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PHYSIOLOGICAL AND AGRONOMICAL RESPONSES TO ENVIRONMENTAL FLUCTUATIONS OF TWO PORTUGUESE GRAPEVINE VARIETIES DURING THREE FIELD SEASONS

RESPOSTAS AGRONÓMICAS E FISIOLÓGICAS DE DUAS VARIEDADES PORTUGUESAS DE VIDEIRA A FLUTUAÇÕES AMBIENTAIS EM TRÊS ÉPOCAS DE PRODUÇÃO

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SUMMARY

Extensive agricultural losses are attributed to heat, often combined with drought. These abiotic stresses occur in the field simultaneously, namely in areas with Mediterranean climate, where grapevine traditionally grows. The available scenarios for climate change suggest an increase in the frequency of heat waves and severe drought events in summer, also affecting the South of Portugal. In this work we monitored several production-related parameters and evaluated the state of the oxidative stress response apparatus of two grapevine varieties, Touriga Nacional (TN) and Trincadeira (TR), with and without irrigation, during three field seasons (2010 to 2012). Overall, results point to a high correlation of most yield and stress-associated parameters with the specific characteristics of each variety and to each season rather than the irrigation treatments. In the season with the driest winter, 2012, the lack of irrigation significantly affected yield in TR, while in the two other seasons the impact of the irrigation regime was much lower. In 2012, the yield of TN was affected by environmental conditions of the previous season. The irrigation treatments significantly affected berry size rather than quality.

RESUMO

Elevadas perdas agrícolas são atribuídas ao calor, frequentemente combinado com seca. Esses stresses abióticos ocorrem simultaneamente no campo, nomeadamente em áreas com clima mediterrâneo, onde tradicionalmente se cultiva a videira. Os possíveis cenários de alterações climáticas apontam para um aumento na frequência de ondas de calor e eventos de seca prolongada no verão, afetando também o Sul de Portugal. Neste trabalho foram monitorizados vários parâmetros relacionados com a produção, e a resposta ao stresse oxidativo foi avaliada em duas variedades de videira, Touriga Nacional (TN) e Trincadeira (TR), com e sem irrigação, durante três épocas sucessivas (2010 a 2012). Em geral, os resultados obtidos apontam para uma elevada correlação da maioria dos parâmetros associados ao stresse e ao rendimento com as características específicas de cada variedade e com as condições ambientais de cada ano e não directamente com os tratamentos de irrigação. No ano cujo inverno foi mais seco, 2012, a falta de irrigação afetou significativamente o rendimento em TR, enquanto nos dois outros anos o impacto do regime de irrigação foi muito menor. Em 2012, o rendimento de TN foi afetado pelas condições ambientais da temporada anterior. Os tratamentos de irrigação não afetaram significativamente a qualidade da uva, apenas o seu tamanho.

Key words: ABA, ascorbate, berry quality, glutathione, irrigation, yield.
Palavras-chave: ABA, ascorbato, glutationa, irrigação, qualidade da uva, rendimento.

INTRODUCTION

As sessile organisms, plants are constantly exposed to changes in the surrounding environment. Temperatures above the normal optimum are sensed as heat stress by living organisms. Heat stress disturbs cellular homeostasis and can lead to severe impairment in growth and development, and even to death. Worldwide, extensive agricultural losses are attributed to heat, often in combination with drought or other stresses. In fact, drought and heat represent an excellent example of two different abiotic stresses often occurring simultaneously (Knight and Knight, 2001), namely in regions of long-time grapevine production, such as the Mediterranean surrounding areas. Furthermore, the available scenarios for climate

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change over the pending decades suggest an increase in aridity and shifts in the amount, seasonality and distribution of precipitation, affecting the Mediterranean region (Pinto *et al.*, 2011). The predicted increase in the frequency of summer heat waves and the simultaneous increase of the duration of the dry season will lead to extended and severe drought events, with a concomitant overexploitation of water resources for agriculture purposes, increasing limitations to plant growth and fruit development and therefore to fulfilling their potential yields (Chaves, 2002; Chaves *et al.*, 2003). Furthermore, it is expected that, as early as 2040, species such as grapevine will have moved their distribution northward and uphill, leading to changes in plant phenology, anticipating flowering and ripening (Fraga *et al.*, 2016; Ramos *et al.,* 2015).

The importance of water supplement in wine grapes was first studied in Portugal *ca.* twenty years ago (Lopes, 1994). In the future it will ensure the long-term sustainability of viticulture, with grapevine plants showing up normal physiological activity and an adequate balance between vegetative and reproductive development, while preserving yield quality (Rodrigues *et al.*, 2008). Vines with no supplemental water or under deficit irrigation have less vegetative growth, smaller berries and lower yields than those with high water availability (Williams *et al.*, 2009). However, grapevine irrigation, unlike in other crops, should usually be sub-optimal, to avoid a decrease in quality (Gaudillère *et al.*, 2002).

The many abiotic stresses that significantly limit the distribution of grapes around the world and reduce crop yield, as is the case of water deficit, can also be used in a positive way to enhance berry flavor and quality characteristics (Chapman *et al.*, 2005). Deficit irrigation with moderate water stress is in fact associated with increased fruit quality, especially for varieties used in wine production (Williams *et al.*, 2009). The resulting reduced shoot vigor, competition for scarce carbon resources, reduced berry size, concentrating flavors and color will improve berry quality (Castellarin *et al.*, 2007; Deluc *et al.*, 2009). In fact, in red wine grapes, the occurrence of some water deficit during the growing season has been interpreted as beneficial for wine quality, leading to wines with more fruity and less vegetal aromas and flavors than vines with higher water status, that show a tendency for vegetal aromas, pepper flavor and astringency.

Typically, crop productivity is dependent on photoassimilates produced at the whole plant level. The decline in stomatal aperture normally observed under drought is accompanied by an adjustment of leaf area at the whole plant level, either through the inhibition of new leaf growth or through the earlier senescence of older leaves, in the case of prolonged stress. This causes a decrease of area available for transpiration but also to lower intercepted radiation throughout the growing season with consequences in biomass production (Pereira and Chaves, 1993). This decrease in foliage can also lead to excessive fruit exposure to sunlight (Pellegrino *et al.*, 2005) with undesirable effects on berry production (sunburn). The plant tries to avoid dehydration and excessive irradiation by changes in the leaf angle, aiming at smaller angles, which protect against excess solar energy but also diminish carbon assimilation (Pinheiro and Chaves, 2011).

