# EFFECTS OF DEFOLIATION AND WATER RESTRICTION ON TOTAL PHENOLS AND ANTIOXIDANT ACTIVITIES IN GRAPES DURING RIPENING

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**Abstract**

**Aim**: To compare the effect of water restriction and defoliation on the phenol contents and oxidant and antioxidant activities of Tempranillo grapes grown in the region of Extremadura, Spain.

**Methods and results**: The results showed that at harvest, the water restriction treatment altered total foliar area, pH, and total soluble solid (TSS) and phenol contents but not polyphenol oxidase (PPO) and superoxide dismutase (SOD) activities. By contrast, the defoliation treatment did not affect TSS and pH, however, titratable acidity and phenol compounds were enhanced and PPO and SOD activities were decreased. Moreover, defoliation resulted in advanced grape ripening and reduced total foliar area.

**Conclusion**: At harvest, water restriction led to alterations in the levels of peroxidation and peroxidase (POX) activity, without any effect on the other parameters. Defoliation induced a lowering of PPO and SOD activities and an increase in phenol content.

**Significance and impact of the study**: Given the environmental characteristics of the Extremadura region, defoliation may be suitable and efficient for regulating Tempranillo grape PPO activity, which is œnologically relevant since it could lead to lower levels of phenol oxidation and hence prevent wine discolouration.

**Key words**: *Vitis vinifera*, defoliation, water restriction, antioxidant activities, flavonoids and phenylpropanoid glycosides, lipid peroxidation

**Résumé**

**Objectif** : Les effets d´une restriction hydrique et d´une défoliation ont été étudiés sur la quantité de composés phénoliques et sur les activitiés oxydantes et anti-oxydantes de raisins de cépage Tempranillo dans la région d´Estrémadure, en Espagne.

**Méthodes et résultats** : Les résultats montrent que, lors de la récolte, la restriction hydrique a eu un effet sur la surface foliaire, le pH, les solides solubles totaux (TSS) et les composés phénoliques, mais n'a eu aucun effet sur les activités de la polyphénol oxydase (PPO) et de la superoxyde dismutase (SOD). Au contraire, la défoliation n´a eu aucun effet sur le pH et les TSS, mais a provoqué des valeurs supérieures en composés phénoliques et en acidité titrable et une réduction des activités PPO et SOD. La défoliation a également provoqué une maturation précoce et une diminution de la surface foliaire.

**Conclusion** : À la récolte, la restriction hydrique conduit à des altérations dans les niveaux de peroxydation et de l'activité de la peroxydase (POX) sans modifier les autres paramètres. La défoliation a provoqué une réduction de l´activité SOD et PPO et une augmentation de la teneur en composés phénoliques.

**Signification et impact de l'étude** : Dans l´environnement caractéristique de l´Estrémadure, la défoliation du cépage Tempranillo peut être une mesure convenable et efficace pour diminuer le niveau d´activité PPO, ce qui est pertinent d'un point de vue œnologique, car cela pourrait conduire à des niveaux inférieurs de l'oxydation des composés phénoliques, évitant ainsi la décoloration des vins.

**Mots clés** : *Vitis vinifera,* défoliation, restriction hydrique, activités anti-oxydantes, flavonoïdes et glycosides de phénylpropanoïde, peroxydation lipidique

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## INTRODUCTION

Traditional indices of grape ripening are based on the content of sugars and acids in the must. However, in the case of red wine grapes, these indices alone are not reliable markers of quality (Saint-Criq de Gaulejac *et al.*, 1998). It is thus essential to understand how the content of phenolic compounds evolves during ripening in order to control the quality of the final product, as these compounds are responsable for the color and astringency of the resulting red wine (Ribéreau-Gayon and Glories, 1987).

Reactive oxygen species (ROS) are produced as a result of normal metabolism and play an important role in the plant life cycle and stress response (Dangl *et al.*, 1996; Kawano, 2003). It has been demonstrated that the plasma membrane NADPH oxidases (RBOH-NOX) and peroxidases are involved in the ROS production (Grant *et al.*, 2000; Bolwell *et al.*, 2002). ROS are potentially dangerous and their overproduction, called "oxidative burst", is part of the plant response to environmental stresses (Miller *et al.*, 2010; Apel and Hirt, 2004). Plants respond to stress by developing a series of oxidative and antioxidative reactions specific to each stress (Mittler *et al.*, 2011). Moreover, the mechanisms involved in the production and scavenging of ROS in these stress responses are also key agents in other physiological processes such as ripening (Jiménez *et al.*, 2003). The antioxidant system plays an important role in ROS homeostasis (Apel and Hirt, 2004) and includes enzymes such as peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD). Increased synthesis of phenolic compounds, including flavonoids and phenylpropanoid glycosides, is a common plant response to stresses (Dixon and Paiva, 1995) and ripening (Fortes *et al.*, 2011). Phenylpropanoid glycosides, in particular, enhance the antioxidant capacity of cells (Grace and Logan, 2000). Many studies have demonstrated the ROS scavenging capacity of flavonoids and phenolic acids (Rice-Evans *et al.*, 1996). This suggests that phenylpropanoid glycosides and flavonoids are involved in protection against oxidative stress (Grace, 2005).

