Nutritional properties of Tempranillo grapevine leaves are affected by clonal diversity, mycorrhizal symbiosis and air temperature regime

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Running title: Minerals and metabolites in Tempranillo leaves

ABSTRACT

Tempranillo grapevine is widely cultivated in Spain and other countries over the world (Portugal, USA, France, Australia, and Argentina, among others) for its wine, but leaves are scarcely used for human or animal nutrition. Since high temperatures affect quality of fruits and leaves in grapevine and the association of Tempranillo with arbuscular mycorrhizal fungi (AMF) enhances the antioxidant properties of berries and leaves, we assessed the effect of elevated air temperature and mycorrhization, separately or combined, on the nutritional properties of Tempranillo leaves at the time of fruit harvest. Experimental assay included three clones (CL-260, CL-1048, and CL-1089) and two temperature regimes (24/14°C or 28/18°C day/night) during fruit ripening. Within each clone and temperature regime there were plants not inoculated or inoculated with AMF. The nutritional value of leaves increased under warming climate: elevated temperatures induced the accumulation of minerals, especially in CL-1089; antioxidant capacity and soluble sugars also increased in CL-1089; CL-260 showed enhanced amounts of pigments, and chlorophylls and soluble proteins increased in CL-1048. Results suggested the possibility of collecting leaves together with fruit harvest with different applications of every clone: those from CL-1089 would be adequate for an energetic diet and leaves from CL-260 and CL-1048 would be suitable for culinary processes. Mycorrhization improved the nutritional value of leaves by enhancing flavonols in all clones, hydroxycinnamic acids in CL-1089 and carotenoids in CL-260.

Keywords: Arbuscular mycorrhizal fungi, global warming, minerals, phenolic compounds, pigments, *Vitis vinifera* cv. Tempranillo

Abbreviations: AMF = arbuscular mycorrhizal fungi; CL = clone; DW = dry weight; -M = non-mycorrhizal plants; +M = mycorrhizal plants; Pro = proline; SLW = specific leaf weight; T = temperature; *T* = transpiration rate; TAC = total antioxidant capacity; TSP = total soluble proteins; TSS = total soluble sugars

1. Introduction

Winter is the main pruning season for grapevines. However, this crop needs regular pruning through the growing season in order to keep it manageable and productive. For example, pruning –manual or mechanical- applied to *Vitis vinifera* cv. Tempranillo vineyards benefits yield and reduces cluster and berry weights without any negative effect on the oenological characteristics of the wines derived from these grapevines (Pérez-Bermúdez et al., 2015). This annual pruning of vineyards produces vegetative residuals (stems and leaves) which are most times left in open fields and, to a lesser extent, used to feed sheep and goats (Gurbuz, 2007). In rural regions of some Mediterranean areas, such as Turkey, Greece and Middle East countries, leaves of grapevines are collected to be used as an ingredient for the preparation of dishes for human consumption (Harb et al., 2015; Lima et al., 2016, 2017). Grapevine leaves can also be found as a marketed food supplement in which case it is very important to know their mineral composition (Pantelić et al., 2017). Nutritional value of grapevine leaves is based on their high levels of minerals, vitamins, carotenoids and phenolic compounds (Andelković et al., 2015). Spain is one of the greatest producers of grapes in the European Union (Eurostat Statistical Books, 2017), being Tempranillo a red grape variety widely cultivated in northern and central regions of the country for its wine of high quality. This variety, which exhibits a broad clonal diversity (Cervera et al., 2002), accounts for the 21% of the total Spanish vineyard surface (OIV Focus, 2017), but leaves are not consumed in

the human diet yet –nor those eliminated in the pruning performed at the vegetative period nor those still present in the plant when fruits are harvested in autumn-. Tempranillo is also cultivated in other countries over the world, although it is known under other synonyms, such as Aragonez in Portugal or Valdepeñas in California.

In many Mediterranean countries, an important part of vineyards are subjected to heat stress from the end of spring till fruit harvest near September. Therefore, the impact of elevated temperatures is one of the environmental factors that most influence both primary and secondary metabolisms and, consequently, the quality of grape berries and leaves (Harb et al., 2015; Torres et al., 2017). Moreover, according to the Intergovernmental Panel on Climate Change (IPCC, 2014), the current situation will aggravate since it is expected that the increase of global average temperature could reach 4°C in the next 100 years. In this challenging context, soil microorganisms may play a crucial role since they can help crops to cope with abiotic stresses (Grover et al., 2011). Amongst these microorganisms, arbuscular mycorrhizal fungi (AMF) have received increasing attention due to their numerous benefits for their host plants. The symbiotic association of plants with AMF is a common phenomenon observed in nearly 80% of plant species, including grapevines (Balestrini et al., 2010; Ocete et al., 2015). The inoculation of grapevines with AMF has been associated with enhanced nutrient uptake and plant vigour (Schreiner, 2005), as well as improved drought tolerance (Nikolau et al., 2003). Recently, Torres et al. (2016) concluded that the association of Tempranillo with AMF may play a relevant role in a future climate change scenario to maintain or even improve fruit quality by enhancing berry antioxidant properties. Moreover, mycorrhizal symbiosis induced the accumulation of antioxidant compounds, such as flavonols and anthocyanins, and enhanced the antioxidant activity in leaves of Tempranillo grapevines subjected to warm temperatures (Torres et al., 2015). All these findings suggest that the nutraceutical value of leaves from Tempranillo may be increased by the association of

grapevines with AMF under stressful conditions. Therefore, the present study has deepen on the effect of mycorrhizal association and elevated air temperature, separately or in combination, on the levels of some primary and secondary metabolites as well as on the concentrations of minerals in leaves of three clones of Tempranillo coming from different geographical areas in order to assess their potential application in the human diet.

2. Materials and methods

2.1. Biological material

Three-node segments of *V. vinifera* (L.) cv. Tempranillo clones were collected in the winter of 2016 from an experimental vineyard of the Institute of Sciences of Vine and Wine (Logroño, Spain). Three clones from different origins and agronomic traits in the field (CL-260, from San Vicente de la Sonsierra, La Rioja; CL-1048, from Laguardia, Álava; and CL-1089, from Bargota, Navarra) were chosen. All of them have short reproductive cycle but different yield: low for CL-260, medium for CL-1048 and high for CL-1089. However, the main reason why they were selected for the present research was their different phenolic content and antioxidant activity in leaves as well as their distinct response to elevated air temperature and mycorrhizal inoculation, applied alone or in combination (Torres et al., 2015). Fruit bearing cuttings were produced as initially described in Mullins (1966) and modified by Ollat et al. (1998) and Antolín et al. (2010). Fruit-bearing cuttings stand out as a useful model to study grapevine physiology under controlled environments (Morales et al. 2016). Moreover, Carbonell-Berejano et al. (2013) concluded that the impact of environmental factors on the secondary metabolism of fruiting cuttings was similar to that reported for conventional vines. Rooting was made in a heat-bed (27°C) kept in a cool room

(4°C). At transplanting, half of the plants (+M) were inoculated with the mycorrhizal inoculum Bioradis Gel (Bioera SLU, Tarragona, Spain). The inoculum consisted in a mixture of five AMF (*Septoglomus deserticola*, *Funneliformis mosseae*, *Rhizophagus intraradices*, *Rhizophagus clarus* and *Rhizophagus aggregatum*), containing 100 spores per g of inoculum and a mixture of rhizobacteria belonging to the genera *Bacillus* and *Paenibacillus* (2×10^6 cfu g^{-1}). The AMF present in the inoculum applied in our study belong to the family *Glomeraceae*, which dominates the composition of the AMF communities in vineyards around the world (Torres et al. 2018). The microbial preparation was diluted in distilled water (1:20) to ensure that each plant could receive 1 g of product. The inoculation was performed by submerging roots of fruit-bearing cuttings in the Bioradis Gel for 15 min. In order to restore rhizobacteria and other soil free-living microorganisms accompanying AMF, uninoculated plants (-M) were submerged for 15 min in a filtrate of the abovementioned mycorrhizal inoculum. The filtrate was obtained by passing mycorrhizal inoculum through a layer of 15-20 mm filter paper with particle retention of 2.5 mm (Whatman 42; GE Healthcare, Little Chalfont, UK). Microorganisms accompanying AMF play an important role in the uptake of soil resources as well as on the infectivity and efficiency of AMF isolates (Agnolucci et al., 2015). On the other hand, the response of grapevines to AMF association in this model that lacks the grafting on a rootstock can be partially different from that of the conventional vines. Then plants were placed in 6.5 L plastic pots containing a mixture of vermiculite-sand-light peat (2.5:2.5:1, v:v:v) and transferred to the greenhouses adapted to simulate climate change conditions (Morales et al. 2014). Peat (N: 70-150 mg L^{-1}; P_2O_5: 80-180 mg L^{-1}; K_2O: 140-220 mg L^{-1}; pH: 5.2-6.0) (Floragard, Vilassar de Mar, Barcelona, Spain) was previously sterilized at 100°C for 1 h on three consecutive days. Initial growth conditions were 25/15°C and 50/90% relative humidity (day/night) regime and natural daylight (photosynthetic photon flux density, PPFD, was on average 850 μmol m^{-2} s^{-1} at

midday) supplemented with high-pressure sodium lamps (SON-T Agro Phillips, Eindhoven, Netherlands) to extend the photoperiod up to 15 h and ensure a minimum PPFD of 350 μmol m^{-2} s^{-1}. Humidity and temperature were controlled by using M22W2HT4X transmitters (Rotronic Instrument Corp., Hauppauge, USA). PPFD was monitored with a LI-190SZ quantum sensor (LI-COR, Lincoln, USA). Plants were watered twice per day (140 mL day^{-1}) with the nutrient solution detailed by Ollat et al. (1998). The electric conductivity of the nutrient solution adjusted to pH 5.5 was 1.46 ± 0.15 mS cm^{-1} as determined with a conductivity meter 524 Crison (Crison Instruments S.A., Alella, Spain) and the phosphorus (P) level was 9.78 mg L^{-1}.