To study the response of the plant to abiotic stress, it is relevant to approach it under field conditions instead of growth-rooms since the most important feature of grapevine production lies in berry production and quality and even phenotypic characteristics are altered (Mishra *et al.,* 2012). Thus, the study of stresses in an artificial environment can elucidate important mechanisms of resistance (Carvalho *et al.*, 2016), but one must always consider the whole environment that a plant grows in to fully comprehend the 'stress resistance' mechanisms build up in the field. In this work we monitored the phenotypical evolution together with several production-related parameters and the status of the oxidative stress response machinery in two Portuguese grapevine varieties, Touriga Nacional (TN) and Trincadeira (TR), with and without irrigation, during three field seasons (2010 to 2012) and integrated the results obtained with the meteorological data registered in the field region. These varieties were chosen for their contrasting responses to abiotic stress, measured in controlled conditions (Carvalho *et al.*, 2015, 2016), that characterized TN as tolerant to stress and adapted to withstand heat waves and combinations of several abiotic stresses while TR is more sensitive and its growth is impaired under a single abiotic stress. The choice of extreme irrigation methods also allowed the clear discrimination of the response behavior of both varieties upon water scarcity and abundance.

MATERIAL AND METHODS

Location and climate

The experiments were conducted at a research vineyard (Centro Experimental de Pegões) located in Pegões (38º40'N; 8º36'W), 70 km east of Lisbon during three seasons (2010 to 2012). The climate is

Mediterranean, with hot, dry summers and mild air temperatures with precipitation concentrated during autumn and winter, although the climate in Pegões is affected by its proximity to the sea and the mountain of Arrábida, which causes low temperatures in the night period. When comparing the 3 years, 2010 was found to be the hottest with maximum and minimum summer temperatures *ca.* 3 ºC higher than those measured in the following years (Figure 1). The year 2012 was characterized by lower precipitation in the winter than the previous years and in 2011, spring and fall temperatures were higher and summer temperatures lower than in the other two years (Figure 1). All these factors may have contributed to a less severe summer drought stress experienced by plants in 2011. Irradiation was measured throughout the seasons using a radiation sensor (LI-250 Light Meter, Li-Cor, Lincoln Nebraska, USA).

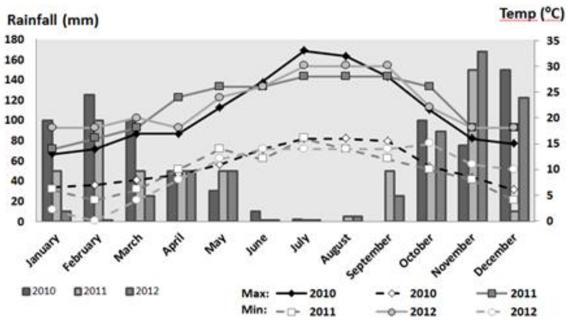

Figure 1. Maximal and minimal monthly temperature and average monthly rainfall in the three seasons monitored (2010 to 2012).

Temperaturas mensais máximas e mínimas e precipitação média mensal nas três épocas de crescimento estudadas (2010 a 2012).

The soil is derived from podzols, with a sandy surface layer and a clay rich (low permeability) horizon at a depth of *ca* 1 m. A mixture of clones resulting from polyclonal selection (certified material with a "standard" designation) of Touriga Nacional and Trincadeira were established in the vineyard. Both were grafted on 1103 Paulsen rootstock in 2002. The plants are spaced 2.5 m between rows and 1 m within rows, resulting in a density of 4000 plants ha^{-1}, and trained on vertical trellises each with a pair of movable foliage wires for upward shoot positioning.

Stress treatments in the Field

Irrigation water was applied with drip emitters, two per vine, positioned 30 cm from the vine trunk (32 L per vine twice a week for FI; Lopes *et al.,* 2011). The water was supplied according to the crop evapotranspiration (ETc), corresponding to 100% ETc (in FI). ETc was estimated from ETo, using the crop coefficients (Kc) proposed by Allen *et al.* (1998). The two irrigation treatments were fully irrigated (FI) and non-irrigated (NI). Six plants in each treatment were pre-selected for yield analysis.

Samples for the quantification of abscisic acid (ABA), hydrogen peroxide (H_2O_2), pigments, ascorbate and glutathione were taken on the 5th August 2010, 18th August 2011 and 23rd August 2012, corresponding to the phenological state 35 (numeric scale of Eichhorn and Lorenz, 1977) and to the values of pre-dawn leaf water potentials (measured with a pressure chamber, Model 600, PMS Instruments Company, Albany, OR) shown on Table III. FI plants were sampled immediately before irrigation. The 3rd, 4th and 5th fully expanded leaves from three individual plants were pooled and frozen in liquid N_2. Four biological replicates from each variety were made.

Soil water content

Volumetric soil water content measurements were conducted with a Diviner 2000 probe (Sentek, Sensor Technologies) placed inside access tubes vertically positioned in the soil at 1 m depth. Quantification of the Fraction of Transpirable Soil Water (FTSW) was made according to the formula FTSW = (HVaccumulated - HVmin)/(HVseason_max – HVseason_min) (Pellegrino *et al.,* 2005). Values of the 30 Year Climate Normal were obtained with the software 'Meu Clima & Solo' developed by Prof. J.P. Melo e Abreu and available at http://home.isa.utl.pt/~jpabreu/downloads.html.

Meteorological measurements

Meteorological data from the area surrounding Pegões was supplied by the "Associação de Viticultores de Palmela" (AVIPE) in 2010 and in the following years was retrieved from Instituto Português do Mar e da Atmosfera (IPMA), at https://www.ipma.pt/pt/oclima/monitorizacao/.

Phenological analysis

The phenological state of the plants was analysed before the start of the irrigation and at regular intervals until harvest. The classification used was according to Baggiolini (1952), later transformed to the numeric scale of Eichhorn and Lorenz (1977) for an easier statistical comparison of the data.

Grapevine yield and vigor

At harvest, grape clusters were counted and weighted. Clusters were separated into clusters from count nodes and clusters from non-count nodes. The incidence of *Botritis* and sunburn was also quantified. Winter pruning was performed in December and all the pruned wood of each plant was weighted and separated into wood from the count and non-count nodes. The crop load calculation, in the form of the

Ravaz index (Yield/Pruning Weight), where the yield from the current harvest is used against the pruning weight in the following dormant season, was also calculated.

Berry analysis

Once harvested and split into groups, determined by treatment and variety, the berries, with their petioles detached, were immediately taken to the laboratory to undergo berry analysis. Triplicate samples of 100 berries each were assessed for berry weight, must volume, pH, total acidity (g/L) and total solids (Brix Index). pH was measured by using a calibrated pH meter, with the must at room temperature. Total acidity was measured through the titratable method with sodium hydroxide, and expressed in terms of tartaric acid.