How a plant's total antioxidant capacity, phenol content, and lipid peroxidation level evolve may be used as an indicator of stress response (Dixon and Paiva, 1995; Ma *et al.*, 2007). For grapes, ripening involves changes in the composition and accumulation of phenols that will subsequently be extracted and transferred to the wine. Antioxidant activity in grapes is positively correlated with the concentration of phenolic compounds (Landbo and Meyer, 2001). Phenolic composition may vary during ripening (Jordao *et al.*, 2001) due to enzymatic alterations, as is the case for the oxidation of phenols by phenol oxidases (Ryan *et al.*, 2002). Doshi *et al.* (2006) reported reduced antioxidant capacity during ripening, as reflected in phenolic compounds. Drăghici *et al.* (2011) described a steady increase in total polyphenol content during ripening until maturity, followed by a slight decline. This evolution in production and accumulation depends, however, on a great variety of factors, including (i) weather conditions, (ii) farming practices (Conde *et al.*, 2007; Singh *et al.*, 2010), such as the input of N and K fertilizers (Delgado *et al.*, 2004), and (iii) water availability at different grapevine phenological stages (Valdés *et al.*, 2008; Yuste *et al.*, 2012). Also, polyphenol synthesis depends partly on the activity of the enzyme phenylalanine ammonia lyase, whose activity is temperature and light dependent (Roubelakis-Angelakis and Kliewer, 1986). This, of course, implies the involvement of a large number of factors (exposure of the clusters to light, the temperature reached by the grapes, etc.), all of which are to a greater or lesser degree directly related to leaf area (Lee and Skinkis, 2013; Tardáguila *et al.*, 2010, 2012). Indeed, it has been shown that defoliation affects grape phenol content (Uriarte *et al.*, 2012).

The objective of the present work was therefore to study the evolution of phenol content and oxidant and antioxidant activities in grapes in response to water stress and defoliation.

## MATERIALS AND METHODS

### 1. Site description

The experiment was carried out during the 2011 season in a Tempranillo vineyard (*Vitis vinifera* L.) planted in 2001 in Ritcher-110 rootstock at a spacing of 2.5 m x 1.2 m (3,333 vines/ha). The vineyard was located in Guadajira (38°N, 6°W, elevation 198 m), in the Extreamadura region (Spain), under Mediterrenean climatic conditions. In the spring of 2011, total rainfall was 122.20 mm, average temperature 20.2 ºC, average relative humidity (HR) 60.87 %, and solar radiation 24.18 MJ/m$^2$ day. The summer was dry (16.74 mm total rainfall) and the averages recorded during ripening were 23.03 ºC, 55.60 % HR and 23.58 MJ/m$^2$ day solar radiation. Vines were trained to a vertical trellis on a bilateral cordon system oriented in the East-West direction. Six spurs per vine, two buds per spur, were retained during winter pruning. The soil had a loam to clay-loam texture. The volumetric water content was 20.4 % at field capacity and 11.4 % at permanent wilting point.

The experimental design was a split-plot with four replicates, irrigation being the main factor and defoliation the second factor. Irrigation started when the stem water potential reached a level of -0.6 MPa (June 21, 2011) and finished on October 10, 2011. The experimental plots had six rows with eighteen vines per row. The irrigation treatments were based on replacing crop evapotranspiration (ETc) at a rate of 0 % ETc (water restriction, R1) or 100 % ETc (non defoliated (R2N) and defoliated (R2D) irrigated plants); the defoliation treatment comprised the selective removal, at pre-bloom, of seven basal leaves from each shoot as well as leaves from secondary shoots.

Four ripening stages were used depending on total soluble solids (TSS): stage I (20.5±0.5 ºBrix); stage II (22.5±0.5 ºBrix); stage III or harvest (23.5±0.5 ºBrix); and stage IV or post-harvest (24.5±0.5 ºBrix).

**2. Field determinations**

The water potential was determined weekly with a Scholander pressure chamber (Soil moisture, Corp. Santa Barbara, CA, USA) on one plant per experimental plot and two leaves per vine (eigth determinations per treatment). According to previous works (Girona *et al.*, 2009; Intrigliolo and Castel, 2011; Valdes *et al.*, 2009), this sampling procedure is representative of the whole experimental treatment. The water stress integral was estimated according to Myers (1988). Leaf area index was determined by means of a canopy analyzer (LAI 2200 LI-Corn inc. Lincoln, Nebraska, USA) on four vines per experimental plot (sixteen vines per treatment). Yield components and winter prunning weight were determined on ten vines per experimental plot. Determinations were carried out according to Valdés *et al.* (2009).

**3. Biochemical analysis**

From veraison to harvest, fresh berry samples (groups of four berries) were chosen randomly from all sides and different parts of the clusters (top, middle and bottom region); thirty-two different vines were used per experimental plot (two rows of sixteen vines, ≈653 clusters) and collected weekly. Must TSS, pH, and acid components were determined in samples of 250 g per experimental plot according to Valdes *et al.* (2009). TSS (°Brix) was determined by refractometry. Juice pH and titratable acidity were determined by an automatic titrator and results were expressed as $g{\cdot}L^{-1}$ of tartaric acid. Tartaric and malic acid concentrations were determined in the harvest samples: tartaric acid was determined according to the Rebelein method (Blouin, 1973) and malic acid by means of an automatic analyzer after enzymatic reactionusing the EasyChem Plus system (Sestea, Italy).

Phenolics, flavonoids and phenylpropanoid glycosides were extracted from grapes by homogenization with methanol, chloroform and 1 % NaCl (2:2:1). The homogenate was filtered and centrifuged at 3200 g for 10 min. Total phenolic content was quantitatively assayed by $A_{765}$ with the Folin-Ciocalteu reagent according to the method of Singleton *et al.* (1985) and expressed as µg of caffeic acid $g^{-1}$ of berry fresh weight (FW).