2.2. Experimental design

From fruit set (Eichhorn and Lorenz (E–L) fruit stage 27) (Coombe, 1995) to harvest (E-L38 stage), -M and +M plants of each clone were exposed to two temperatures (24/14°C and 28/18°C day/night). Temperature regimes were chosen according to the average temperature registered in La Rioja during the growing season (1981-2010) (AEMET, Spain) and the projected rise of 4°C for 2081-2100 (IPCC 2014). The excessive soil warming, which can negatively affect AMF infection, was avoided by wrapping the pots with a reflecting material (Passioura, 2006; Poorter et al., 2012). Soil temperature was measured at 5 cm soil depth using probes PT100 (Coreterm, Valencia, Spain) and reached 23 ± 0.5°C and 28 ± 0.5°C for 24/14°C and 28/18°C temperatures, respectively. Leaves were harvested coinciding with commercially ripe berries (approximately 22°Brix, E-L38 stage) and immediately frozen at -80°C for further analysis.

2.3. Determination of plant growth, transpiration rate (T) and mycorrhizal colonization

At harvest (E-L38), leaves were collected and weighted. Total leaf area was measured with a portable area meter (model LI-3000, Li-Cor, Lincoln, Nebraska, USA) and total leaf dry weight (DW) was estimated by applying the ratio of fresh to dry weight of a representative leaf sample for every grapevine cultivar and treatment. DW was calculated after drying leaf samples in oven at 70°C for 48 h. Specific leaf weight (SLW) was calculated by dividing the total leaf DW by the total leaf area of every plant. Transpiration rates (*T*) were measured with a portable photosynthesis system (ADC-LCi, BioScientific Ltd., Hoddesdon, UK) under the abovementioned greenhouse conditions with a photosynthetically active photon flux density (PPFD) of 1200 μmol m^{-2} s^{-1}.

Root samples were cleared and stained following the procedure described by Koske and Gemma (1989). 10% potassium hydroxide solution (w:v) was added to the roots which were placed in an oven at 70°C for 2 h. After rinsing with water, roots were clarified by the addition of 3% H_2O_2 (v:v) and subsequently washed with water. Then, they were acidified by soaking in 1% HCl (v:v) for 5-15 min and stained in a solution of 1% methyl blue: lactic acid (w:v) at 70°C for 1 h. Stained roots were stored in a mixture of glycerol, water and 1% HCl (500:450:50, v:v:v) until mycorrhizal quantification. The percentage of mycorrhizal colonization was determined under a stereoscopic microscope by the grid intersect method (Giovannetti and Mosse, 1980).

2.4. Fluorimetric sensor measurements in leaves through berry ripening

The evolution of the concentration of leaf chlorophylls, the plant nitrogen status and the epidermal levels of flavonols and anthocyanins in leaves was estimated *in situ* by using a

hand-held, non-destructive fluorescence based proximal Multiplex3^{TM} sensor (Force A, Orsay, France) at four stages of berry ripening: 1) onset of softening (E-L34 stage, green berries); 2) beginning of berry coloration and enlargement (E-L35 stage, veraison); a week after veraison (E-L36 stage); and 4) two weeks after veraison (E-L37 stage). Multiplex3^{TM} records twelve signals and several signal ratios that are linked to plants constituents. Thus, SFR_G index is positively correlated with grapevine leaf chlorophylls (Diago et al., 2016). The Nitrogen Balance Index (NBI_1) was designed to use a single emission signal (FRF) in order to avoid the influence of the variable chlorophyll fluorescence under certain conditions and has been shown to respond to nitrogen nutrition of the plant (Agati et al., 2013a). Finally, the ANTH_RG and FLAV indexes are proportional to the anthocyanin and flavonols concentration in the epidemic cells, respectively (Agati et al., 2013b; Diago et al., 2016). For the present experiment, the chlorophylls fluorescence signals RF_G and FRF_G, excited with green (G) light, FRF_UV, excited with ultraviolet (UV) radiation and FRF_R, excited with red (R) light were used to calculate the abovementioned indexes as:

SFR_G = FRF_G / RF_G

NBI_1 = FRF_UV x FRF_G / FRF^2_R)

ANTH_RG = log (FRF_R / FRF_G)

FLAV = log (FRF_R/FRF_UV)

2.5. Minerals in leaves at fruit harvest (E-L38)

Leaf samples (0.5 g DW) were dry-ashed and dissolved in HCl according to Duque (1971). Phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), manganese (Mn), iron (Fe), zinc (Zn) and copper (Cu) were determined using a Perkin Elmer Optima 4300 inductively coupled plasma optical emission spectroscopy (ICP-OES) (Perkin Elmer, USA).

The operating parameters of the ICP-OES were: radio frequency power, 1300 W; nebulizer flow, 0.85 L min^{-1}; nebulizer pressure, 30 psi; auxiliary gas flow, 0.2 L min^{-1}; sample introduction, 1 mL min^{-1} and three replicates per sample. Total nitrogen (N) and carbon (C) were quantified after combustion (950°C) of leaf DW with pure oxygen by an elemental analyzer provided with a thermal conductivity detector (TruSpec CN, Leco, USA).

2.6. Total soluble proteins (TSP), proline (Pro), total soluble sugars (TSS) and starch in leaves at fruit harvest (E-L38)

Determination of TSP, TSS and starch was performed on 0.5 g of fresh leaves which were ground in an ice-cold mortar and pestle containing potassium phosphate buffer (50 mM, pH 7.0). The homogenates were filtered through four layers of cheese cloth and centrifuged at 28,710 × *g* at 4°C for 15 min. The supernatant was collected and stored at 4°C for TSP and TSS determinations. The pellet was used to determine starch after iodine reaction (Jarvis and Walker, 1993). TSP were analyzed with the protein dye-binding method (Bradford, 1976) and TSS with the anthrone reagent (Yemm and Willis, 1954) using, respectively, bovine serum albumin (BSA) and glucose as standards. Proline was analyzed as described by Rienth et al. (2014). 500 mg of fresh leaves were powdered in liquid nitrogen, diluted 5 fold with deionized water and centrifuged at 3,000 × *g* for 10 min at 4°C. 750 µL of the supernatant were mixed with the same volume of formic acid in a vortex for two min. Then, 750 µL of 3% ninhydrin in dimethylsulfoxide (daily prepared) were added and the mixture was heated at 100°C for 15 min. The absorbance was read at 520 nm.

2.7. Chlorophylls and carotenoids in leaves at fruit harvest (E-L38)

Total chlorophylls (a + b) and total carotenoids were extracted according to Sèstak et al. (1971) by immersing samples of fresh leaves (1 cm^2, approximately equivalent to 20 mg) in 5 mL of 96% ethanol at 80°C for 10 min. The absorbance of extracts was measured at 470, 649, 665 and 750 nm. Estimation of total chlorophylls (a + b) and total carotenoids was performed by using the extinction coefficients and equations described by Lichtenthaler (1987).

2.8. Phenolic compounds and total antioxidant activity (TAC) in leaves at fruit harvest (E-L38)

2.8.1. Extraction of phenolic compounds

Samples of 0.5 g of fresh leaves were ground to a powder in a mortar with liquid nitrogen. After adding 3 mL 80% aqueous acidified methanol (2% HCl 12N) (Revilla et al., 1998) to each sample, phenolics were extracted by shaking samples overnight at room temperature in the dark. Then, samples were centrifuged at 13,200 × *g* for 15 min at ambient temperature. The residues were re-extracted other two more times (for 3 h every re-extraction) under similar conditions. Supernatants were combined (9 mL in total for each sample) before determining phenolic compounds and total antioxidant capacity (TAC).

2.8.2. Determination of phenolics and TAC

Flavonoids were analysed according to Kim et al. (2003). 4 mL of deionized water was added to 1 mL of each sample. After adding 300 µL of $NaNO_2$ samples were shaken for 5 min, and 300 µL of $AlCl_3$ were added. After 6 min, 2 mL of 1M NaOH were added to the flask. Immediately, the mixture was diluted with 2.4 mL of deionized water and the

absorbance was read at 510 nm using catechin as a standard. Flavonols and hydroxicinnamic acids were spectrophotometrically determined as described by Boulanouar et al. (2013). Samples (0.5 mL) were diluted (1:2) with aqueous ethanol (95% v:v) acidified with 0.1% HCl. Then other 4 mL of 2% HCl were added until a total final volume of 5 mL. The absorbance was measured at 360 and 320 nm, and quercetin and caffeic acid were used as standards for flavonols and hydroxycinnamic acid derivatives, respectively. Procyanidin monomers (flavan-3-ols) were analysed by the *p*-dimethylaminocinnamaldehyde (DMACA) method (Arnous et al., 2001). One mL of DMACA solution (0.1% in 1 N HCl in MeOH) was added to 0.2 mL of 1:20 diluted sample with 80% aqueous acidified methanol (2% HCl 12N). The mixture was vortex-mixed and kept at room temperature for 10 min. Afterwards the absorbance was read at 640 nm. Catechin was used as a standard. Absorbance values were always read in a UV-VIS spectrophotometer (UV 1800, Shimadzu, Tokyo) with a range of 190-1100 nm, and results were expressed as mg of the standard used for each group of phenolics per gram of leaf DW.

Total antioxidant capacity (TAC) in leaves was evaluated by the free radical scavenging activity (*α, α*-diphenil-*β*-picrylhydrazyl (DPPH•)) assay (Brand-Williams et al., 1995). The variation of the absorbance at 515 nm was measured after 30 min. The reaction started after adding 20 μL of the sample to the cuvette containing 980 μL of 80 μM DPPH• in methanol in parafilm-sealed glass cuvettes (Llorach et al., 2004). TAC was estimated by interpolation on a linear regression curve made with gallic acid.