H_2O_2 quantification

H_2O_2 production was detected using a fluorometric horseradish peroxidase (HRP) linked assay (Amplex Red assay kit, Invitrogen). Leaf material (0.1 g) was collected at each time point and ground over activated charcoal in the presence of liquid N_2 as described by Creissen *et al.* (1999). Samples were centrifuged 10 min at maximum speed and the supernatants were kept on ice until measurements. H_2O_2 concentrations in purified extracts were determined according to the manufacturer's instructions. Absorbance was then measured with a microplate reader at 570 nm. H_2O_2 concentrations were expressed in µmol/g fresh weight.

Pigment quantification

Chlorophyll, carotenoids and anthocyanins were extracted from four leaf disks (total area 113 mm^2). Disks were incubated in 1 mL acetone in Tris-HCl 100mM buffer pH 7.5 (80:20) and centrifuged at 12000 *g* 15 min. The supernatants were used for quantification. Absorbance was measured at 537, 647 and 663 nm for chlorophylls and anthocyanins and at 470 nm for carotenoids. Pigment concentrations were calculated using the equations described by Sims and Gamon (2002): Chl*a* (µmol/mL) = 0,01373xA663 - 0,000897xA_{537} – 0,003046xA_{647}; Chl*b* (µmol/mL) = 0,02405xA_{647} - 0,004305xA_{537} – 0,005507xA_{663}; tot Chl (µmol/mL) = Chl*a* + Chl*b*, carotenoids (µmol/mL) = (A_{470} – 17,1xtot Chl – 9,479xanthocyanins) / 119,26; anthocyanins (µmol/mL) = 0,08173xA_{537} - 0,00697xA_{647} – 0,002228xA_{663}. Values were converted to mg/cm^2 leaf area (Richardson *et al.*, 2002).

Abscisic acid

The extracts for ABA quantification were carried out as described by Vilela *et al.* (2007). ABA was quantified through immunoassay by indirect enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies, using a commercial kit (Olchemim Enzyme Immunoassay, Olomouc, Czech Republic), according to the manufacturers recommendations.

Antioxidative metabolite quantification

Reduced and oxidized glutathione and ascorbate concentrations were determined in leaves collected at the end of each stress treatment. Leaf material (0.5 g) was frozen in liquid N_2. Each sample was homogenised in 5 mL of ice-cold 6% meta-phosphoric acid (pH 2.8), containing 1 mM EDTA, in the presence of liquid N_2. Homogenates were centrifuged at 27 000 *g* for 15 min at 4 ºC and the resulting acid extract was stored at –80 ºC.

Reduced (GSH) and oxidised (GSSG) glutathione were analysed colorimetrically by the 2-vinylpiridine method described by Anderson *et al.* (1995). GSH and GSSG concentrations were expressed in µmol/g fresh weight. Percentage of reduction corresponds to the percentage of total glutathione pool present as GSH and is defined as GSH/ (GSH + GSSG) x 100.

Ascorbic (AsA) and dehydroascorbic (DAsA) acids were assayed using a method adapted from Okamura (1980). To determine AsA and total ascorbate, 125 µL of the acid extract was neutralized with 25 µL of 1.5 M triethanolamine. After thorough mixing, 150 µL of 150 mM sodium phosphate buffer pH 7.4 were added. For the quantification of total ascorbate 75 µL of 10 mM DTT were added. This was followed by 15 min incubation at 25 ºC to reduce the DAsA present in the extract. To remove excess DTT, 75 µL of 0.5% (w/v) N-ethylmaleimide were added. The samples were then mixed and incubated 30 s at 25 ºC. For the quantification of AsA, water was added instead, so that the volumes of both samples were equal. To both samples the following reagents were added successively: 300 µL of 10% (w/v) trichloroacetic acid, 300 µL of 44% (v/v) phosphoric acid, 300 µL of 4% (w/v) 2,2'-dipyridyl in 70% ethanol and 150 µL of 3% (w/v) $FeCl_3$. After mixing, the samples were incubated for 1 h at 37 ºC. Absorbance was recorded at 525 nm. The concentration of DAsA was calculated by subtracting the AsA concentration measured from the total ascorbate determined. Standard curves of AsA in the range of 10-60 µM were prepared in 5% metaphosphoric acid.

Statistical analysis

To study the influence of the water regime on indicators of production, for each variety and each season a completely randomized design with two replicates (each with three plants) was adopted. For data analysis the mean of the three plants of each plot was considered and the model included the effects of the water regime factor (two levels, FI and NI), the effects of the season factor (three levels, 2010, 2011 and 2012) and the respective interaction. ANOVAs were performed to assess the effect of the water regime and the seasons and all analyses were done separately per variety. The effects of the season, water regime and respective interaction were considered significant when the P-value of the test to the respective effects was lower than 0.05. Additionally, a Tukey test was performed to compare the means of each combination season/water regime and statistically significant differences were accepted for a p-value lower than 0.05. For several pairs of possible agronomically-related parameters, Pearson correlations were also performed. ANOVAs, the post-hoc tests and the Pearson correlations were made in R (version 2.15.1 Copyright (C) 2012 The R Foundation for Statistical Computing).

RESULTS AND DISCUSSION

Weather conditions and soil water status in the three seasons

In 2010 there was abundant rainfall in the winter and it rained until June and the maximum summer temperatures were the highest of the three seasons (33 ºC in August, Figure 1). The season of 2011, although without rain from June to August, had high levels of rainfall in the spring (Figure 1), allowing the soil water profile to be fully refilled in May (Figure 2) and also had the mildest summer temperatures (average maximum temperature from July to September was 28 ºC, Figure 1). In fact, maximum temperatures in July and August of 2011 were below the 30 year average. The season with the lowest amount of rainfall was 2012, with 137 mm of rainfall until June, less than half the quantity of the two previous years. Comparing these data with the 30 year average, it is possible to note that, in fact, rainfall in 2012 was below the average expected for the area. Monitoring the soil water content through the quantification of the Fraction of Transpirable Soil Water (FTSW) in the 0-1.0 m soil profile during the growth season allowed to clearly separate the well watered treatment (FI) from the non-irrigated treatment (NI) in the field (Figure 2) in 2010 and 2012.

In a climate with Mediterranean characteristics, although with Atlantic influence, the most prominent individual abiotic stress in non-irrigated crops is drought. This was the case in the three studied seasons, particularly from June to August, where rainfall was almost absent. The maximum temperatures did not exceed 33ºC (July 2010) and light intensity reaching the canopy was, in average, 2500 µmol quanta $m^{-2}s^{-1}$. The three seasons monitored were, overall, typical representatives of the Mediterranean weather, with some specificities that made each unique, and thus the majority of the parameters quantified was more significantly affected by the season than by the irrigation treatments. In these three seasons it was possible to verify the different strategies of response to abiotic stress already analyzed in previous greenhouse experiments with the two varieties under study, namely a rapid and efficient reaction of TN, that was able to boost the buffering capacity of the cell's redox pool upon heat stress while TR was more intensely affected by stress for a longer period of time (Carvalho *et al.,* 2015).