Total flavonoid content was measured colorimetrically following the method described by Kim *et al.* (2003). Total flavonoids were calculated using the standard rutin curve and expressed as µg of rutin $g^{-1}$FW.

Total phenylpropanoid glycoside content was determined by a colorimetric method ($A_{525}$) using the Arnow reagent as described in Gálvez *et al.* (2005). The concentration was calculated on the basis of the standard 3,4-dihydroxyphenylalanine curve and expressed as µg of verbascoside $g^{-1}$FW.

Ferric reducing antioxidant power (FRAP) determination was performed at $A_{593}$ as described in Rios *et al.* (2008). Calibration was done against a standard curve using freshly prepared ammonium ferrous sulfate (Benzie and Strain, 1996), and the concentration was expressed as µg of ferrous sulfate $g^{-1}$FW.

Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation using the thiobarbituric acid (TBA) method described by Madhava Rao and Sresty (2000); the MDA concentration was calculated by using an $\varepsilon$=155 $mM^{-1}$ $cm^{-1}$ and expressed as µmol of MDA $g^{-1}$FW.

**4. Enzyme determinations**

Assays were carried out on a crude extract of grape extracts. The grapes (2 g $ml^{-1}$) were homogenized at 4 °C in 50 mM phosphate buffer, pH 6.0. The homogenate was filtered and centrifuged at 39000 g for 30 min at 4 °C, the pellet was discarded, and the supernatant was filtered and collected as an enzyme extract. The protein content was determined by method of Bradford (1976).

Polyphenol oxidase activity (PPO, EC 1.14.18.1) was determined as described by Thipyapong *et al.* (1995). Briefly, PPO activity was recorded by measuringas $A_{390}$ at 30 °C, in a reaction medium composed of the enzyme extract, 100 mM phosphate buffer, 0.58 % (v/v) Triton X-100, and 30 µM caffeic acid. A unit of

PPO is defined as the amount of enzyme required to cause a $\Delta A_{390}$ of 0.001 units $min^{-1}$.

Superoxide dismutase activity (SOD, EC 1.15.1.1) was determined as $A_{560}$ in 50 mM phosphate buffer pH 7.8, 0.1 mM EDTA, 1.3 µM riboflavine, 13 mM methionine and 63 µM nitroblue tetrazolium (NBT) (Beauchamp and Fridovich, 1971). A unit of SOD is defined as the amount of enzyme required to cause 50% inhibition of NBT reduction.

Peroxidase activity (POX, EC 1.11.1.7) was measured at $A_{590}$ ($\varepsilon$=47.6 $mM^{-1}$ $cm^{-1}$) (Ngo and Lenhoff, 1980) in a reaction mixture containing 3.3 mM 3-dimethylaminobenzoic acid (DMAB) and 66.6 µM 3-methyl-2-benzothiazolinonhydrazon (MBTH) in 50 mM phosphate buffer pH 6.0. A unit of nonspecific POX is defined as the amount of enzyme required to cause the formation of 1 nmol DMAB-MBTH (indamine dye) per minute at 25 °C, pH 6.0. The coniferyl alcohol peroxidase (CA-POX) activity was recorded by measuring the decrease in absorbance as $A_{265}$ in a reaction medium composed of 0.1 mM CA in 25 mM acetate buffer pH 5.0 ($\varepsilon$=7.5 $mM^{-1}$ $cm^{-1}$). A unit of CA-POX is defined as the amount of enzyme required to cause the oxidation of 1 nmol CA per minute at 25 °C, pH 5.0.

### 5. Statistical analysis

The data presented are the means±SD of at least 10 replicates obtained from three independent experiments. The data obtained were statistically analyzed by the Mann-Whitney U test.

## RESULTS AND DISCUSSION

Three treatments were compared: water restriction (R1), irrigation-no defoliation (R2N) and irrigation-defoliation (R2D). For all treatments, harvesting was performed when the TSS content had reached 23-24 °Brix (i.e., ripening stage III), so that treatments could be compared at similar technological ripeness. As shown in Table 1, R1 displayed the greatest degree of stress, as reflected by its pre- and post-veraison water stress integral values relative to those of the two irrigation treatments (R2N and R2D), between which there were no significant differences. Nevertheless, the leaf area (LA) was not significantly reduced by this water stress but, as expected, was considerably reduced in R2D relative to R2N. Defoliation (R2D) reduced berry weight to a greater extent than water stress (R1) (Table 2). This decrease in berry weight due to water stress is consistent with the findings of Williams and Matthews (1990), who showed that a reduction in vegetative growth results in lower cluster and berry weight, an effect that is enhanced when the water deficit takes place at pre-veraison (Girona *et al.*, 2009), as was the case in the present experiments. The stress caused by defoliation reduced berry weight even more than the pre-veraison water stress. The ripening stages occurred at similar time in R1 and R2N, while in R2D ripening and harvesting was advanced by two weeks.

In order to compare the effects of the different treatments on antioxidant activities and phenolic compounds, we have taken into account the respective ripening stages, and not the corresponding harvest dates. Figure 1A-B shows how R2N and R2D influenced titratable acidity and pH. The monotonic decrease in titratable acidity was substantially less in the berries of the irrigation treatments. The acids that most contribute to titratable acidity are tartaric and malic acids, especially the latter in the case of berries with high pH values (Esteban *et al.*, 2002). During ripening, malic acid is mainly broken down by combustion phenomena (Conde *et al.*, 2007), so that in vines with greater LA index there is less degradation of this acid. The data presented in Table 1, which lists the values found at harvest for these acids, confirm these findings. Similar results have been reported by Intrigliolo and Castell (2008) and Valdés *et al.* (2009).