2.9. Statistical analysis

Statistical analyses were carried out using statistical software the Statistical Package for the Social Sciences (SPSS) (SPSS Inc., Chicago, IL, USA) version 21.0 for Windows. After

establishing the normal distribution of the residuals with the Kolmogorov-Smirnov normality test due to the small sample size (n= 3-5) and the homogeneity of variance with the Levene test, data within each clone were subjected to a two-way analysis of variance (ANOVA) with or without Welch correction, taking into account if the requirement of the homogeneity of variances was fulfilled or not. The test allowed assessing the main effect of the factors temperature (T) (24/14°C, 24 and 28/18°C, 28), and AMF inoculation (M, +M and –M) and the interaction between them. Means ± standard errors (SE) were calculated and when the F ratio was significant ($P \leq 0.05$), a Duncan test was applied. Two-way ANOVA was performed to determine significant differences in measured parameters. To determine general trends within the different samples, a principal component analysis (PCA) was performed over the leaf minerals, pigments, phenolic compounds and TAC.

3. Results and discussion

3.1. Plant growth, transpiration rates (T) and mycorrhizal colonization at fruit harvest (E-L38)

The model of fruiting cuttings implies selective pruning in order to get one well developed bunch per plant. All plants cultivated in our experimental assay were subjected to equal pruning. Therefore, the differences in leaf DW and leaf area shown in Table 1 were due to mycorrhizal symbiosis in the case of CL-260 and, mainly, to elevated air temperature. Grapevines from CL-1048 and CL-1089 had lower leaf DW and leaf area at 28/18°C than at 24/14°C. Mycorrhizal plants (+M) from CL-260 had lower leaf DW than –M plants when cultivated at 24/14°C, but did not reduce significantly their leaf DW after applying elevated temperatures. The proportional decrease in leaf DW and foliar area under 28/18°C derived in

the maintenance of SLW in comparison to SLW values achieved at 24/14°C (Table 1). Only in the CL-1048, mycorrhizal association was the factor that influenced SLW, being its value lower in +M plants than in –M plants, which suggests less amount of cell wall components or nonstructural carbohydrates in cells of leaves from +M plants (Brown and Byrd, 1997). Results on TSS shown in Fig. 2C and further discussed, however, do not support the hypothesis of decreased amount of nonstructural sugars in leaves of +M plants from CL-1048. We did not observe a clear effect of high air temperatures on the transpiration rates (*T*) expressed by unit of leaf area and time (Table 1), which may be in part due to the interaction between temperature and mycorrhizal association (T × M, $P \leq 0.05$ in CL-1048 and $P \leq 0.001$ in CL-1089). The decreased total leaf area in plants undergoing 28/18°C would have presumably derived in reduced total plant transpiration as an adaptation to withstand with elevated air temperatures. The limited soil volume available for roots may have reinforced this adaptation in plants cultivated at 28/18°C. Together with aspects related to leaf conductance, the control of leaf water losses by adjusting the total leaf area per plant based on water availability is another critical factor which can be crucial at the reproductive stage of crops (Vadez et al., 2014).

Microscopic observations of cleared and stained roots revealed the presence of mycorrhizal structures in roots from +M plants. In contrast, fungal structures never were found in roots of –M plants. Percentages of mycorrhizal colonization in +M plants ranged from 41.9% in CL-1048 grown at 24/14°C to 67.7% in CL-260 cultivated at 28/18°C (Fig. 1). Only in CL-1048 elevated temperature was significantly linked to an enhanced mycorrhizal colonization. Increased mycorrhizal colonization has been found in most studies performed under warming temperatures, although fungal activity can decrease under those conditions (Mohan et al., 2014). Contrariwise, some authors have reported decreased AMF colonization as a direct effect of elevated temperature (Wilson et al., 2016).

3.2. Chlorophylls, nitrogen balance, anthocyanins and flavonols in leaves during fruit ripening

Optical sensing technologies may be implemented to provide frequent and spatially widespread monitoring of plant nutrient status as well as, a faster and non-destructive phenotyping tool (Diago et al., 2016). To the best of our knowledge, this is the first study in which this tool has been used to monitor the combined effect of elevated temperatures and mycorrhizal symbiosis on grapevine and it has demonstrated the different behavior of each Tempranillo clone (Table 2).

In CL-260 the levels of chlorophylls in leaves, estimated as SFR_G, were positively influenced by mycorrhizal association at early stage of fruit ripening (E-L34) and by warming air temperatures in a later stage (E-L36). In CL-1048 elevated temperature increased the concentration of chlorophylls in leaves during fruit ripening (E-L35, E-L36 and E-L37). In CL-1089, chlorophylls were significantly affected by the interaction between elevated temperature and mycorrhization, this effect being opposite depending on the stage of fruit ripening: chlorophylls decreased at the beginning (E-L34) and increased later (E-L36).

The NBI_1 in leaves is related to the nitrogen nutrition of plants and corresponds to the ratio between chlorophylls and flavonols (Agati et al., 2013a). Only in CL-260 this index was influenced by the association of plants with AMF and the positive effect was observed at an intermediate stage of fruit ripening (E-L36). Elevated temperature enhanced NBI_1 in leaves of CL-260 and CL-1089 at the final stage of fruit ripening (E-L37).

High air temperatures exerted a negative effect on the anthocyanins (ANTH_RG) present in the epidermal cells of leaves, especially at final stages of fruit ripening (E-L36, E-L37), CL-1048 being the most sensitive clone to warming temperatures (Table 2). Accordingly, Rowan et al. (2009) demonstrated that the loss of anthocyanins due to high temperatures was

explained by the inhibition of the transcription of anthocyanin biosynthetic genes and increased rates of degradation in *Arabidopsis thaliana* leaves. However, this negative effect was not clearly observed in previous studies carried out in Tempranillo subjected to warming temperatures at berry maturity (E-L38) (Torres et al., 2015). Mycorrhizal symbiosis counteracted the decrease in anthocyanins in leaves of CL-1089 subjected to elevated temperatures at stage E-L37 of berry ripening (Table 2), which is of high interest because these pigments are regarded as important components in human nutrition due to their antioxidant capacities (Stintzing and Carle, 2004). Similarly, Torres et al. (2015) observed a positive effect of mycorrhizal inoculation on the levels of anthocyanins in leaves of CL-260 and CL-1048 at stage E-L38.

When compared the epidermal levels of flavonols (FLAV) between -M and +M plants under elevated temperatures, we found higher amount of these phenolic compounds in leaves of plants associated with AMF (Table 2). In CL-260 +M28 plants showed higher FLAV levels than –M28 plants from E-L34 till E-L36. In CL-1048 this beneficial effect of AMF was restricted to stage E-L36. In contrast, Torres et al. (2015) found reduced FLAV content at stage E-L38 in leaves of Tempranillo inoculated with AMF and subjected to elevated temperatures. The disagreement between both studies can be also due to differences in the type of mycorrhizal inocula and in the level of mycorrhizal colonization achieved in roots of grapevines. While Torres et al. (2015) used a commercial inoculum derived from an *in vitro* culture of *Rhizophagus intraradices*, in the present study grapevines received a mixture of five AMF (*S. deserticola, F. mosseae, R. intraradices, R. clarus* and *R. aggregatum*). The percentages of mycorrhizal colonization achieved when applied this mixture of AMF (Fig. 1) were significantly higher than those observed after inoculating *R. intraradices* alone (15% or less). Similarly, Eftekhari et al. (2012) also reported different effectiveness of different

mycorrhizal species applied to different grapevine varieties for inducing the accumulation of a given compound in leaves.

3.3. Minerals in leaves at fruit harvest (E-L38)

Phenotypic differences within cv. Tempranillo clones were highlighted in their foliar mineral composition. Even in plants grown at 24/14°C and not inoculated with AMF (-M24) the concentrations of some macro and micronutrients in leaves differed between clones (Table 3). Despite the equal mineral nutrition and water regime, CL-260 showed around 40% and 30% higher amounts of Mg and Mn, respectively, than CL-1048, which suggests different uptake and translocation rates of water and mineral nutrients from soil to the aerial part among clones. Similarly, the concentration of Zn in leaves of CL-260 was 40% higher than that found in leaves of CL-1089. In contrast, warming day/night temperatures produced similar effects on the three tested clones: the amount of several minerals increased in leaves, regardless grapevines were or not associated with AMF (Table 3). Such behavior agrees with findings of Martins et al. (2014) in *Coffea arabica* subjected to increased air temperature and it was attributed to an enhanced transpiration in order to promote leaf cooling. Leaf *T* measured in our study at fruit harvest and the total transpiration per plant estimated through *T* and the total leaf area (Table 1) cannot explain the increased levels of some minerals in leaves of plants subjected to 28/18°C. In addition, according to results obtained by Iglesias-Acosta et al. (2010), soil temperature (28 ± 0.5°C) recorded in pots of plants undergoing elevated air temperature does not seem to be high enough to cause significant changes in plasma membrane fluidity and/or in the abundance of aquaporins in root cells, two factors closely related to soil water (and minerals) uptake. Since leaf DW decreased in grapevines under elevated temperatures (Table 1), increased mineral concentrations (expressed on a DW basis)

may respond to a concentration effect. However, despite this general behavior under warming temperatures, there was also intravarietal diversity in the response, being CL-1089 the most sensitive to high air temperatures. Levels of Ca, P, Mg, Cu, Zn and Mn significantly increased in leaves of CL-1089 after applying elevated temperatures. The accumulation of Cu, Zn and Mn would have reinforced the defense mechanisms of these plants against oxidative stress (Ramalho et al., 2013). From a human point of view, the increased levels of Ca, Mg, Cu and Zn found in leaves of CL-1089 cultivated under warming temperatures clearly improve their nutritional value because their consumption may reduce the risk of the called 'hidden hunger'. 'Hidden hunger' is the term used to describe the malnutrition inherent in human diets that are adequate in calories but lack in vitamins and/or mineral nutrients, and refers to a nutritional problem also present in developed countries (White and Broadley, 2009). For example, many people in United Kingdom or USA do not consume adequate quantities of Cu (Copper Development Association, 2011), and nearly 50% of the world's population is at risk of inadequate Zn intake (FAOSTAT, 2002). Other minerals that are sometimes scarce in the diet of people from developed countries are Ca, Mg, Fe, Se and I (White and Broadley, 2009).