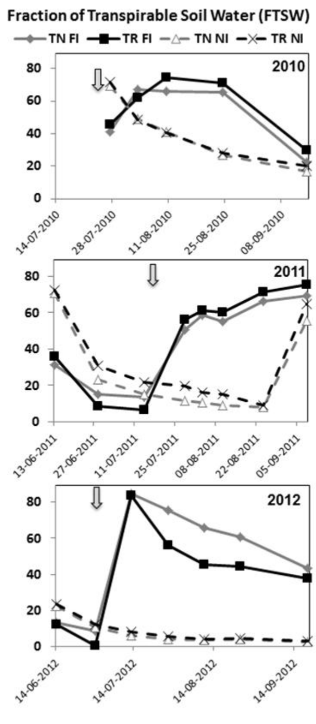

Figure 2. Fraction of Transpirable Soil Water (FTSW) in the three seasons monitored in the soil of Touriga Nacional (TN) and Trincadeira (TR), in the non-irrigated (NI) and irrigated (FI) treatments. Vertical arrows indicate the beginning of irrigation in each season.

Fração de água Transpirável (FTSW) nas três épocas de crescimento estudadas no solo de Touriga Nacional (TN) e Trincadeira (TR), nos tratamentos não-irrigado (NI) e totalmente irrigado (FI). Setas verticais indicam o início da rega em cada época.

Furthermore TN showed a greater ability to withstand a combination of two or three abiotic stresses simultaneously when compared to TR (Carvalho *et al.*, 2016).

Yield and vigor

The duration of the main phenological stages was unaffected by the irrigation regimes during the three growing seasons (Figure 3). Yield, however, experienced changes with the water regime, TR showing a significantly larger range of yield and number of clusters than TN, varying between 0.38 and 7.43 kg per vine and 4.7 and 19.7 clusters per vine (Table I).

Table I

Effect of season (2010 to 2012) and irrigation treatment (FI/NI) on indicators of production measured in Touriga Nacional (TN) and Trincadeira (TR)

Efeito da época (2010 a 2012) e do tratamento de irrigação (FI/NI) sobre indicadores de produção medidos em Touriga Nacional (TN) e Trincadeira (TR)

Season	Water regime	Yield (kg/vine)	Number of clusters	% Sunburn incidence	% *Botrytis* incidence	% Clusters in non count nodes	Harvest weight (kg)	% Wood in non count nodes	Ravaz Index
Touriga Nacional									
Season		***	***	**	***	**	***	*	***
Water regime		*	ns	ns	ns	ns	ns	ns	*
Interaction		ns	ns	ns	ns	ns	ns	ns	ns
2010	FI	2.00^{a}	24.3^{a}	20.3	8.3^{a}	20.2	0.70^{ab}	42.9	2.89^{a}
	NI	2.02^{a}	24.5^{a}	20.4^{a}	8.1^{ab}	22.8^{ab}	0.73^{a}	43.1	2.78^{a}
2011	FI	1.87^{a}	27.3^{a}	7.8	36.1^{b}	31.7	0.93^{b}	24.9	2.04^{a}
	NI	1.42^{a}	23.0^{a}	1.2^{a}	23.6^{b}	35.4^{b}	1.10^{b}	31.8	1.37^{a}
2012	FI	4.83^{b}	52.0^{b}	9.8	0.0^{a}	21.7	0.52^{a}	30.7	9.7^{b}
	NI	3.63^{b}	52.2^{b}	10.4^{ab}	0.0^{a}	17.3^{a}	0.66^{a}	22.1	6.25^{*b}
Trincadeira									
Season		***	***	***	**	*	**	ns	***
Water regime		***	ns	***	ns	ns	ns	ns	***
Interaction		***	ns	*	ns	ns	ns	ns	***
2010	FI	3.02^{b}	9.8^{a}	0.0^{a}	34.5	43.5	1.16^{b}	36.5	3.07^{a}
	NI	1.12*	4.7^{a}	3.0^{a}	30.8^{b}	43.0	1.09	38.6	1.04*
2011	FI	0.39^{a}	8.0^{a}	28.3^{b}	19.8	38.2	0.74^{ab}	23.9	0.69^{a}
	NI	0.38	7.3^{a}	44.8^{b}	37.6^{b}	14.9	0.76	41.7	0.53
2012	FI	7.43^{c}	19.7^{b}	2.6^{*a}	9.3	24.1	0.63^{a}	46.6	12.65b
	NI	2.25*	16.7^{b}	39.9^{b}	0.0^{a}	15.1	0.75	26.8	2.85*

The parameters measured were yield per vine, number of clusters per vine, incidence of sunburn and of *Botrytis*, percentage of clusters in non count nodes, harvest weight, percentage of wood from non count nodes, the Ravaz Index.
Significance levels of the factors in the ANOVA: *** 0.001; ** 0.01; * 0.05; "ns" not significant. Statistically significant differences after Tukey's multiple comparison tests for a p value lower than 0.05 are the following: * indicates significant differences between irrigation treatments in the same season; lower case letters indicate significant differences between seasons in the same irrigation treatment.

TR was more affected by season and water regime ($p<0.001$), with higher yield in FI plants in 2010 and 2012, the seasons in which soil water availability was lowest in NI. In 2011 the differences observed between FI and NI were not significant in both varieties. In TN the major factor influencing yield and number of clusters was the season ($p<0.001$), and there were no significant differences between irrigated and non irrigated vines. TN consistently produced more clusters than TR, a reflection of its higher production capacity, a well documented intrinsic varietal characteristic (IVV, 2011). The percentage of sunburn and *Botrytis* incidence were affected by the environmental conditions ($p<0.01$). The highest percentage of *Botrytis* incidence occurred in 2011, the season with lower maximum temperatures and higher humidity (Table I). Sunburn incidence affected TN in the drier warmer years while TR was mostly affected in 2011 (Table I), with similar tendencies in FI and NI. The percentage of clusters from non-count shoots was affected by the season to a lesser extent than the previous parameters (p < 0.05), showing significantly higher values in TN-NI-2011 (Table I), while wood in non-count nodes was unaffected by season and irrigation.