Others have found decreases in titratable acidity as a result of defoliation (Tardáguila *et al.*, 2008). In the present work, however, berries from R2D presented the highest values of titratable acidity throughout the cycle studied (Table 1). This may be because,

**Table 1 - Agronomic parameters and must quality at harvest in the different treatments**

| Treatment | Int ¥ Pre-veraison (Mpa*day) | Int ¥ Post-veraison (Mpa*day) | Total Leaf Area ($m^2$/vine stock) | Total Soluble Solids (ºBrix) | pH | Titratable Acidity (g $L^{-1}$) | Malic acid (g $L^{-1}$) | Tartaric acid (g $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| R1 | 21.50$^{b}$ | 24.90$^{b}$ | 9.64$^{ab}$ | 24.00$^{a}$ | 3.84$^{b}$ | 4.86$^{a}$ | 1.51$^{a}$ | 4.64$^{a}$ |
| R2N | 13.75$^{a}$ | 10.07$^{a}$ | 11.14$^{b}$ | 23.18$^{a}$ | 3.69$^{ab}$ | 5.20$^{ab}$ | 1.86$^{b}$ | 4.91$^{b}$ |
| R2D | 11.18$^{a}$ | 8.62$^{a}$ | 8.73$^{a}$ | 23.40$^{a}$ | 3.66$^{a}$ | 5.70$^{b}$ | 1.96$^{b}$ | 4.63$^{a}$ |

The data are means of triplicate determinations (n = 15). Differences between treatment means are based on Mann-Whitney U test at $p \leq 0.05$. R1, water restriction; R2N, irrigation-no defoliation; R2D, irrigation-defoliation; Int ¥, water stress integral.

although the total LA was lesssmaller, since defoliation was carried out during flowering, new leaves emerged on the vines so that there was ultimately no alteration in the microclimate affecting the clusters. This is supported by the fact that the malic acid content of these berries was similar to that of the R2N berries. Defoliation did, however, succeed in increasing the amounts of flavonoids and total phenols at harvest (Figure 2).

Figure 2 shows the evolution of various families of phenols with respect to the accumulation of sugars. Total phenolic content, in particular, differed between treatments (Figure 2A). Thus, with the R1 treatment, there was a sharp increase in these compounds to reach stage II, then a decrease until stage IV. In the R2N treatment, the total phenol content remained practically constant at around 22 °Brix (stage II), with the stage IV value coinciding with that observed for the R1 treatment. This lack of accumulation of phenols in this treatment could be because there is hardly any cessation of vegetative growth. and not being subjected to stress. In contrast, R2D induced a gradual

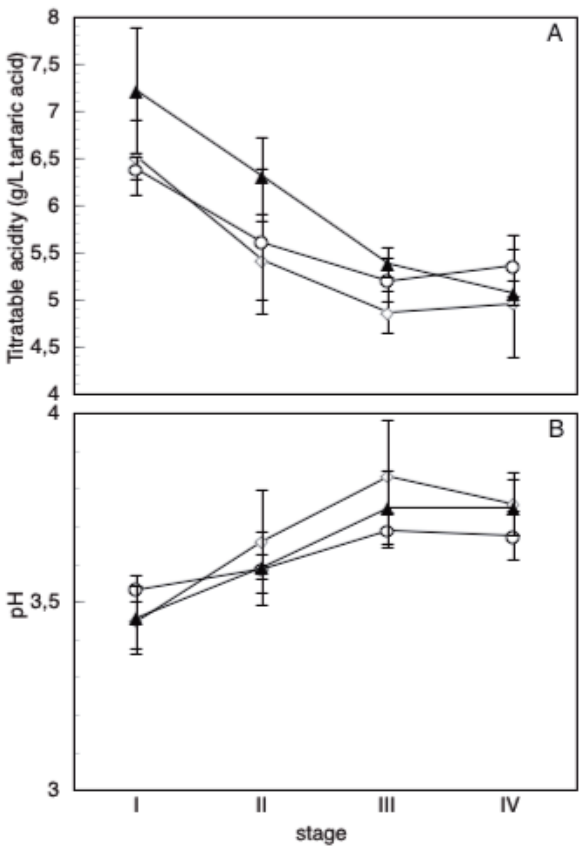


**Figure 1 - Effects of different treatments (-◇- R1, -◯- R2N and -▲- R2D) on titratable acidity (A) and pH (B) in grapes harvested at different stages of ripening. The data are means of triplicate determinations±SD.**

decrease in these compounds up to stage III, followed by an increase at stage IV to a value greater than that of the other treatments. The total flavonoid content showed a similar pattern of behaviour (Figure 2B). The R2D treatment, apart from an initial flavonoid content greater than that of the other treatments, induced a sharp increase in these metabolites at stage IV. The evolution of total phenylpropanoid glycosides was similar, but with more pronounced oscillationslarger amplitudes, and again the R2D treatment led to the highest levels at stage IV (Figure 2C). There is some debate in the literature as to the cause of alteration shift in total phenol content. The accumulation of these compounds may be a response to abiotic stressors such as water deficit or light. Thus, Nadal and Arola (1995) and Dry *et al.* (1999) proposed the hypothesis that the accumulation of anthocyanin-type phenols is a result of water stress. Data from this study are in agreement with those of the cited works. Kennedy *et al.* (2000), however, argued that water deficit does not affect the biosynthesis of these compounds, but rather that their variation in response to water deficit is a simple consequence of reduction in the size of the berries, thus concentrating the flavonoids rather than affecting their biosynthesis (Roby *et al.*, 2004). Nonetheless, even though our results showed decreased berry size in the R1 vines, the evolution of the dry weight to fresh weight ratio in the different treatments was insufficient to explain this greater accumulation under water restriction conditions (Table 2).