On the other hand, CL-1048 was the most responsive to the combination of elevated temperatures and mycorrhizal symbiosis (Table 3). The interaction between these two factors (T × M) was significant for the concentrations of N ($P \leq 0.05$), P ($P \leq 0.01$), Mg ($P \leq 0.01$) and Mn ($P \leq 0.01$), but only the amount of N increased when both factors were applied together, which may be a surprising result. Since grapevine root has low density and large diameter fine roots, mycorrhizal symbiosis is expected to be very beneficial for the mineral nutrition of grapevines by extending the volume of the explored soil allowing an adequate uptake of water and mineral nutrients (Trouvelot et al., 2015). Our experiment, however, was performed with potted plants. Consequently, the limited soil volume would have restricted the ability of AMF for enhancing the absorption of minerals, which could explain the low impact

of mycorrhizal inoculation on the accumulation of mineral nutrients in leaves of grapevines (Table 3; M, ns). In a review that included papers published over three decades, Schreiner (2005) reported that mycorrhizal symbiosis always improved the growth of grapevines cultivated under controlled conditions in pots but the concentrations of mineral nutrients in leaves only increased in few of these studies. In addition, as previously commented, the increased percentage of mycorrhizal colonization under warming temperatures is not always associated with enhanced fungal activity (Mohan et al., 2014).

3.4. Primary metabolites in leaves at fruit harvest (E-L38)

Except for CL-1089, the application of elevated temperatures provoked significant increases in the concentrations of soluble proteins in leaves, being these increases especially marked in CL-1048 (Fig. 2A). Although heat stress down-regulates proteins involved in the photosynthetic electron transport, carbon metabolism and glycolytic pathway, high temperatures increase the abundance of chaperones and enzymes implied in the antioxidant metabolism of plants (Rocco et al., 2013).

Proline concentrations in leaves were significantly affected by air temperature and mycorrhizal symbiosis in CL-1089 (Fig. 2B). In these plants proline decreased under elevated temperatures, being the reduction more pronounced in +M than in –M plants. This behavior contrasts with the enhanced proline levels found by Torres et al. (2017) in berries of CL-1089 subjected to high air temperature. Since leaves were the site of synthesis of proline accumulated in citrus fruits during cold hardening (Purvis and Yelenosky, 1982), we can hypothesize that proline synthetized in grapevine leaves may be translocated to fruits when plants are undergoing elevated temperatures.

When compared the levels of soluble sugars in leaves of grapevines collected from semiarid and temperate regions in Palestine, Harb et al. (2015) did not find big differences. Similarly, the concentrations of sugars (TSS and starch) (Figs. 2C and 2D) in leaves of Tempranillo were not drastically affected by elevated temperatures and/or mycorrhizal inoculation. The only exception was CL-1089, in which high temperatures induced an increase in the levels of TSS in leaves, especially in -M plants (Fig. 2C), in accordance with the enhanced concentrations of glucose and fructose found in berries of CL-1089 under high temperatures (Torres et al., 2017). Those increases in TSS, however, were not associated with a decrease in the concentrations of starch (Fig. 2D). Changes in sugars may reflect changes in the rate of photosynthesis and/or acclimation in response to stressful conditions (Harb et al., 2015). Accumulation of TSS can be also a consequence of decreased levels of glycolytic enzymes or proteins implied in energy-generating reactions when plants undergo heat stress (Rocco et al., 2013). In our study, the most relevant differences between the amounts of sugars were due to intravarietal diversity. At moderate temperatures (24/14°C), the concentration of sugars accumulated in leaves of CL-260 (around 50 mg g^{-1} DW) was more than double than that in leaves of CL-1089 (below 20 mg g^{-1} DW) (Fig. 2C). Therefore, from a nutritional point of view, leaves from CL-260 would be adequate for supplying energy through the diet whereas leaves from CL-1048 and CL-1089 would be a better food source for diabetic people.

3.5. Chlorophylls and carotenoids in leaves at fruit harvest (E-L38)

Natural and semi-synthetic chlorophyll derivatives are mainly used as food colorants but they could also be used as food supplements that may delay the development of several chronic diseases (Fernandes et al., 2007). Moreover, they have shown anti-inflammatory

activity *in vitro* (Mulabagal et al., 2010). Carotenoids are thought to be responsible for the beneficial properties of fruits and vegetables in preventing, among others, cardiovascular dysfunctions and cancer in human beings (Rao and Rao, 2007).

In agreement with the information collected through the fruit ripening process (Table 2), data obtained at fruit harvest (E-L38) (Fig. 3) showed that the application of elevated temperatures (28/18°C) enhanced the levels of chlorophylls and carotenoids in CL-260, increased those of chlorophylls in CL-1048 and had not significant effect on the concentrations of pigments in CL-1089. The levels of carotenoids also increased in leaves of CL-260 as a consequence of mycorrhizal inoculation, which agrees with findings of Baslam et al. (2011) working with lettuce. All these observations corroborate that the clonal diversity of Tempranillo results in different abilities to respond to elevated temperatures and AMF inoculation (Torres et al., 2016). Together with their potential benefits for human health, chlorophylls and carotenoids are also intrinsically related to the color, a relevant aspect that consumers take into account. However, the contents of carotenoids and chlorophylls in grapevine leaves are drastically reduced with boiling during cooking processes (Lima et al., 2017). Therefore, an increased level of those pigments in fresh leaves may counteract, to some extent, the loss during culinary treatments. Regarding a potential use of leaves from Tempranillo for nutritional purposes, information on the intra-varietal differences in the basal levels of chlorophylls and carotenoids and their responsiveness to biotic or abiotic factors could be used as criteria for deciding which clones would be the most suitable to be consumed in fresh or to be submitted to culinary process.

3.6. Phenolic compounds and antioxidant activity in leaves at fruit harvest (E-L38)

The term 'flavonoids' includes a large number of pigments (flavonols, flavan-3-ols, flavones, anthocyanidins, flavanones, isoflavones) which are present in fruits, vegetables, nuts and beverages consumed in the human diet. These secondary metabolites are small organic compounds with anti-inflammatory, anti-cancer and antiviral properties, so that they are seen as one of the safest non-immunogenic drugs (Lee et al., 2007). According to epidemiological studies and data from animal models and clinical trials, flavonoids may beneficially affect disease etiology and pathophysiology (Graf et al., 2005).

At ambient temperatures (24/14°C), the highest amount of flavonoids (59.99 mg g^{-1} DW) was found in leaves of non-mycorrhizal plants (-M24) belonging to CL-1048 (Table 4). However, this concentration was reduced by half (29.14 mg g^{-1} DW) under elevated temperatures (28/18°C). The levels of flavonoids in leaves of -M plants from CL-260 also tended to diminish when applied high temperatures because the concentration of these compounds decreased from 31.89 mg g^{-1} DW at 24/14°C till 26.34 mg g^{-1} DW at 28/18°C, which meant a reduction of 17%. In contrast, flavonoids in leaves from CL-1089 were not significantly affected by elevated temperature. Loss of flavonoids in leaves from CL-1048 under elevated temperatures was avoided by the association of plants with AMF.

The flavonols reported in leaves of some red varieties of grapevine are quercetin and derivatives, rutin, luteoline-glucoside, myricetin-glucoside and kaempferol (Andelković et al., 2015). Torres et al. (2017) found myricetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-glucoside, laricitrin-3-O-glucoside, kaempferol-3-O-glucoside and isorhamnetin-3-O-glucoside in berries from CL-1089 and CL-843 of Tempranillo. The present study shows the levels of total flavonols in leaves. At E-L38 stage the concentrations in leaves from mycorrhizal plants grown at ambient temperatures (+M24) were 22.28, 25.03

and 39.14 mg g^{-1} DW in CL-260, CL-1048 and CL-1089, respectively, while the levels of these compounds in leaves of their respective non-mycorrhizal controls (-M24) were 16.13, 22.64 and 19.68 mg g^{-1} DW (Table 4). This means that mycorrhizal symbiosis induced the accumulation of flavonols in leaves of Tempranillo cultivated at 24/14°C day/night temperatures, which agrees with findings of Eftekhari et al. (2012), who measured higher levels of quercetin in Keshmeshi and Shahroodi varieties of grapevine following inoculation with AMF. This positive effect of mycorrhizal symbiosis on the levels of flavonols was also observed under elevated temperatures, and it was especially evident in CL-1089 (Table 4). Moreover, in CL-1048, mycorrhizal association counteracted the negative effect of elevated temperature on the amount of flavonols in leaves.

Among the flavonoids subgroups, flavan-3-ols were less abundant than flavonols in leaves of all studied Tempranillo clones (Table 4), which agrees with findings of Doshi et al. (2006). Except for the CL-1048, these phenolic compounds increased as a consequence of mycorrhizal symbiosis or elevated temperatures. Andelković et al. (2015) observed that the infection of red grapevines with the fungus *Plasmopara viticola*, the causal agent of downy mildew, induced the accumulation of flavan-3-ols in leaves. However, when combined high temperatures and mycorrhization, there was a significant interaction ($T \times M$, $P \leq 0.01$) that reduced the enhancement of flavan-3-ols observed when these factors acted separately.