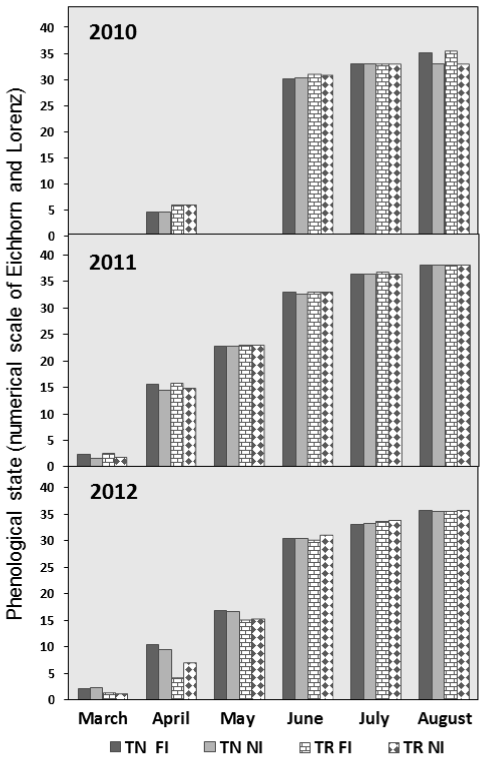

Figure 3. Phenological state of Touriga Nacional (TN) and Trincadeira (TR), in the non-irrigated (NI) and irrigated (FI) treatments during the three seasons studied (from March to August). Values are presented according to the numeric scale of Eichhorn and Lorenz (1977).

Estados fenológicos de Touriga Nacional (TN) e Trincadeira (TR), nos tratamentos não-irrigado (NI) e totalmente irrigado (FI) entre Março e Agosto nas três épocas de crescimento estudadas. Os valores são apresentados de acordo com a escala numérica de Eichhorn and Lorenz (1977).

In this experiment, phenology did not change with irrigation nor variety, while yield was affected by the interaction of the factors at play (water regime, weather variability in the three seasons). The most significant yield decreases with drought (70%) occurred in TR in 2010 and 2012. In deficit irrigation treatments the decreases in yield are *ca.* 25% (Oliveira *et al.,* 2013), the same value that was obtained in TN-NI in 2011 and 2012, while in 2010 this variety had no decrease in yield. Gouveia *et al.* (2012) found significant differences in yield between FI and NI TN vines while deficit irrigated ones had similar yield than NI. The results obtained in 2010 can be explained by the availability of water in the soil until further in the summer than in the other seasons studied. As the number of clusters is defined in the season previous to the harvest, this parameter was fairly constant with weather/irrigation conditions and only changed with the variety. Also, in all the seasons, the weight and volume of each berry was significantly affected by drought ($p<0.001$) in both varieties with the exception of 2011, the season in which the differences were attenuated. The sunburn incidence was unaffected by the water regime in TN while in TR it was higher in NI in all the seasons ($p<0.001$) accompanying the significant decreases in yield. Thus, the low values of Ravaz index in TR-NI do not reflect an increase of vigor but a decrease of yield. In 2012, the Ravaz index gave an indication of overcropping in TR-FI, mostly due to the yield of clusters in non-count nodes. Harvest weight changed with the season in TN ($p<0.001$), with the highest value in 2011 (Table I). In TR this parameter was also affected by season ($p<0.01$) but he highest values were quantified in 2010. The yield to pruning weight ratio (Ravaz index) was significantly affected by irrigation and season in both varieties, but with significant interaction only in TR (Table I). In TN-2012, the values indicate balanced vines, both in FI and NI (between 4 and 10, Santos *et al.*, 2003), the value for TR-FI in 2012 indicated overcrop (>10) and all the other values showed excessive vegetative growth (<4). In 2012 values were higher in FI than in NI and these differences were more evident in TR.

Both berry volume and weight varied significantly with the season and the water regime in TN and TR (Table II). In TN, FI yielded heavier berries with higher water content than NI in 2010 and 2012 while there were no significant differences between NI and FI berries in 2011. This was probably due to the higher soil water availability in that season (Figure 2). In TR, there were significant differences between irrigation regimes in all seasons, and 2011 was the season with the highest berry weight and volume. Overall TR berries were heavier and had higher water content than TN berries, and total soluble sugars (ºBrix) was slightly higher in TR than in TN in the three years studied. The influence of the water regime on ºBrix was contrasting in the varieties studied, with TR showing no effects of water regime and TN having influence of irrigation in NI (Table II), with TN-NI showing higher ºBrix in 2011. In TR there was only a very significant effect of season ($p<0.001$), with 2011 showing the highest values and 2012 the lowest in both FI and NI. Titratable acidity and pH showed the same tendency in both varieties and changed significantly with the water regime and the season. In 2012 both varieties had significantly higher pH values and the lowest titratable acidity in FI and NI. In 2011 both varieties had significantly different values between FI and NI, with NI showing the highest pH and the lowest acidity.

Typically, TN has lower bunch weight than TR, with smaller berries, although, in general its production capacity was higher and more regular (IVV, 2011). In fact TR is known for its low basal fertility and high vegetative vigor. Berry weight and volume was, in average, higher in TR through the three seasons and the number of clusters was higher in TN in both irrigation treatments. Pruning weight, however, was only higher in TR in 2010, a season with plentiful water and mild temperatures in the winter.

Table II

Effect of season (2010 to 2012) and irrigation treatment (FI/NI) on berry characteristics measured in the three seasons monitored in Touriga Nacional (TN) and Trincadeira (TR)

Efeito da época (2010 a 2012) e do tratamento de irrigação (FI/NI) sobre características do bago medidos em Touriga Nacional (TN) e Trincadeira (TR nas três épocas de crescimento)

Season	Water regime	Berry weight (g)	Berry volume (mL)	pH	Brix index (%)	Total acidity (g/L)
Touriga Nacional						
Season		***	**	***	*	***
Water regime		***	***	**	ns	**
Interaction		*	ns	***	**	***
2010	FI	1.66^{b}	1.04	3.7^{b}	21.9	4.0^{b}
	NI	$1.26*^{b}$	$0.69*^{a}$	3.6^{a}	20.2^{a}	4.3^{c}
2011	FI	1.70^{b}	1.01	$3.6*^{a}$	21.8	4.4^{c}
	NI	1.61^{c}	0.92^{b}	3.8^{b}	23.2^{b}	$3.8*^{b}$
2012	FI	1.43^{a}	0.93	$4.2*^{c}$	22.2	2.7^{a}
	NI	$1.13*^{a}$	$0.64*^{a}$	4.3^{c}	20.2^{a}	2.3^{a}
Trincadeira						
Season		***	***	***	***	***
Water regime		***	***	***	ns	***
Interaction		ns	*	**	*	**
2010	FI	2.40^{b}	1.57^{a}	3.9^{a}	22.5^{a}	3.6^{b}
	NI	$1.34*^{a}$	$0.77*^{a}$	3.9^{a}	24.0^{b}	3.6^{b}
2011	FI	3.40^{c}	2.11^{b}	$3.7*^{a}$	25.4^{b}	4.1^{b}
	NI	$2.83*^{b}$	$1.74*^{c}$	4.0^{a}	26.7^{c}	$3.3*^{b}$
2012	FI	2.18^{a}	1.34^{a}	4.2^{b}	20.6^{a}	2.6^{a}
	NI	$1.33*^{a}$	$0.84*^{b}$	4.3^{b}	19.3^{a}	2.2^{a}

