between 89-92.2% and 7.8-11%, respectively, all cultivars included. The mean concentrations of di-oxygenated anthocyanins at harvest were 0.13, 0.34, 0.69 and 0.10 mg g^{-1} skin f.w. for Xinomavro, Agiorgitiko, Syrah and Grenache noir, respectively, while for tri-oxygenated derivatives, the higher content was found in Syrah (5.40 mg g^{-1}) and the lower in Xinomavro (1.47 mg g^{-1}) and Grenache noir (1.19 mg g^{-1}).

Regarding irrigation effects on anthocyanin levels, NI skins had the highest anthocyanin content, in almost all samplings, varieties and years (Figure 2). Anthocyanin levels of FI and DI treatments differed significantly mostly in 2012, while in 2013 they reached similar levels at harvest (except for Agiorgitiko). Previous studies have shown that the response of grape anthocyanins to water conditions is cultivar dependent; according to Niculcea *et al*. (2015), Tempranillo and Graciano berry anthocyanins were affected differently by irrigation under the same experimental conditions, with an increasing trend with water limitation for Tempranillo but not for Graciano. In our experimental conditions, the four cultivars had a similar response to irrigation, with values increasing with water deficit.

Irrigation also affected the profile of anthocyanins, however, these changes were dependent on the variety. Xinomavro, Agiorgitiko and Grenache noir berries from NI treatments had higher concentration of non-acylated anthocyanins (Dp, Cy, Pt, Pn, Mv) and MvC compared to DI and FI treatments (Table 4). In Syrah grapes, the absence of irrigation affected positively Mv and its derivatives (MvA, MvC), while no clear effect was observed for most of the non-acylated anthocyanins. It was reported in previous studies that water stress can lead to a significant increase in the content of p-coumaroylated forms of anthocyanins (Ollé *et al*., 2011; Ortega-Regules *et al*., 2006). In the conditions of this study, a proportional increase in MvC under NI conditions was observed only for Xinomavro (data not shown). Year × irrigation interaction content was significant for Dp, Pt, Pn, Mv and MvC in Xinomavro, Cy in Syrah and MvA in Grenache noir. Analyzing data separately for each year, in the case of Pt and Pn in Xinomavro and Cy in Syrah, this was related to the fact that differences were only significant for 2013, while in the other cases, it was due to the differentiation (or not) between FI and DI

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TABLE 4. Irrigation and year effects on the content of individual anthocyanins in berry skins (mg g^{-1} skin fresh weight) at harvest.

		Dp	Cy	Pt	Pn	Mv	MvA	MvC
Xinomavro	**2012**	0.03 b	0.01	0.04 b	0.10 b	1.1 b	0.12	0.5 b
	2013	0.05 a	0.01	0.08 a	0.14 a	1.6 a	0.12	0.7 a
	FI	0.02 c	0.01 b	0.04 c	0.08 c	0.6 c	0.06 b	0.2 c
	DI	0.03 b	0.01 b	0.06 b	0.12 b	1.2 b	0.10 b	0.4 b
	NI	0.06 a	0.02 a	0.09 a	0.16 a	2.3 a	0.20 a	1.2 a
	Y×I	*	*ns*	**	*	*	*ns*	***
Agiorgitiko	**2012**	0.05 b	0.02 b	0.12	0.19 b	2.4 b	0.07	0.9 b
	2013	0.11 a	0.03 a	0.16	0.46 a	4.0 a	0.12	1.4 a
	FI	0.03 b	0.01 b	0.04 b	0.11 c	1.3 c	0.07	0.8 c
	DI	0.04 b	0.02 b	0.09 b	0.25 b	2.7 b	0.10	1.1 b
	NI	0.16 a	0.03 a	0.29 a	0.61 a	5.6 a	0.11	1.6 a
	Y×I	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Syrah	**2012**	0.12 b	0.02 b	0.23 b	0.45 b	4.3 b	0.47	3.0
	2013	0.22 a	0.03 a	0.53 a	0.87 a	5.4 a	0.56	2.9
	FI	0.17	0.03	0.35 ab	0.73	3.4 c	0.35 b	2.4 b
	DI	0.15	0.02	0.33 b	0.59	4.6 b	0.47 b	2.9 b
	NI	0.19	0.03	0.47 a	0.67	6.6 a	0.74 a	3.6 a
	Y×I	*ns*	*	*ns*	*ns*	*ns*	*ns*	*ns*
Grenache noir	**2012**	0.06 b	0.02	0.06	0.07	0.9 b	0.03 b	0.09 b
	2013	0.07 a	0.02	0.07	0.09	1.2 a	0.07 a	0.27 a
	FI	0.03 c	0.01 b	0.04 c	0.06 b	0.6 b	0.02 c	0.1 a
	DI	0.06 b	0.01 b	0.07 b	0.07 b	0.7 b	0.05 b	0.08 b
	NI	0.1 a	0.04 a	0.1 a	0.1 b	1.9 a	0.09 a	0.4 a
	Y×I	*ns*	*ns*	*ns*	*ns*	*ns*	*	*ns*