Hydroxycinnamic acids are polyphenolic compounds that possess antioxidant, anti-inflammatory, anti-collagenase, antimicrobial and anti-tyrosinase activities. All these properties make these natural compounds good potential candidates to fight against obesity and the associated health problems (Alam et al., 2016), to apply as cosmeceutical ingredients in skin anti-aging and hyperpigmentation-correcting products (Taofiq et al., 2017), and to use as additives to new functional foods (Budryn and Rachwal-Rosiak, 2013). In our study, the amount of flavonols and hydroxycinnamic acids in leaves of different Tempranillo clones

were quite similar (Table 4), which contrasts with the lower levels of hydroxycinnamic acids compared with those of flavonols found by Lima et al. (2016) in leaves comparing ten white and red varieties of grapevines. Application of elevated temperatures had not a significant effect on the concentrations of hydroxycinnamic acids in leaves of Tempranillo, although a slight increase was observed in CL-260 and CL-1089 (Table 4). By contrast, the association of grapevine with AMF clearly induced the accumulation of these phenolic compounds in leaves from CL-1089 (Table 4). Hydroxycinnamic acids can exert an antifungal activity in some plant-pathogen interactions (Morrissey and Osbourn, 1999), although not in others (Latouche et al., 2013). Host plants can react by activating defense mechanisms in response to the colonization of their roots by AMF (García Garrido and Ocampo, 2002) and this defense response may include the accumulation of phenolic substances in the aerial part (Baslam et al., 2011). In our study, it is necessary to take into account that the results obtained with the model of fruit-bearing cuttings may be modulated by the presence of a rootstock. Indeed, grapevine root development is rootstock-dependent (Smart et al., 2006), thus, the expected benefit of the AMF symbiosis would also depend on the rootstock. However, the available data show that small differences exist among rootstocks in the ability to form AMF associations (Schreiner, 2003). In addition, there is recent evidence that AMF associated to rootstock can induce the accumulation of phenolic compounds in leaves of grafted cuttings of several *V. vinifera* cultivars (Bruisson et al., 2016).

Król et al. (2015) reported that the application of chilling diminished the ability to scavenge the DPPH• radical in grapevine leaf extracts. Contrariwise, the TAC in CL-260 showed an increasing tendency when applied high temperatures, mycorrhizal inoculum or both together (Table 4). Moreover, CL-1089 was the most sensitive to the elevated temperatures and the radical-scavenging capacity of its leaf extracts significantly increased at 28/18°C. In CL-1048, mycorrhizal symbiosis was the factor that improved TAC in leaves.

3.7. Principal component analysis of minerals and metabolites in leaves at fruit harvest (E-L38)

In order to obtain general trends concerning the effects of elevated temperatures and mycorrhizal symbiosis on primary and secondary metabolites as well as on mineral nutrients present in leaves of Tempranillo clones a principal component analysis (PCA) was performed. Fig. 4 shows the score (A) and the loading (B) plots of the PCA. The first principal component (PC1) covered about 22.92 % of the total variance and it clearly separated temperature treatments (Fig. 4A), some minerals (Ca, Mg, P and Mn) and proline (Fig. 4B). Otherwise, CL-1089 was separated from the other two clones (CL-260 and CL-1048) by the second principal component (PC2) which accounted for the 15.60 % of the variance (Fig. 4A). CL-1089 clone is characterized in the PCA by a higher content in flavonols, flavan-3-ols and hydroxycinnamic acids and lower content in Zn. Mycorrhizal symbiosis was not distinguished by PCA. Thus, clonal diversity mainly affected the secondary metabolism and Zn concentration while air temperature modified primary metabolism and the concentrations of several minerals in Tempranillo leaves. Phenolic compounds can function as antioxidants in plants subjected to stressful conditions (Oh et al., 2009). In our study, however, the PCA showed a strong correlation between TAC and carotenoids and chlorophylls in leaves and, to a lesser extent, a relationship between TAC and phenolic compounds (Fig. 4B).

In summary, elevated air temperatures induced the accumulation of several mineral nutrients in leaves of Tempranillo grapevines, especially in the CL-1089. In this clone, also TAC and TSS increased in leaves under warming temperatures. Leaves from CL-260 showed higher amounts of chlorophylls and carotenoids when subjected to high temperatures, while chlorophylls and TSP increased in leaves of CL-1048 under those conditions. Mycorrhizal symbiosis induced the accumulation of flavonols in leaves of the three studied clones,

increased the levels of hydroxycinnamic acids in leaves from CL-1089 and those of carotenoids in leaves of CL-260.

4. Conclusion

Our results support the interest of collecting Tempranillo grapevines leaves at fruit harvest in order to use them for nutritional purposes. In general, the nutritional value of leaves from Tempranillo grapevines may enhance under the predicted warming climate. However, the diversity in the response to increased temperatures suggests different applications for each clone: leaves from CL-260 and CL-1048 would be more adequate than those of CL-1089 for diabetic people and leaves from CL-260 -and to a lesser extent those from CL-1048- may be more suitable for culinary processes than leaves from CL-1089. The association of Tempranillo grapevines with AMF may provide an additional improvement of the nutritional value of leaves because it can induce the accumulation of flavonols in these three clones. Our results highlight differences in the leaf composition in terms of minerals, primary and secondary metabolites within Tempranillo grapevine cultivar due to clonal diversity and to the sensitivity of each clone to mycorrhizal colonization and/or air temperatures. However, further studies including rootstocks are needed to corroborate the conclusions obtained with the model of fruit bearing cuttings.

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Figure legends

Fig. 1. Mycorrhizal colonization of roots from fruit-bearing cuttings of Tempranillo clones (CL) grown at 24/14°C (24) or 28/18°C (28) (day/night) temperatures (T) during berry ripening. Data were collected at final fruit harvest (E-L38). Values are means ± SE (n = 3-5). Within each CL, histograms with the same letter indicate that values are not significantly different ($P > 0.05$) between treatments according to Duncan's test. One-way ANOVA was performed to evaluate the effect of temperature (T). ns, and * indicate non-significance or significance at 5% probability levels, respectively.

Fig. 2. Total soluble proteins (TSP) (mg g^{-1} DW) (A), proline (Pro) (µmol g^{-1} DW) (B), total soluble sugars (TSS) (mg g^{-1} DW) (C), and starch (mg g^{-1} DW) (D) in leaves from fruiting cuttings of Tempranillo clones (CL) grown at 24/14°C or 28/18°C day/night temperatures (T) during berry ripening. Data were collected at final fruit harvest (E-L38). Values represent means ± SE (n = 3-5). Two-way ANOVA analysis was made to evaluate temperature (T), mycorrhizal inoculation (M) and interaction (T×M) effects within each CL. ns, *, and ** indicate non-significance or significance at 5%, and 1% probability levels, respectively. Within each graph (A, B, C or D) and CL, different letters indicate significant differences ($P \leq 0.05$) according to Duncan's test. DW= dry weight.

Fig. 3. Total chlorophylls (a+b) (mg g^{-1} DW) (A) and total carotenoids (mg g^{-1} DW) (B) in leaves from fruiting cuttings of Tempranillo clones (CL) grown at 24/14°C or 28/18°C day/night temperatures (T) during berry ripening. Data were collected at final fruit harvest (E-L38). Values represent means ± SE (n = 3-5). Within each CL, two-way ANOVA analysis was made to evaluate temperature (T), mycorrhizal inoculation (M) and their interaction

(T×M) effects. ns, and * indicate non-significance or significance at 5% probability levels, respectively. Within each graph (A or B) and CL, different letters indicate significant differences ($P \leq 0.05$) according to Duncan's test. DW= dry weight.

Fig. 4. Principal component analysis score (A) and loading plot (B) obtained from the statistical analysis of minerals, primary and secondary metabolites, and total antioxidant capacity of the 60 studied samples in leaves from fruit-bearing cuttings of Tempranillo clones (CL) inoculated (+M) or not (-M) with arbuscular mycorrhizal fungi and grown at 24/14°C or 28/18°C day/night temperatures. Data were collected at final fruit harvest (E-L38). TAC = total antioxidant capacity; TSP = total soluble proteins; TSS = total soluble sugars.

Author contributions

Nazareth Torres performed experiments and analyzed data.

M Carmen Antolín designed research, supervised experiments, read and improved the manuscript.

Idoia Garmendia analyzed minerals, read and improved the manuscript.

Nieves Goicoechea designed research, supervised experiments and wrote the paper.

Table 1. Plant growth parameters and transpiration rate (*T*) of fruit-bearing cuttings of Tempranillo clones (CL) inoculated (+M) or not (-M) with arbuscular mycorrhizal fungi and grown at 24/14°C (24) or 28/18°C (28) day/night temperatures (T). Data were collected at final fruit harvest (E-L38).