The characteristics measured were berry volume and weight, brix index, pH and total acidity.
Significance levels of the factors in the ANOVA: *** 0.001; ** 0.01; * 0.05; "ns" not significant. Statistically significant differences after Tukey's multiple comparison tests for a p value lower than 0.05 are the following: * indicates significant differences between irrigation treatments in the same season; lower case letters indicate significant differences between seasons in the same irrigation treatment.

Contrary to what happens in roots, leaf growth is usually significantly impaired under drought conditions, due to a rapid decrease in the extensibility of expanding leaf cell walls (Hsiao and Xu, 2000). This drought-response process is ABA-mediated as the upkeep of leaf expansion is dependent on the presence of endogenous ABA, regardless of water status (Sharp *et al.*, 1994). The increase of ABA content verified in 2012 in TR-NI was not accompanied by changes in pruning weight; however, the Ravaz index indicated excessive vegetative vigor. These differences in results obtained in the chemical signaling in response to water deficit may be caused by differences in the susceptibility of the varieties to water stress (Carvalho *et al.*, 2016; Rocheta *et al.*, 2016) but environmental factors are also likely to intervene (Dodd, 2009; Romero *et al.*, 2012), such as sporadic summer showers, temperature and evaporative demand in the area and the type of soil.

When irrigation is in excess the number of viable buds and their fruitfulness is reduced in Thompson seedless grapevines (Williams *et al.,* 2009). In the current study, even though irrigation was set to compensate 100% ETc, cluster number was not affected by the irrigation regime in both varieties. Berry size (volume and weight) were most affected by irrigation in all seasons, showing that a late imposition of water deficit significantly affects berry size, possibly through a reduction of cell expansion. Reports usually indicate that an earlier water deficit (post flowering water-stress) is more effective in influencing this berry characteristic, though the reduction of cell number as reported in berries of Syrah (Mccarthy, 1997). In Cabernet Franc, early water stress increased anthocyanin and phenolics content at harvest (Matthews and Anderson, 1988) and in Bobal, this increase was accompanied by a decrease of berry size (Salón *et al.,* 2005). These decreases of berry weight and volume were more significant in TR, reaching 50% of the FI values in 2010, while TN-NI showed decreases typical of deficit irrigated vines (circa 80% the FI values) as the ones obtained by Oliveira *et al.* (2013) working with the Sardinian variety Cannonau. Balanced growth of the vines was affected by irrigation in 2012, the season with the driest and warmest winter, with contrasting behaviours of both varieties: on TN, NI and FI treatments showed balanced vines, with FI closing in on overcropping while TR FI had clearly overcropped vines and NI showed an excessive vegetative growth. As a consequence, TN phenological development was slightly anticipated without affecting vegetative growth or berry composition. Relationships between winter temperature and phenological events have been reported in South Africa, France, Australia and Spain

(Conradie *et al.,* 2002; Petrie and Sadras, 2008; de Cortázar-Atauri *et al.*, 2009; Ramos *et al.*, 2015). In Portugal, strong correlations between budburst and winter temperatures were found (Fraga *et al.*, 2016). In the current work, a significant increase in the number of clusters also accompanied the anticipation of phenology, probably as a result of the abundance of water and mild spring temperatures of the previous season (2011).

Oxidative stress, antioxidative defence system and pigments

Plant water status was evaluated through the assessment of the pre-dawn leaf water potential (ψ_{pd}) at the moment of sampling for pigments, H_2O_2 and status of the antioxidative defence system (Table III). Sampling was done on the phenological stage 35 (numeric scale of Eichhorn and Lorenz, 1977) and irrigated plants showed ψ_{pd} values around -0.2 MPa in the three seasons, indicating a similar water supply throughout the experiment. In the non-irrigated treatments, 2012 was the season with the highest water deficit (ψ_{pd} = -1.17 MPa in TN-NI) while in the previous seasons values were similar and ranging from -0.7 to -0.8 MPa, values consistent with severe water stress.

Table III

Effect of season (2010 to 2012) and irrigation treatment (FI/NI) on pre dawn leaf water potential (ψpd), Reduced (AsA) and oxidized (DAsA) ascorbate, reduced (GSH) and oxidized (GSSG) glutathione, abscisic acid (ABA) and hydrogen peroxide (H_2O_2) concentrations, and percentage reduction of ascorbate and of glutathione, in leaves of Touriga Nacional (TN) and Trincadeira (TR)

Efeito da época (2010 a 2012) e do tratamento de irrigação (FI/NI) sobre o potencial hídrico de base(ψpd), teor de ascorbato (AsA) e glutationa reduzidos (GSH) e oxidados (respetivamente, DAsA e GSSG), ácido abscísico (ABA) e peróxido de hidrogénio (H_2O_2, e percentagem de redução de ascorbato e de glutationa, em folhas de Touriga Nacional (TN) e Trincadeira (TR)