Irrigation treatments: FI, 100% of ET_c; DI, 50% of ET_c; and NI, non-irrigated.
Anthocyanins: delphinidin-3-*O*-glucoside (Dp), cyanidin-3-*O*-glucoside (Cy), petunidin-3-*O*-glucoside (Pt), peonidin-3-*O*-glucoside (Pn), malvidin-3-*O*-glucoside (Mv), malvidin-3-*O*-acetylglucoside (MvA) and malvidin-3-(6-*O*-p-coumaroyl) glucoside (MvC).
Significant differences between years and among irrigation treatments are indicated by different letters (Tukey's test, $p<0.05$). *, ** and *** represent significance of the year × irrigation treatment (Y × I) interaction at $p<0.05$, $p<0.01$ and $p<0.001$, respectively; *ns*, not significant.

treatments. In all cases, however, the increasing order among treatments was not altered (data not shown).

According to Table 5, the contribution of acylated anthocyanins to the total anthocyanin content of NI berries was higher compared to DI and FI for Xinomavro (and to a lesser degree in Grenache noir) but it was the opposite in Agiorgitiko; no clear trend was observed for Syrah regarding the degree of acylation of anthocyanins. Non-acylated anthocyanins are more sensitive to oxidation reactions in berries and wines (He *et al*., 2010), while acylated ones represent a more stable form of anthocyanins to oxidative effects (Rinaldo *et al*., 2015) such as those caused by berry exposure to solar radiation and to high temperatures (Downey *et al*., 2004). In a previous work with Grenache noir (Oliveira *et al*., 2013), water deficit increased the contribution of acylated anthocyanins, while Esteban *et al*. (2001) reported that irrigation affected positively the concentration of non-acylated anthocyanins in Tempranillo grapes. According to Ollé *et al*. (2011), in Syrah berries, the application of water deficit after veraison resulted in a significant increase in the content of the p-coumaroylated forms of anthocyanins as compared to acetylated derivatives.

It has been reported that tri-oxygenated forms are more stable than di-oxygenated anthocyanins

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TABLE 5. Irrigation and year effects on anthocyanin composition (% total anthocyanin content) at harvest

		Non-acylated	Acylated	Acylated/ Non-acylated	di-O	tri-O	di-O/tri-O
Xinomavro	**2012**	69.1 b	30.9 a	0.45 a	6.5	93.5	0.07
	2013	74.0 a	26.0 b	0.36 b	6.4	93.6	0.07
	FI	75.3 a	24.7 b	0.33 b	8.5 a	91.5 c	0.09 a
	DI	74.0 a	26.0 b	0.36 b	6.4 b	93.6 b	0.07 b
	NI	65.4 b	34.6 a	0.53 a	4.5 c	95.5 a	0.05 c
	Y×I	*	*	*ns*	*ns*	*ns*	*ns*
Agiorgitiko	**2012**	70.1	29.9	0.45	5.3 b	94.7 a	0.06 b
	2013	72.4	27.6	0.39	7.0 a	92.9 b	0.08 a
	FI	61.9 c	38.1 a	0.63 a	5.2 b	94.7 a	0.05 b
	DI	72.9 b	27.0 b	0.37 b	6.1 ab	93.9 ab	0.06 ab
	NI	78.9 a	21.1 c	0.27 b	7.2 a	92.7 b	0.08 a
	Y×I	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Syrah	**2012**	58.9 b	41.1 a	0.70 a	5.7 b	94.3 a	0.06 b
	2013	66.7 a	33.3 b	0.50 b	8.9 a	91.1 b	0.10 a
	FI	61.9	38.1	0.62	9.8 a	90.2 b	0.11 a
	DI	62.5	37.5	0.60	6.7 b	93.3 a	0.07 b
	NI	63.9	36.1	0.56	5.5 b	94.5 a	0.06 b
	Y×I	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Grenache noir	**2012**	89.7 a	10.1 b	0.11 b	7.3	92.5	0.08
	2013	82.4 b	17.6 a	0.21 a	6.6	93.3	0.07
	FI	86.1 ab	13.8 ab	0.16 ab	7.4	92.6	0.08
	DI	87.9 a	11.9 b	0.14 b	7.2	92.6	0.08
	NI	84.1 b	15.9 a	0.19 a	6.4	93.6	0.07
	Y×I	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