Our results showed increases in the contents of all the phenols in response to the altered thermal and light conditions and the source-sink balance caused by defoliation (Pastore *et al.*, 2013). Santesteban *et al.* (2011) found that berry thinning increases phenol concentrations. These compounds are known to be involved in defence processes against both biotic and abiotic stressors (Zhang *et al.*, 2003; Falcone-Ferreyra *et al.*, 2012), which would explain the observed behaviour in R2D compared to the R1 and R2N treatments without defoliation. Flavonoids and phenylpropanoid glycosides are also known to participate in the scavenging of ROS (Williams *et al.*, 2004), in protection against UV light (Zhang *et al.*, 2003), and in the response of ripening berries to light exposure (Downey *et al.*, 2004) and defoliation (Kliewer and Antcliff, 1970; Hunter *et al.*, 1995; Pastore *et al.*, 2013). Pilati *et al.* (2007) have reported the occurrence of an oxidative stress during berry ripening. It should be also taken into account that grapes accumulate many phenylpropanoids that can play an antioxidant role. A similar role could be played by these compounds in R1 and R2D.

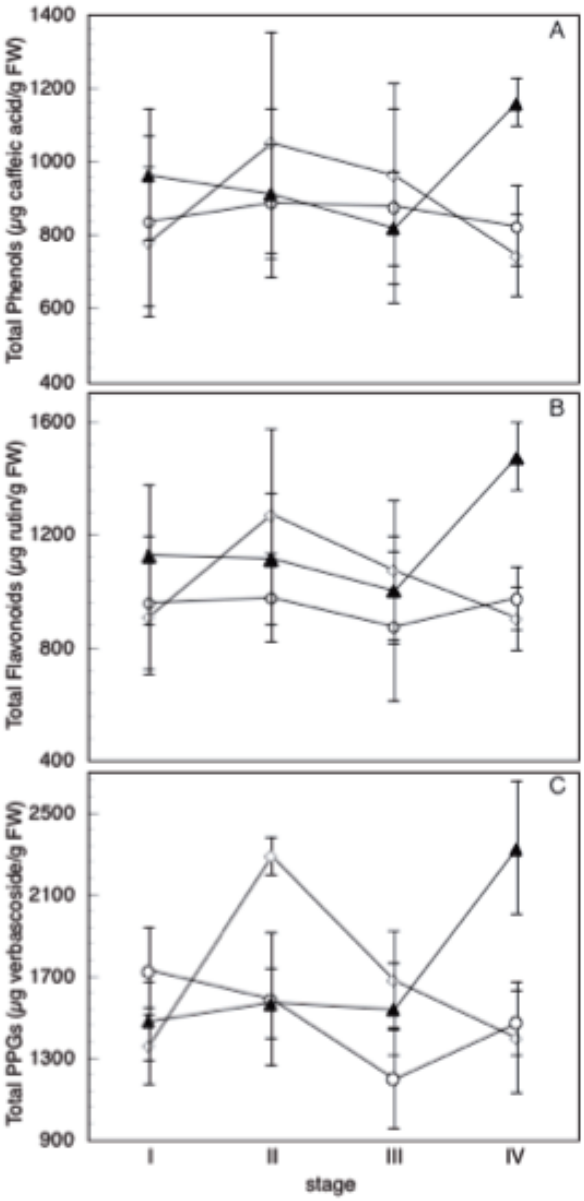


**Figure 2 - Effects of different treatments (-◇- R1, -○- R2N and -▲- R2D) on total phenolic (A), flavonoid (B) and phenylpropanoid glycoside (PPGs) (C) content in grapes harvested at different stages of ripening. The data are means of triplicate determinations±SD.**

We then determined FRAP at different stages of ripening and found that in R1 and R2N, there was a slight increase in FRAP as the berries ripened from stage I to stage II, followed by a subsequent decrease (Figure 3A). Overall, the general trend in these two treatments was thisa reducing capacity to decreasereduction in the antioxidant capacity. In contrast, R2D showed a clear and strong increase in FRAP with increasing ripeness, up to a final value much higher than with the other treatments. The

**Table 2 - Effect of different treatments and dates of berry harvest on berry weight, dry weight to fresh weight ratio (DW/FW), total soluble solids (TSS), total phenolics (TP), total flavonoids (TF), total phenylpropanoid glycosides (TPPGs), antioxidant capacity (FRAP), lipid peroxidation (MDA), and polyphenol oxidase (PPO), superoxide dismutase (SOD), conyferil alcohol peroxidase (CA-POX) and nonspecific peroxidase (POX) activities.**