Season	Water regime	ψpd (MPa)	AsA	DAsA	GSH	GSSG	%redAsA	%redGSH	H_2O_2 (µmol/g FW)	ABA (µmol/g DW)
			(µmol/g FW)							
Touriga Nacional										
Season		ns	***	*	ns	***	ns	***	***	**
Water regime		***	ns	ns	ns	ns	ns	ns	***	ns
Interaction		**	***	*	ns	ns	ns	ns	ns	ns
2010	FI	-0.2*	5.33^{a}	0.96^{a}	266.77*^{ab}	62.35^{a}	85.30	84.95^{ab}	1.23*^{a}	36.63
	NI	-0.8^{a}	3.60^{a}	1.55	95.25^{a}	21.41	76.36	72.44^{ab}	6.77^{a}	34.62^{b}
2011	FI	-0.2*	7.68^{b}	13.91*^{b}	191.55^{a}	216.24^{b}	48.71	55.94^{a}	3.53*^{a}	29.04
	NI	-0.8^{a}	5.12^{a}	2.08	149.87^{a}	122.13	76.81	60.54^{a}	9.82^{ab}	18.07^{ab}
2012	FI	-0.1*	4.43^{a}	0.17^{a}	399.92^{b}	9.54^{a}	96.54	96.92^{b}	9.11^{b}	16.41
	NI	-1.2^{b}	10.47^{b}	1.07	390.58^{b}	25.72	91.02	95.36^{b}	12.45^{b}	9.42^{a}
Trincadeira										
Season		**	***	***	*	***	***	*	*	ns
Water regime		***	*	***	**	***	*	**	ns	**
Interaction		**	*	**	**	***	***	**	*	*
2010	FI	-0.2*	3.25^{a}	1.05^{a}	186.50^{a}	80.80^{a}	84.67	68.61^{a}	10.88	24.54
	NI	-0.7^{a}	4.12	0.12	232.50	69.30	97.06^{b}	77.60	15.91^{b}	20.47^{a}
2011	FI	-0.3*	5.10^{a}	2.34*^{ab}	180.50^{a}	42.70^{a}	75.13	91.34^{b}	9.65	22.31*
	NI	-0.9^{ab}	6.81	11.13	112.40	18.60	52.0*^{a}	85.00	5.48^{a}	52.55^{b}
2012	FI	-0.2*	17.48^{b}	3.26*^{b}	312.85*^{b}	142.55*^{b}	84.28	68.69*^{a}	10.27	15.39*
	NI	-1.1^{b}	8.02*	1.79	107.00	9.60	84.69^{b}	92.47	10.03^{ab}	40.01^{ab}

Each value is the mean of four independent samples measured in triplicate (n=4). Significance levels of the factors in the ANOVA: *** 0.001; ** 0.01; * 0.05; "ns" not significant. Statistically significant differences after Tukey's multiple comparison tests for a p value lower than 0.05 are the following: * indicates significant differences between irrigation treatments in the same season; lower case letters indicate significant differences between seasons in the same irrigation treatment.

H_2O_2 concentration in TN was significantly lower in FI-2010 and 2011 and increased from 2010 to 2012 in both irrigation regimes (Table III). In TR, H_2O_2 concentration changed with the season in NI ($p<0.1$), with the highest values in 2010 and the lowest in 2011. ABA concentration in the leaves (Table III) was not significantly affected by the water regime in TN, only by the season ($p<0.01$) in NI, decreasing from 2010 to 2012. In TR, irrigation was the main factor of variation ($p<0.01$) of ABA contents, with NI showing significantly higher values than FI in 2011 and 2012.

The operational status of the mechanisms of defence against oxidative stress was assessed through the quantification of the oxidized and reduced forms of ascorbate and glutathione and of photosynthetic and protective pigments (Table III). In TN, AsA was affected by season ($p<0.001$) and not by irrigation, a trend similar to that of DAsA, GSSG and of the percentage reduction of glutathione (%redGSH). There was no influence of the factors studied in the concentration of GSH and in the percentage reduction of ascorbate (%redAsA) in TN. The values of %redGSH were lowest in 2011 and highest in 2012, in both irrigation treatments. In TR both metabolites were significantly affected by season and irrigation, with Fi-2012 showing very high values of AsA, GSH and GSSG. The percentage reduction of glutathione (%redGSH) was also influenced by both factors and showed significant interaction, with the highest values inFI-2011 and NI-2012.

In both varieties, the content of all pigments was significantly affected by season ($p<0.001$) while the water regime did not affect anthocyanins and chl*a*/chl*b* ratio in TN and in TR chlorophylls and anthocyanins were unaffected (Table IV). In TR-210 and TN-2012 chl*a*/chl*b* ratio was higher in NI while in TN-2011 it was higher in FI.

Oxidative stress was highest in the most water stressed season (2012) in TR, with a concomitant response of the antioxidative response system, with repercussions in the shift of ascorbate to the oxidized form, as opposed to its unchanged status in TN, which showed high % reduction of both ascorbate and glutathione. These intrinsic differences between varieties, that are evident in imposed abiotic stress conditions (Carvalho *et al.,* 2016), were not as striking with season variation although TN invested in increasing total glutathione and its redox status while TR invested in increasing its levels of ascorbate, especially in 2012. Also, photosynthetic pigments increased in the seasons of 2010 and 2012 in the NI treatments, indicating an attempt to optimize photosynthetic ability in an environment that favors stomatal closure.

Correlations between winter meteorological data and the following year's yield and between agronomically relevant quantifications

To investigate whether the environmental conditions, namely precipitation and temperature during the winter months, affected the following year's production, and if summer irrigation was able to overcome eventual production impairments, we performed correlations between meteorological data and yield. There were significant positive correlations between the maximum winter temperatures and the number of clusters in both varieties while the correlation between the accumulated precipitation in the same period and the number of clusters was significantly negative (Figure 4).

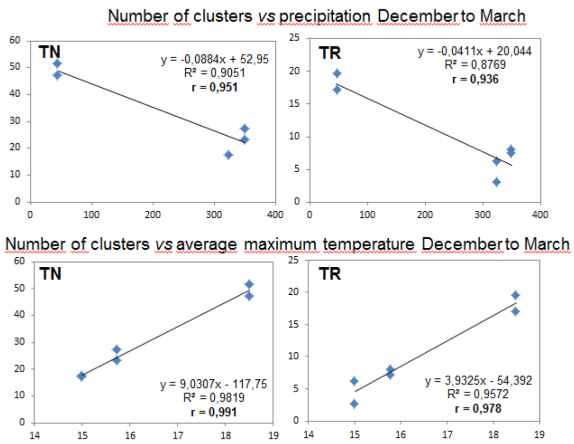

Figure 4. Correlations between the number of clusters and the accumulated precipitation from December to March of the previous year and the average maximum temperatures in the same period. The correlation coefficient (r) is indicated within each frame, together with the equation of the curve and the determination coefficient (R^2).

Correlações entre o número de cachos e a precipitação acumulada de dezembro a março do ano anterior e as médias das temperaturas máximas no mesmo período. O coeficiente de correlação (r) está indicado em cada gráfico, juntamente com a equação da respetiva reta e o coeficiente de determinação (R^2).

Pearson correlations between several production indicators and environmental parameters were performed in order to assess the influence of the environment on the production/quality of both varieties (Table V). It is interesting to notice that in TR ABA content was significantly correlated with ψ_{pd} and with FTSW at the time of ABA quantification while in TN it was not. Also, in TN harvest weight was negatively correlated with FTSW while in TR there was no correlation between soil water content during the season and wood production but there was a positive correlation between winter rainfall and wood production. Expectably, in both varieties berry weight and volume were significantly influenced by water availability in the soil and by ψ_{pd}, with TR showing larger and heavier berries per unit of water available.