Irrigation treatments: FI, 100% of ET_c; DI, 50% of ET_c; and NI, non-irrigated.
Non-acylated anthocyanins (Sum of Dp, Cy, Pt, Pn and Mv); acylated anthocyanins (Sum of MvA and MvC); di-O (Sum of Cy and Pn); tri-O (Sum of Dp, Pt, Mv, MvA and MvC).
Significant differences between years and among irrigation treatments are indicated by different letters (Tukey's test, $p<0.05$). * represents significance of the year × irrigation treatment (Y × I) interaction at $p<0.05$; *ns*, not significant.

to cultural or environmental conditions (Guidoni *et al*., 2008). Regarding the relative concentrations of di-oxygenated and tri-oxygenated anthocyanins, a different response to water availability between Xinomavro and Agiorgitiko berry skins was observed (Table 5). In Xinomavro (similarly to Syrah), NI regime increased the proportion of tri-oxygenated anthocyanins over di-oxygenated (Table 5) as well as the proportion of methoxylated on the B-ring (Mv and esters, and Pn) over hydroxylated ones (Dp, Cy and Pt) (the ratio -OH/-OCH_3 derivatives was 0.074 for FI and 0.044 for NI, $p<0.05$). A similar response was reported by Castellarin *et al*. (2007) in Merlot berries, related to an up-regulation of the genes coding for flavonoid 3',5'-hydroxylase (F3'5'H) and *O*-methyltransferase (OMT) in the water stressed plants. Cook *et al*. (2015) also reported higher tri-oxygenated anthocyanins over di-oxygenated ones in Merlot skins under increased water deficit during the fruit-set to veraison phase. An opposite trend with irrigation was observed for Agiorgitiko, where berry skins of non-irrigated plants had increased proportion of di-oxygenated (Table 5) and hydroxylated anthocyanins (the ratio -OH/-OCH_3 derivatives was 0.035 for FI and 0.061 for NI, $p<0.05$). In Grenache noir, no significant differences in anthocyanin composition were observed among treatments. We could therefore suggest that, in the conditions of this experiment, non-irrigated regime may increase color intensity and stability of Xinomavro and Syrah wines as a result of increased participation rate of more stable forms

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of anthocyanins (increased substitution and methoxylation of the B-ring). On the contrary, the different trend with water supply of the relative distribution of B-ring substitution derivatives in Agiorgitiko might imply a need for a different irrigation strategy in this variety.

The accumulation of anthocyanins in response to stress has been previously reported to be cultivar specific. According to Hochberg *et al*. (2015), significant increases in the less stable hydroxylated anthocyanins (Dp, Cy and Pt) were measured in Shiraz under deficit irrigation as compared to the irrigated control, while in Cabernet-Sauvignon only the Pt form was increased. Moreover, deficit irrigation significantly reduced the more stable acylated forms in Syrah, while the opposite was true for Cabernet-Sauvignon. In the same study, Mv was not affected by the water regime, unlike our results. It could be also of interest to examine whether the response of cultivars in the context of this experiment was also influenced by local temperature conditions (Gaiotti *et al*., 2018), i.e. maximum temperatures reaching 30-35°C and minimum temperatures frequently exceeding 20°C. It is well documented that anthocyanin biosynthesis is favored at temperatures around 25°C (Mori *et al*., 2007) and under night temperatures below 15°C (Mori *et al*., 2005b). According to the same author (Mori *et al*., 2005a), hydroxylated anthocyanins (Dp, Cy and Pt) are the most sensitive to high night temperatures (30°C) compared to malvidin. However, since the anthocyanin profile was found to be similar between the two Greek cultivars, their different response of anthocyanin composition to water conditions cannot be associated to a different resilience to high temperatures.

CONCLUSIONS

Under the summer conditions of the Greek climate, the four cultivars had a similar response regarding vigor and yield parameters, with values increasing with water supply. Anthocyanin concentration was maximized under non-irrigated conditions, but anthocyanin profile and relative distribution of single glucosides did not respond uniformly to irrigation in all cultivars. Especially regarding the two indigenous Greek cultivars, Xinomavro seemed to favor the synthesis of more stable forms of anthocyanins under limited water supply (acetylated vs. non-acetylated, tri-oxygenated on the B-ring vs. di-oxygenated and methoxylated on the B-ring vs. hydroxylated), while Agiorgitiko had an opposite behavior. Syrah had a similar response to Xinomavro (in most cases), while anthocyanin composition of Grenache noir berries had a less marked response to irrigation. The difference in the response of anthocyanin composition to water conditions between Xinomavro and Agiorgitiko possibly implies the adaptation of irrigation strategy. While a sustained water deficit may be beneficial for both the total amount and the stability of color in Xinomavro grapes, in the case of Agiorgitiko, a moderate deficit irrigation regime might provide a better compromise between anthocyanin levels and color stability.

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