	Treatments				*Main effects*				*ANOVA*
CL-260	-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
					24	28	-M	+M	T × M
Total leaf DW (g plant^{-1})	12.34 a	9.55 b	6.15 c	7.55 bc	10.94	6.85	9.25	8.55	*
Total leaf area (dm^2 plant^{-1})	19.37 a	16.69 ab	9.36 c	13.72 bc	18.03	11.54	14.36	15.20	*
SLW (g m^{-2})	63.65	57.04	69.10	55.57	60.35	60.33	66.38	56.31	ns
T (mmol H_2O m^{-2} s^{-1})	3.34	2.29	3.67	2.54	2.81	3.10	3.50 a	2.41 b	ns
	Treatments				*Main effects*				*ANOVA*
CL-1048	-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
					24	28	-M	+M	T × M
Total leaf DW(g plant^{-1})	12.88	11.44	9.87	6.60	12.16 a	8.23 b	11.38	9.02	ns
Total leaf area (dm^2 plant^{-1})	22.01	22.07	16.34	14.01	22.04 a	15.16 b	19.18	18.04	ns
SLW (g m^{-2})	58.20	51.60	60.21	47.84	54.90	54.03	59.21 a	49.73 b	ns
T (mmol H_2O m^{-2} s^{-1})	2.79 b	1.78 c	3.15 ab	3.87 a	2.29	3.51	2.97	2.83	*
	Treatments				*Main effects*				*ANOVA*
CL-1089	-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
					24	28	-M	+M	T × M

Total leaf DW (g plant^{-1})	16.47	14.06	8.49	9.18	15.26 a	8.83 b	12.48	11.62	ns
Total leaf area (dm^2 plant^{-1})	25.67	23.78	14.39	15.88	24.72 a	15.14 b	20.03	19.82	ns
SLW (g m^{-2})	66.15	60.84	58.98	58.03	63.49	58.50	62.56	59.44	ns
T (mmol H_2O m^{-2} s^{-1})	1.91 a	2.57 a	2.71 a	1.81 a	2.24	2.26	2.31	2.19	***

Values represent means (n = 3-5) separated by Duncan's test (at $P \leq 0.05$). Within lines and clones, means followed by different letters are significantly different as affected by the main factors temperature (T, 24, 28), mycorrhization (M, +M, -M) and their interaction (T × M). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns, not significant ($P > 0.05$). DW, dry weight; SLW, specific leaf weight; T, transpiration rate.

Table 2. Evolution of chlorophylls (SFR_G), plant nitrogen status (NBI_1), epidermal anthocyanins (ANTH_RG) and epidermal flavonols (FLAV) in leaves of fruit-bearing cuttings of Tempranillo clones (CL) inoculated (+M) or not (-M) with arbuscular mycorrhizal fungi and grown at 24/14°C (24) or 28/18°C (28) day/night temperatures (T) during berry ripening.

		Treatments				*Main effects*				*ANOVA*
CL-260		-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
						24	28	-M	+M	T × M
SFR_G	E-L34	1.88	2.29	2.04	2.31	2.09	2.18	1.96 b	2.30 a	ns
	E-L35	1.94 a	1.58 b	2.03 a	2.13 a	1.76	2.08	1.99	1.85	*
	E-L36	1.91	1.86	2.56	2.56	1.88 b	2.56 a	2.23	2.21	ns
	E-L37	2.35	2.20	2.29	2.34	2.27	2.32	2.32	2.27	ns
NBI_1	E-L34	1.14 a	0.73 bc	0.59 c	0.84 b	0.93	0.72	0.86	0.79	***
	E-L35	0.86	1.01	0.79	0.93	0.93	0.86	0.82	0.97	ns
	E-L36	0.81	0.92	0.7	0.93	0.86	0.82	0.76 b	0.93 a	ns
	E-L37	0.75	0.65	0.83	0.9	0.70 b	0.86 a	0.79	0.77	ns
ANTH_RG	E-L34	0.98	0.98	1.01	0.98	0.98	1.00	1.00	0.98	ns
	E-L35	0.98 b	1.05 a	0.97 b	0.95 b	1.02	0.96	1.00	0.99	**
	E-L36	0.97	1.01	0.93	0.93	0.99 a	0.93 b	0.95	0.97	ns
	E-L37	0.96	1.00	0.97	0.95	0.98	0.96	0.96	0.97	ns
FLAV	E-L34	1.17 a	0.78 ab	0.01 c	0.53 bc	1.01	0.29	0.66	0.63	**
	E-L35	0.59 bc	1.05 a	0.40 c	0.96 ab	0.82	0.68	0.50	1.01	*
	E-L36	0.42 ab	0.80 a	0.03 b	0.68 a	0.61	0.33	0.19	0.74	**
	E-L37	0.21	1.00	0.43	0.59	0.46	0.51	0.32	0.72	ns
		Treatments				*Main effects*				*ANOVA*
CL-1048		-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
						24	28	-M	+M	T × M
SFR_G	E-L34	2.71 a	2.50 ab	2.27 b	2.59 ab	2.00	2.43	2.49	2.54	*
	E-L35	1.63	1.95	2.16	2.30	1.79 b	2.23 a	1.89	2.12	ns
	E-L36	2.10	2.03	2.53	2.46	2.06 b	2.49 a	2.29	2.25	ns
	E-L37	1.95	2.04	2.50	2.49	1.99 b	2.50 a	2.23	2.26	ns
NBI_1	E-L34	0.59 b	0.64 b	0.96 a	0.67 b	0.61	0.82	0.77	0.66	**

	E-L35	1.01	1.04	0.85	1.04	1.03	0.94	0.93	1.04	ns
	E-L36	0.85	1.02	0.76	0.77	0.93 a	0.76 b	0.81	0.89	ns
	E-L37	0.91	0.81	0.82	1.01	0.86	0.92	0.87	0.91	ns
ANTH_RG	E-L34	0.95 b	0.96 ab	1.00 a	0.93 b	0.96	0.97	0.98	0.94	*
	E-L35	1.01	1.09	0.95	0.94	1.05 a	0.95 b	0.98	1.02	ns
	E-L36	0.99	1.00	0.95	0.95	0.99 a	0.95 b	0.97	0.98	ns
	E-L37	0.98	1.02	0.94	0.93	1.00 a	0.94 b	0.96	0.97	ns
FLAV	E-L34	0.05	0.65	0.86	0.27	0.35 b	0.62 a	0.59	0.43	ns
	E-L35	0.99	1.23	0.85	0.88	1.11	0.87	0.93	1.06	ns
	E-L36	0.61	1.41	0.26	1.10	0.95	0.62	0.47	1.28	ns
	E-L37	0.99	0.77	0.56	0.87	0.87	0.71	0.75	0.82	ns
		Treatments				*Main effects*				*ANOVA*
CL-1089		-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
						24	28	-M	+M	T × M
SFR_G	E-L34	1.46 c	1.84 b	2.17 a	1.85 b	1.67	2.03	1.85	1.85	***
	E-L35	1.75	1.96	2.05	1.93	1.85	2.00	1.90	1.94	ns
	E-L36	1.87 b	1.87 b	2.13 b	2.63 a	1.87	2.35	2.00	2.20	*
	E-L37	2.12	2.07	2.39	2.39	2.09	2.39	2.25	2.21	ns
NBI_1	E-L34	0.94 a	0.98 a	0.97 a	0.65 b	0.96	0.83	0.96	0.83	***
	E-L35	1.03 ab	1.16 a	0.94 bc	0.81 c	1.09	0.88	0.98	1.00	**
	E-L36	1.04 b	1.16 a	0.88 c	0.90 c	1.10	0.89	0.96	1.04	*
	E-L37	0.85	0.83	0.99	0.99	0.84 b	0.99 a	0.92	0.90	ns
ANTH_RG	E-L34	1.07 a	1.03 a	0.97 b	1.05 a	1.05	1.00	1.01	1.04	***
	E-L35	1.04 a	0.97 b	0.98 ab	0.99 ab	1.00	0.99	1.01	0.98	*
	E-L36	0.99	0.98	0.98	0.93	0.99 a	0.96 b	0.99 a	0.96 b	ns
	E-L37	0.97	1.02	0.92	0.93	0.99 a	0.92 b	0.95 b	0.98 a	ns
FLAV	E-L34	1.02 a	0.97 a	0.86 a	0.14 b	0.99	0.54	0.93	0.61	**
	E-L35	1.10 a	1.20 a	0.80 b	0.50 c	1.15	0.66	0.95	0.89	**
	E-L36	1.05 b	1.27 a	0.72 c	0.60 c	1.16	0.67	0.88	0.97	***
	E-L37	0.71	0.80	0.76	0.83	0.75	0.80	0.73	0.82	ns

Values represent means (n = 3-5) separated by Duncan's test (at $P \leq 0.05$). Within lines and clones, means followed by different letters are significantly different as affected by the main factors temperature (T, 24, 28), mycorrhization (M, +M, -M) and their interaction (T × M). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns, not significant ($P > 0.05$).

Table 3. Mineral concentration in leaves of fruit-bearing cuttings of Tempranillo clones (CL) inoculated (+M) or not (-M) with arbuscular mycorrhizal fungi and grown at 24/14°C (24) or 28/18°C (28) day/night temperatures (T). Data were collected at final fruit harvest (E-L38).