Table IV

Effect of season (2010 to 2012) and irrigation treatment (FI/NI) on chlorophyll *a* and *b* (Chl*a* and Chl*b*), carotenoid, anthocyanin concentration, chlorophyll *a*/ chlorophyll *b* ratio and total chlorophyll/carotenoid ratio in leaves of Touriga Nacional (TN) and Trincadeira (TR)

Efeito da época (2010 a 2012) e do tratamento de irrigação (FI/NI) sobre o teor de clorofila a e b (Chla e Chlb), carotenóides, antocianinas, razão clorofila a/ clorofila b e razão clorofila total /carotenóides, em folhas de Touriga Nacional (TN) e Trincadeira (TR)

Seaso n	Water regime	Chlorophyll *a* (mg/cm LA)	Chlorophyll *b* (mg/cm LA)	Anthocyanins (mg/cm LA)	Carotenoids (mg/cm LA)	Chlorophyll *a* / Chlorophyll *b*	Chlorophylls/ Carotenoids
Touriga Nacional							
Season		***	***	***	***	***	***
Water regime		***	ns	ns	***	*	**
Interaction		ns	*	ns	ns	***	*
2010	FI	152.89^{a}	72.58	155.92	117.73	2.13^{b}	1.92^{a}
	NI	78.79^{a}	32.67^{a}	149.59^{a}	76.15^{a}	2.42^{b}	1.47^{a}
2011	FI	258.02^{b}	116.57	259.70	154.93	2.21^{b}	2.40^{ab}
	NI	191.21^{b}	149.27^{b}	301.68^{b}	127.49^{b}	$1.60*^{a}$	2.52^{b}
2012	FI	208.12^{ab}	135.12	260.21	127.76	$1.56*^{a}$	2.68^{b}
	NI	$118.44*^{ab}$	$58.46*^{a}$	255.91^{ab}	99.27^{ab}	2.06^{ab}	$1.81*^{ab}$
Trincadeira							
Season		***	***	***	***	***	***
Water regime		ns	ns	ns	**	***	ns
Interaction		***	*	ns	***	ns	ns
2010	FI	136.74	60.13^{a}	144.59^{a}	90.71^{a}	$2.27*^{b}$	2.18
	NI	95.30^{a}	33.88^{a}	142.01^{a}	87.4^{a}	2.82^{b}	$1.46*^{a}$
2011	FI	161.22*	101.78^{b}	251.05^{b}	118.24^{b}	1.71^{a}	2.25
	NI	228.77^{c}	111.52^{b}	293.52^{b}	175.95^{b}	2.04^{a}	1.92^{ab}
2012	FI	148.98	80.96^{ab}	197.82^{ab}	104.48^{ab}	1.99^{ab}	2.22
	NI	162.06^{b}	70.08^{c}	187.45^{a}	97.64^{a}	2.33^{a}	2.45^{b}

Each value is the mean of four independent samples measured in triplicate (n=4). Significance levels of the factors in the ANOVA: *** 0.001; ** 0.01; * 0.05; "ns" not significant. Statistically significant differences after Tukey's multiple comparison tests for a p value lower than 0.05 are the following: * indicates significant differences between irrigation treatments in the same season; lower case letters indicate significant differences between seasons in the same irrigation treatment.

Table V

Agronomically/physiologically relevant Pearson correlations between several indicators of production and/or stress and environmental conditions and the respective p-values in the three seasons (2010 to 2012) and two irrigation treatments (FI and NI) in Touriga Nacional (TN) and Trincadeira (TR)

Correlações de Pearson agronomicamente/fisiologicamente relevantes entre vários indicadores de produção e/ou stresse e as condições ambientais e os respetivos valores de p-value nas três épocas (2010 a 2012) e nos dois tratamentos de irrigação (FI e NI) em Touriga Nacional (TN) e Trincadeira (TR)

Variety		**TN**		**TR**	
Parameters in the correlation		**Correlation**	**p-value**	**Correlation**	**p-value**
Winter temperature	Number of clusters per vine	0.77	0.00	0.69	0.00
Winter rainfall	Number of clusters per vine	-0.74	0.00	-0.66	0.00
Winter temperature	Yield per vine	0.76	0.00	0.56	0.00
Winter rainfall	Yield per vine	-0.71	0.00	-0.49	0.00
Winter temperature	Ravaz Index	0.77	0.00	0.63	0.00
Winter temperature	Harvest weight	-0.40	0.02	-0.43	0.01
Winter rainfall	Ravaz Index	-0.71	0.00	-0.58	0.00
Winter rainfall	Harvest weight	0.30	0.07	0.49	0.00
Incidence of sunburn	Harvest weight	-0.31	0.07	-0.24	0.07
ψ_{pd}	Berry volume	0.79	0.00	0.42	0.00
ψ_{pd}	Berry weight	0.66	0.00	0.44	0.01
ψ_{pd}	ABA	0.23	0.18	-0.50	0.00
Harvest weight	FTSW-Fen35	-0.38	0.02	0.14	0.43
Berry volume	FTSW-Harv	0.61	0.00	0.79	0.00
Berry weight	FTSW-Harv	0.67	0.00	0.84	0.00
ABA	FTSW-Fen35	0.06	0.72	-0.46	0.01

Indicators of production and/or stress are: yield per vine, number of clusters per vine, incidence of sunburn, percentage of clusters in non count nodes, harvest weight, Ravaz Index, berry volume and weight, abscisic acid (ABA), FTSW-Fen 35 (Fraction of Transpirable Soil Water in the phenological stage 35 of the numeric scale of Eichhorn and Lorenz, 1977), FTSW-Harv (Fraction of Transpirable Soil Water at harvest). Indicators of the environmental conditions are: predawn leaf water potential (ψ_{pd}), Winter temperature (average temperature between December and March of the previous season), Winter rainfall (accumulated precipitation between December and March of the previous season).

CONCLUSIONS

In TN, yield was significantly affected by environmental conditions during winter, especially in 2012. Summer irrigation was able to increase berry weight but was unable to increase the number of clusters and berries, as they had already been established well before irrigation started. The irrigation treatments affected berry size and volume significantly, while other berry characteristics changed with the severity of the season, but overall this variety can withstand lack of irrigation without decreasing quality, as already observed in the region of ‘Dão’ (Gouveia *et al.*, 2012).

Overall, larger differences in plant behavior were obtained between seasons rather than among treatments within a variety and season. Such differences are due to the specific characteristics of each variety. Thus, when analyzing the effects of irrigation practices in field conditions, multi-year studies should be undertaken (Intrigliolo and Castel, 2010) and varieties should be carefully compared with one another in order to understand which are better suited for each specific environmental conditions.

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