| Days from veraison/ sampling date | Treatment | Berry weight (g) | DW/FW | TSS (°Brix) | TP (µg/g FW) | TF (µg/mg DW) | TPPGs (µg/mg DW) | FRAP (nmol/mg DW) | MDA (µg/mg DW) | PPO (U/mg prot) | SOD (U/mg prot) | CA-POX (U/mg prot) | POX (U/mg prot) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R1 | 1.087$^{b}$ | 0.105$^{a}$ | 18.05$^{a}$ | 918.52$^{ab}$ | 1005.88$^{b}$ | 1733.41$^{b}$ | 12.01$^{a}$ | 75.00$^{b}$ | 1270.87$^{b}$ | 100.32$^{a}$ | 176.10$^{a}$ | 4.39$^{b}$ |
| 7 days/08-07-2011 | R2N | 1.103$^{b}$ | 0.112$^{a}$ | 18.48$^{a}$ | 823.82$^{a}$ | 803.18$^{a}$ | 1469.52$^{a}$ | 12.04$^{a}$ | 60.97$^{a}$ | 998.95$^{a}$ | 146.50$^{b}$ | 223.75$^{b}$ | 4.14$^{ab}$ |
| | R2D | 0.943$^{a}$ | 0.125$^{b}$ | 20.63$^{b}$ | 964.16$^{b}$ | 1129.12$^{c}$ | 1781.02$^{b}$ | 14.19$^{b}$ | 66.53$^{a}$ | 948.07$^{a}$ | 131.12$^{b}$ | 195.23$^{a}$ | 3.65$^{a}$ |
| | R1 | 0.908$^{a}$ | 0.099$^{a}$ | 20.53$^{a}$ | 763.29$^{a}$ | 930.38$^{a}$ | 1540.25$^{a}$ | 10.87$^{a}$ | 45.00$^{a}$ | 533.82$^{a}$ | 120.40$^{c}$ | 201.91$^{b}$ | 2.15$^{c}$ |
| 21 days/08-21-2011 | R2N | 1.064$^{a}$ | 0.108$^{a}$ | 20.66$^{a}$ | 837.37$^{b}$ | 960.00$^{a}$ | 1657.38$^{a}$ | 12.03$^{a}$ | 61.29$^{b}$ | 479.56$^{a}$ | 40.89$^{a}$ | 109.09$^{a}$ | 0.96$^{b}$ |
| | R2D | 0.878$^{a}$ | 0.135$^{b}$ | 23.86$^{b}$ | 820.40$^{b}$ | 1004.92$^{a}$ | 1538.75$^{a}$ | 18.17$^{b}$ | 73.35$^{c}$ | 1446.11$^{b}$ | 101.21$^{b}$ | 190.64$^{b}$ | 0.30$^{a}$ |
| | R1 | 0.947$^{a}$ | 0.138$^{a}$ | 23.37$^{a}$ | 964.74$^{b}$ | 889.33$^{b}$ | 1488.49$^{a}$ | 11.79$^{a}$ | 44.24$^{a}$ | 2879.25$^{b}$ | 144.94$^{a}$ | 162.97$^{a}$ | 6.22$^{b}$ |
| 35 days/09-04-2011 | R2N | 1.086$^{a}$ | 0.129$^{a}$ | 23.58$^{a}$ | 877.37$^{a}$ | 746.83$^{a}$ | 1416.28$^{a}$ | 11.38$^{a}$ | 70.04$^{b}$ | 1944.53$^{a}$ | 118.18$^{a}$ | 157.59$^{a}$ | 2.75$^{a}$ |
| | R2D* | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- | ----- |

The data are means of triplicate determinations (n = 15). Differences between treatment means are based on Mann-Whitney U test at $p \leq 0.05$. (*)R2D advances ripening and harvesting by two weeks (08-21-2011).

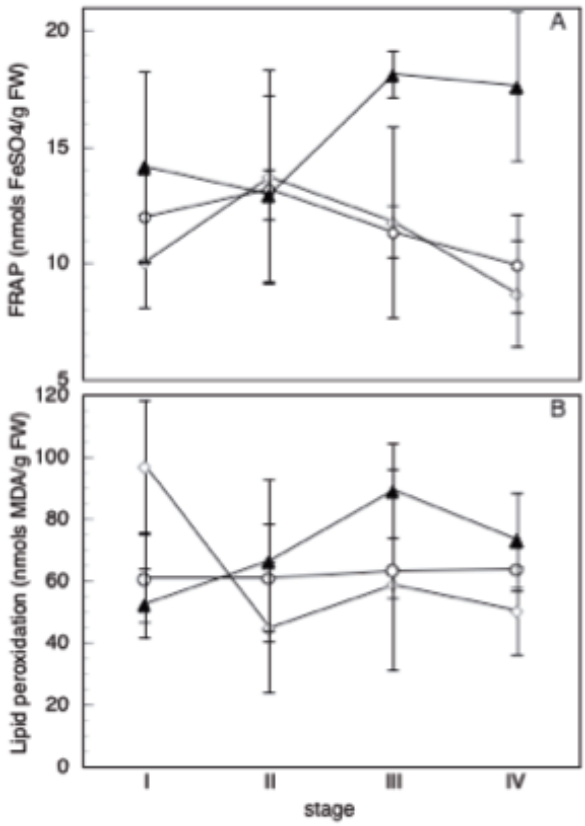


**Figure 3 - Effects of different treatments (-◇- R1, -○- R2N and -▲- R2D) on ferric-reducing antioxidant power (FRAP) (A) and lipid peroxidation (B) in grapes harvested at different stages of ripening. The data are means of triplicate determinations±SD.**

decrease in FRAP in R1 and R2N could be due to the accumulation of water in the berries in these final stages, as evidenced by the increase in their fresh weight (Table 2). This accumulation of water could enhance the hydrolysis of high molecular weight phenols (Cimato *et al.*, 1990). In contrast, the maintenance of high FRAP values in R2D would be consistent with the lower accumulation of water in the berries, as reflected in their lower fresh weight, with the consequent lower level of hydrolysis of high molecular weight phenols. Thus, this treatmentthe defoliation treatment gave the highest levels of total phenols, flavonoids, and phenylpropanoid glycosides at post-harvest. These results are similar to those described by Doshi *et al.* (2006) and Cao *et al.* (1997), who found a direct correlation between total phenol levels and FRAP values.