		Treatments				*Main effects*				*ANOVA*
CL-260		-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
						24	28	-M	+M	T × M
Macronutrients	C	473.86	478.36	476.16	475.00	476.11	475.58	475.01	476.68	ns
(mg g^{-1} DW)	N	37.66	40.58	40.10	42.48	39.12	41.29	38.88	41.53	ns
	Ca	16.75	17.18	21.85	22.07	16.97 b	21.96 a	19.30	19.63	ns
	K	9.39	9.47	9.13	10.41	9.43	9.77	9.26	9.94	ns
	P	2.95	2.78	4.60	4.66	2.87 b	4.63 a	3.78	3.72	ns
	Mg	2.87	3.26	4.45	4.14	3.07 b	4.30 a	3.66	3.70	ns
Micronutrients	Cu	6.36	7.60	7.24	7.43	6.98	7.34	6.80	7.52	ns
(mg kg^{-1} DW)	Zn	35.63	28.90	30.07	34.29	32.27	32.18	32.85	31.60	ns
	Fe	86.00 b	80.38 b	82.81 b	111.98 a	83.19	97.40	84.41	96.18	*
	Mn	212.67	203.13	270.74	300.75	207.90 b	285.75 a	241.71	251.94	ns
		Treatments				*Main effects*				*ANOVA*
CL-1048		-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
						24	28	-M	+M	T × M
Macronutrients	C	472.64	472.08	472.52	477.66	472.36	475.09	472.58	474.87	ns
(mg g^{-1} DW)	N	39.16 b	37.62 b	40.26 b	42.94 a	38.39	41.60	39.71	40.28	*
	Ca	15.19	16.91	23.12	18.97	16.05 b	21.05 a	19.16	17.94	ns
	K	9.56	9.24	8.46	8.62	9.40	8.54	9.01	8.93	ns
	P	2.38 c	3.20 b	4.61 a	3.99 a	2.79	4.30	3.50	3.60	**
	Mg	1.79 c	1.89 c	4.26 a	3.20 b	1.84	3.73	3.03	2.55	**

Micronutrients	Cu	5.19	4.87	6.75	7.06	5.03 b	6.91 a	5.97	5.97	ns
(mg kg $^{-1}$ DW)	Zn	38.65	41.21	46.57	37.34	39.93	41.96	42.61	39.28	ns
	Fe	77.94	73.13	99.67	72.87	75.54	86.27	88.81 a	73.00 b	ns
	Mn	147.56 c	218.52 b	296.33 a	246.87 b	183.04	271.6	221.95	232.70	**
		Treatments				*Main effects*				*ANOVA*
CL-1089		-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
						24	28	-M	+M	T × M
Macronutrients	C	477.80 a	468.96 b	472.70 ab	475.70 ab	473.38	474.20	475.25	472.33	*
(mg g^{-1} DW)	N	39.50	38.10	41.58	41.58	38.80	41.58	40.54	39.84	ns
	Ca	15.76	19.09	21.72	24.29	17.43 b	23.01 a	18.74	21.69	ns
	K	8.84	9.35	8.01	8.43	9.10	8.22	8.43	8.89	ns
	P	2.32	2.81	3.86	4.24	2.57 b	4.05 a	3.09	3.53	ns
	Mg	2.18	2.43	4.07	3.83	2.31 b	3.95 a	3.13	3.13	ns
Micronutrients	Cu	4.79	3.76	6.92	6.26	4.28 b	6.59 a	5.86	5.01	ns
(mg kg $^{-1}$ DW)	Zn	20.65	20.52	28.30	23.68	20.60 b	25.99 a	24.50	22.10	ns
	Fe	74.55	79.67	77.94	84.79	77.11	81.37	76.25	82.23	ns
	Mn	190.26	234.85	253.93	293.68	212.56 b	273.81 a	222.10	264.27	ns

Values represent means (n = 3-5) separated by Duncan's test (at $P \leq 0.05$). Within lines and clones, means followed by different letters are significantly different as affected by the main factors temperature (T, 24, 28), mycorrhization (M, +M, -M) and their interaction (T × M). * $P \leq 0.05$; ** $P \leq 0.01$; ns, not significant ($P > 0.05$). DW, dry weight.

Table 4. Phenolic composition and total antioxidant capacity (TAC) in leaves of fruit-bearing cuttings of Tempranillo clones(CL) inoculated (+M) or not (-M) with arbuscular mycorrhizal fungi and grown at 24/14°C (24) or 28/18°C (28) day/night temperatures (T). Data were collected at final fruit harvest (E-L38).

		Treatments				*Main effects*				*ANOVA*
CL-260		-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
						24	28	-M	+M	T × M
Phenolic compounds										
(mg g^{-1} DW)	Flavonoids	31.89	30.70	26.34	32.26	31.30	29.30	29.11	31.48	ns
	Flavonols	16.13	22.28	20.03	23.75	19.20	21.89	18.25 b	23.02 a	ns
	Flavan-3-ols	4.37 b	7.49 a	8.48 a	6.64 ab	5.93	7.56	6.43	7.07	**
	Hydroxycinnamic acids	16.74	23.24	24.45	24.78	19.99	24.62	20.60	24.01	ns
TAC										
(mg g^{-1} DW)		1.55	2.12	2.42	2.08	1.83	2.25	1.99	2.10	ns
		Treatments				*Main effects*				*ANOVA*
CL-1048		-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
						24	28	-M	+M	T × M
Phenolic compounds										
(mg g^{-1} DW)	Flavonoids	59.99 a	28.81 b	29.14 b	32.63 b	44.40	30.88	44.57	30.72	**
	Flavonols	22.64	25.03	16.47	22.14	23.84 a	19.30 b	19.55 b	23.59 a	ns
	Flavan-3-ols	5.85	4.11	3.60	4.05	4.98	3.82	4.72	4.08	ns
	Hydroxycinnamic acids	24.07	29.28	20.05	24.31	26.68	22.18	22.06	26.80	ns
TAC										
(mg g^{-1} DW)		1.67	2.85	1.94	2.60	2.26	2.27	1.80 b	2.73 a	ns
		Treatments				*Main effects*				*ANOVA*
CL-1089		-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
						24	28	-M	+M	T × M
Phenolic compounds										
(mg g^{-1} DW)	Flavonoids	35.46	42.19	38.65	40.36	38.83	39.51	37.06	41.28	ns

	Flavonols	19.68	39.14	28.07	41.34	29.41 b	34.71 a	23.88 b	40.24 a	ns
	Flavan-3-ols	6.12 c	9.61 b	12.65 a	9.20 b	7.87	10.93	9.38	9.41	**
	Hydroxycinnamic acids	21.73	42.27	29.59	45.80	32.00	37.69	25.66 b	44.03 a	ns
TAC (mg g^{-1} DW)		1.62	1.90	2.24	2.51	1.76 b	2.37 a	1.93	2.20	ns

Values represent means (n = 3-5) separated by Duncan's test (at $P \leq 0.05$). Within lines and clones, means followed by different letters are significantly different as affected by the main factors temperature (T, 24, 28), mycorrhization (M, +M, -M) and their interaction (T × M). ** $P \leq 0.01$; ns, not significant ($P > 0.05$). DW, dry weight.

Figure

Figure 1

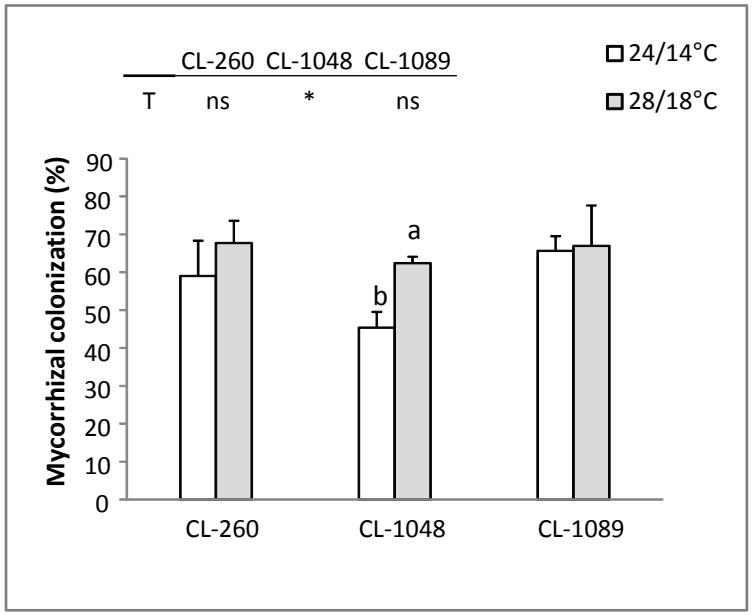
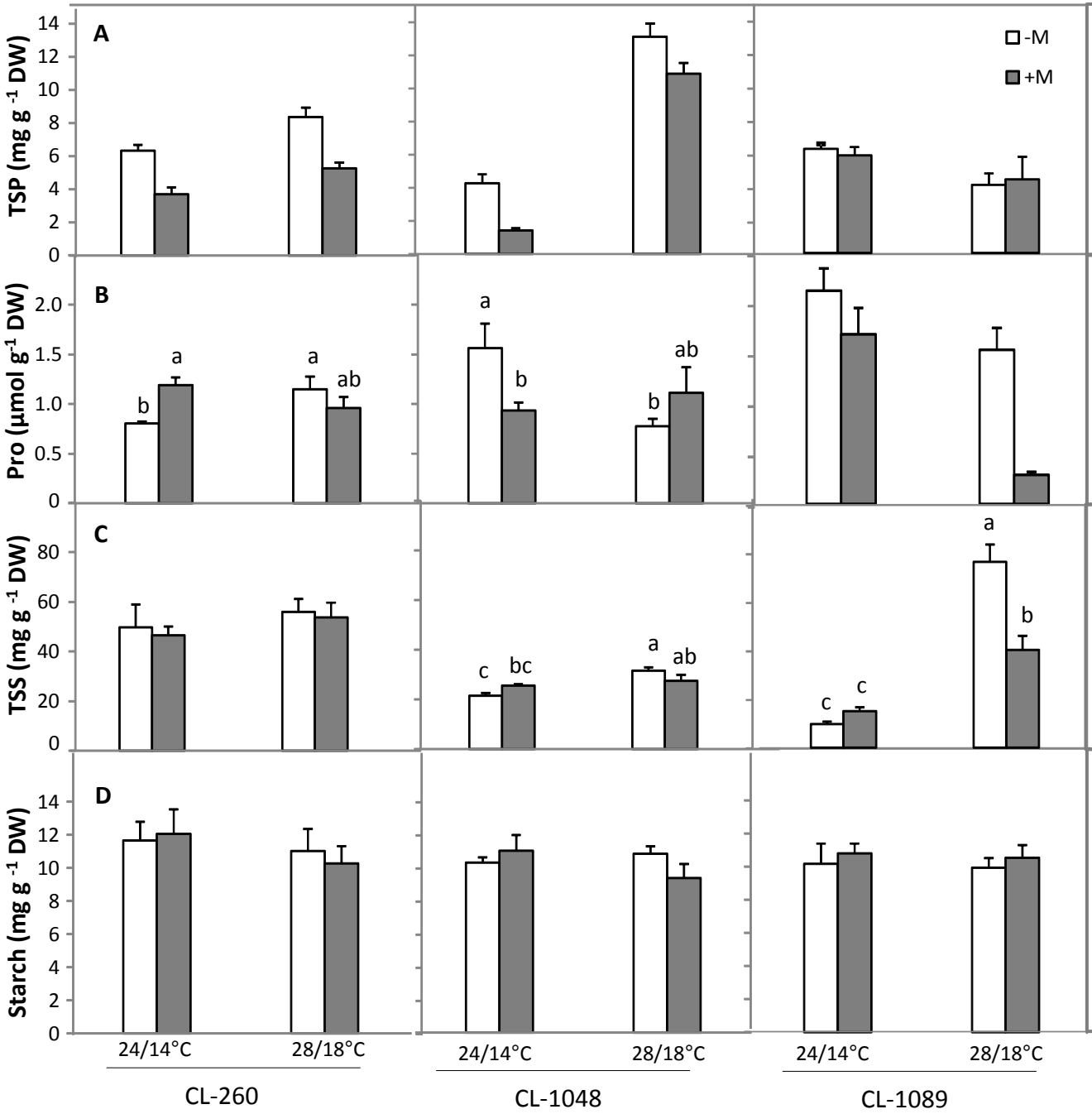