Membrane lipid peroxidation, as measured by the MDA content, was practically constant from stage I to IV in R2N (Figure 3B). The other two treatments presented different patterns. Thus, the R1 treatment presented very high peroxidation levels at stage I, which decreased at stage II and remained low up to stage IV(as seen in Figure 3B). In contrast, the R2D treatment presented a steady increase until stage III, followed by a slight decrease up to stage IV, but still maintained the highest levels of lipid peroxidation of all treatments, indicative of greater stress.

Phenolic compounds are thought to be accumulated in the cell vacuoles and are mostly derived from the shikimate and phenylpropanoid pathways (Gomez *et al.*, 2009). Under the conditions of our study, none of the treatment had a considerable effect on fruit phenolic content at the time of harvest (stage III), although both R1 and R2D increased flavonoid and phenylpropanoid glycoside contents. This indicates an accumulation of antioxidant compounds in response to stresses. In post-harvest berries, R2D increased total phenol, flavonoid and phenylpropanoid glycoside contents and FRAP, but not lipid peroxidation.

Figure 4 shows the behaviour of a series of enzymatic activities associated with oxidative and antioxidative processes. The PPO activity increased during ripening in the R2N and R1 treatments, while this activity was very much lower in R2D (Figure 4A). When the berries reached stage II, R2N and R1 gave values much higher than those obtained for R2D. At stage IV, the value for this last treatment was much lower than for R1 berries. The reduction in PPO activity caused by defoliation is especially important œnologically since it implies less exposure of berry phenolics to oxidation, a process that causes a loss of quality due to the resulting polymerizations and colour changes of the wines to tones of orange. This could explain the results obtained by Tardáguila *et al.* (2012) showing that defoliation leads to more coloured wines. Furthermore, a direct technological consequence is that lower doses of antioxidants (mainly sulfites) will be required for berries from plants subjected to defoliation.

We also studied three antioxidant systems involved in the scavenging of hydrogen peroxide, which otherwise may later affect the quality of wines. These were SOD, CA-POX, and nonspecific POX. Figure 4B shows the SOD activity for the different treatments. Plant cells actively produce ROS at low levels, but many stresses that disrupt cell homeostasis enhance ROS production. SOD dismutates the $O_2^-$ produced during oxidative stress to $H_2O_2$. The R1 treatment maintained high levels of SOD activity throughout ripening, with no major changes from stage I to stage IV. R2N is the only treatment that gave very low initial levels, but these increased in the final

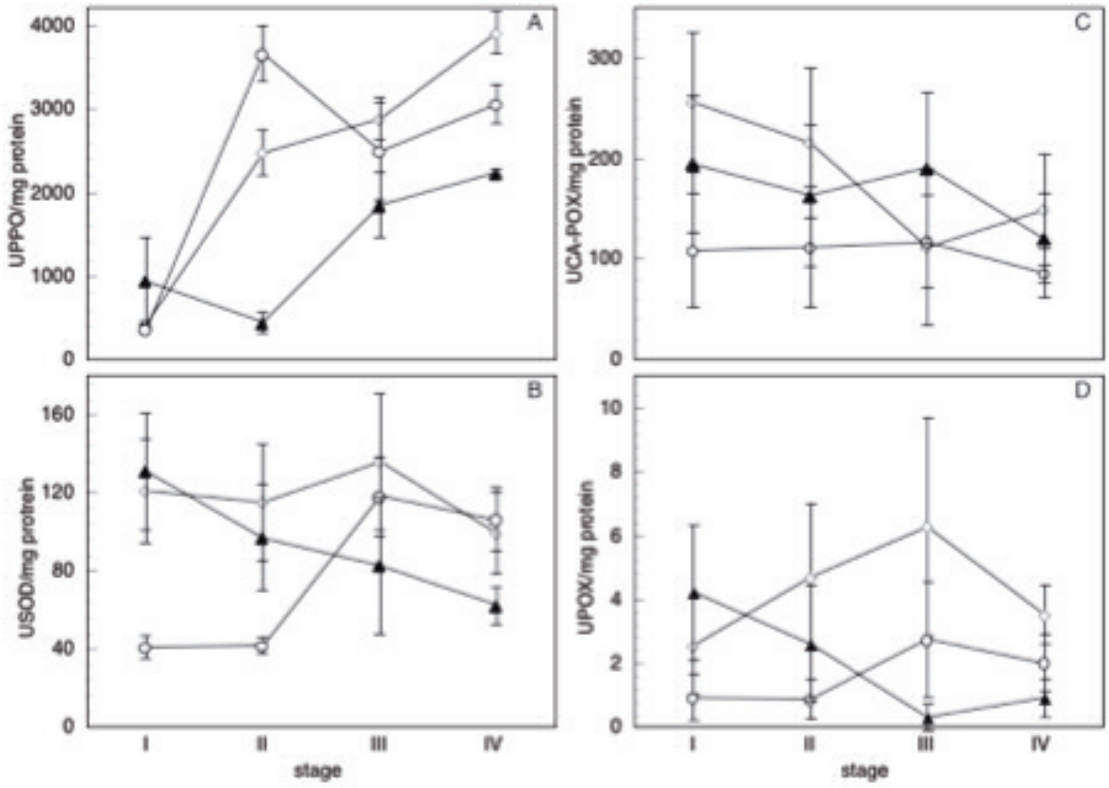


**Figure 4 - Effects of different treatments (-◇- R1, -○- R2N and -▲- R2D) on antioxidant activities (expressed as units per mg of protein): polyphenol oxidase (PPO) (A), superoxide dismutase (SOD) (B), coniferyl alcohol peroxidase (CA-POX) (C) and nonspecific POX (D) activities in grapes harvested at different stages of ripening. The data are means of triplicate determinations±SD.**

phase of ripening, especially from stage II onwards, to reach stage IV activity levels similar to those of R1. However, R2D led to a different SOD activity behaviour, with initially high levels declining with ripening to post-harvest levels far below those of the other treatments. Yildirim *et al.* (2003) observed a decrease in SOD activity and an increase in lipid peroxidation from veraison to ripening, which was attributed to increased oxidative activity as a result of the ripening process. Here, a similar behaviour was observed in R2D (except for stage IV) as a result of defoliation. However, this did not occur in R2N due to the strong increase in SOD activity observed at the end of the ripening process, contrary to what was observed by Yildirim *et al.* (2003), thus offsetting the increase of ROS and preventing oxidative damage.

The CA-POX activity, which is involved in cell wall lignification processes, stayed almost constant throughout ripening in the R2N treatment (Figure 4C). In R1, the initial levels (stage I) were 2.45-fold higher compared with irrigated berries. As ripening advanced, there was a decrease in this activity up to stage III and then a slight increase at stage IV to reach values just slightly higher than those of the irrigated berries. For R2D, the initial values were intermediate between those of the other two treatments (possibly indicative of a response to the stress induced by defoliation), followed by a behaviour similar to that of the R2N berries. After harvest, the CA-POX activity in the R2D berries was intermediate. The behaviour of the CA-POX activity during ripening in stressed berrries in berries under some kind of stress (water restriction in R1 growing, defoliation) indicates that this activity may be related to the levels of of lignin polymerization from monolognolspolymerization of monolignols to form lignin. Under stress, this activity would strengthen the cell walls, which may explain the initially high levels relative to the berries from the R2N (unstressed) treatment. At the end of ripening, when further lignification is not required, the different treatments presented very similar activities.

The nonspecific POX activity of R1 berries increased with ripening, and eventually decreased after harvest (Figure 4D). In contrast, the R2N berries presented low initial POX levels, although these increased in the final phase of ripening to values similar to R2D and lower than R1, which may be indicative of a lesser degree of stress. Again, defoliation led to different

behaviour, with initially very high values that decreased steadily with ripening to reach the lowest POX activity of all treatments at both stage III and stage IV. The high initial values could be a response to the immediate stress caused by defoliation, but then this treatment presented very low levels of POX activity similar to those of the other two treatments, in agreement with the results reported by Okuda and Yokotsuka (1999) and López *et al.* (2010). By contrast, Arnnok *et al.* (2010) observed an increase in both PPO and POX during ripening. Our results indicate an increase in PPO but not in POX.

In R2D, the SOD and nonspecific POX activities were low at stage III, suggesting that ROS could have accumulated and caused lipid peroxidation in this treatment. R1 induced lipid peroxidation during ripening (stage I), but the increase in SOD and nonspecific POX activities may have kept lipid peroxidation at values similar to R2N at stage III.

The POX activities changed from stage I to stage III, but with opposite behaviour in both peroxidases. At stage I, nonspecific POX showed a high levels of activity in R1 and R2D. However, at stage III R1 showed a maximum and R2D a minimum value with respect to the R2N control. On the contrary, CA-POX showed initially high values, dropping in R1 (similar to R2N) but not in R2D. At stage I (7 days from veraison), higher values were observed for R1 and R2D, which could be related to stress. During ripening there is an increase in cell volume without wall synthesis, and the cell wall becomes in fact thinner at the end of ripening. The decrease in CA-POX activity during ripening would be related to softening of the grapes.

At stages III and IV, only the R2D treatment significantly affected PPO, SOD and nonspecific POX activities, decreasing them, so that the final content of these grapes contains less oxidized polyphenols.

The evolution of phenolic compounds during ripening showed that the stress caused by water restriction induced an increase in the accumulation of these compounds (stage II; see Figure 2), hence reducing and reduced lipid peroxidation (see Figure 3). In more avanced stages of ripening, the levels of these compounds were similar to R2N. Defoliation altered these levels from stage III to stage IV, thereby showing higher antioxidant capacity.

## CONCLUSION

Water restriction led to alterations in the levels of peroxidation and peroxidase activity, but only in the first weeks after veraison since the post-harvest levels were similar to those of the unstressed R2N treatment. Defoliation led to changes relative to the other two treatments. In particular, at stage IV it led to higher contents of total phenols, flavonoids and phenylpropanoid glycosides which were also reflected in a greater total antioxidative capacity. Defoliation also caused lower PPO and SOD activities, which is important œnologically since it could lead to lower levels of phenol oxidation, thus preventing discolouration of the wines. Further studies are needed to analyze the impact of these farming practices on phenolic compounds and oxidant and antioxidant activities in order to improve wine quality.

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