Figure 2



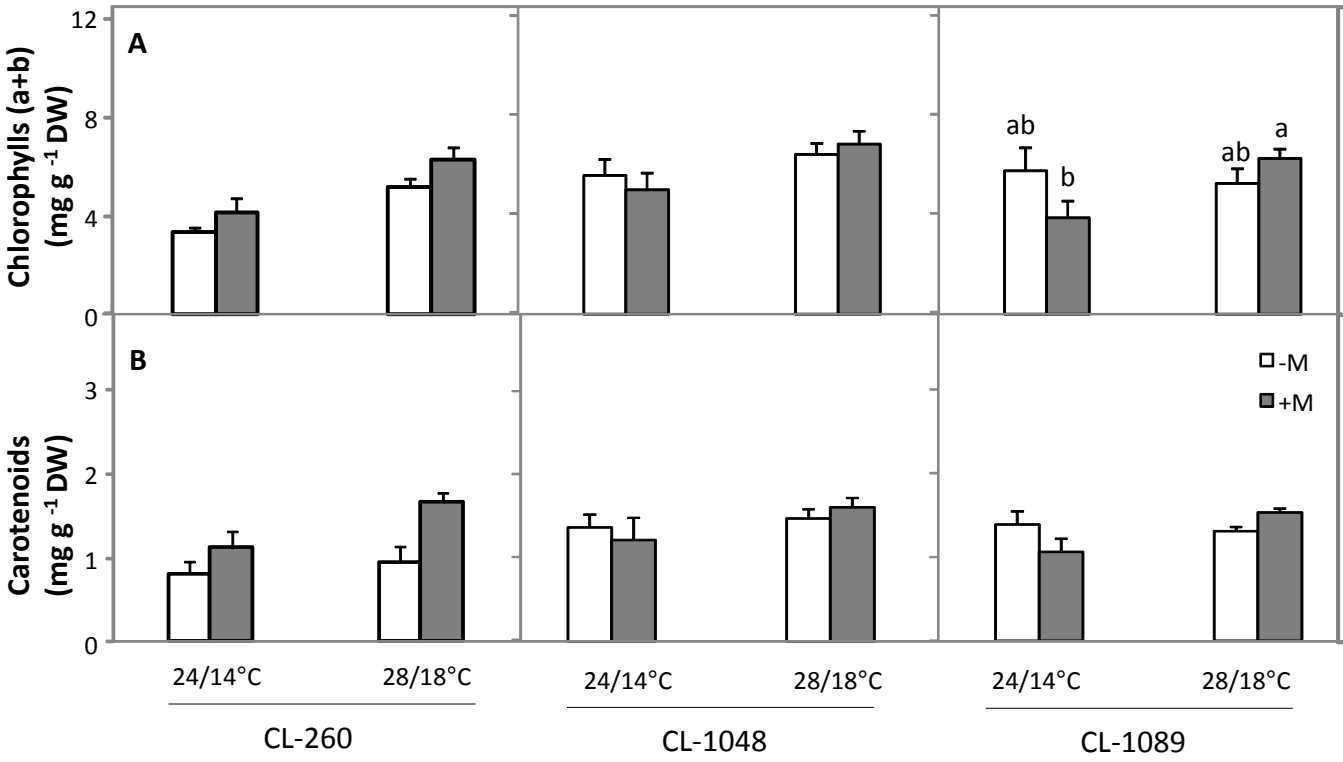
TSP (mg g^{-1} DW)	Main effects: T 24	T 28	M -M	M +M	ANOVA TxM
CL-260	5.02 b	6.83 a	7.35 a	4.50 b	ns
CL-1048	2.85 b	12.01 a	8.70 a	6.16 b	ns
CL-1089	6.02 a	4.23 b	5.14	5.11	ns

Pro (µmol g^{-1} DW)	Main effects: T 24	T 28	M -M	M +M	ANOVA TxM
CL-260	1.00	1.05	0.98	1.08	*
CL-1048	1.25	0.94	1.17	1.02	*
CL-1089	1.93 a	0.92 b	1.85 a	1.00 b	ns

TSS (mg g^{-1} DW)	Main effects: T 24	T 28	M -M	M +M	ANOVA TxM
CL-260	48.11	54.76	52.80	50.08	ns
CL-1048	23.51	29.51	26.54	26.47	*
CL-1089	12.17	57.10	42.18	27.09	**

Starch (mg g^{-1} DW)	Main effects: T 24	T 28	M -M	M +M	ANOVA TxM
CL-260	11.87	10.65	11.35	11.17	ns
CL-1048	10.62	10.07	10.54	10.15	ns
CL-1089	10.49	10.24	10.05	10.67	ns

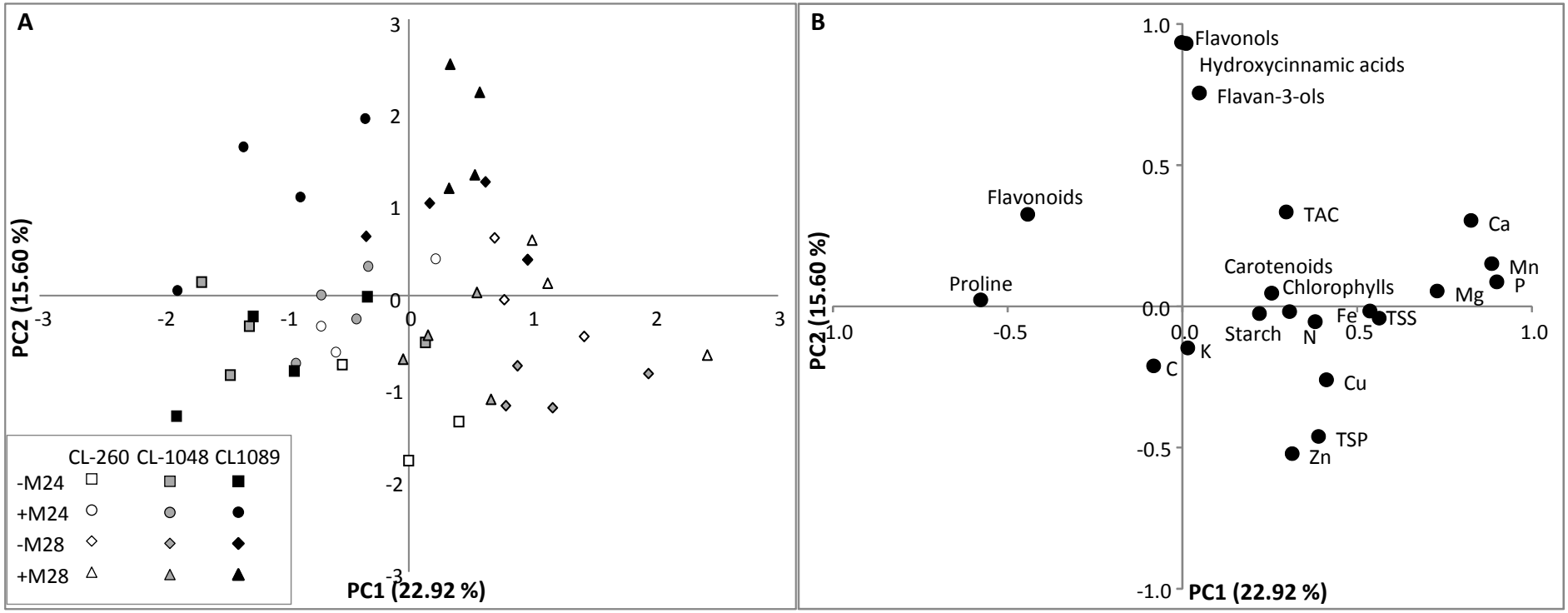
Figure 3



Chlorophylls (mg g^{-1} DW)	*Main effects*				*ANOVA*
	T		M		TxM
	24	28	-M	+M	
CL-260	3.76 b	5.73 a	4.28	5.21	ns
CL-1048	5.26 b	6.61 a	5.98	5.89	ns
CL-1089	4.79	5.74	5.49	5.04	*

Carotenoids (mg g^{-1} DW)	*Main effects*				*ANOVA*
	T		M		TxM
	24	28	-M	+M	
CL-260	0.95 b	1.30 a	0.87 b	1.38 a	ns
CL-1048	1.29	1.53	1.41	1.40	ns
CL-1089	1.22	1.42	1.35	1.29	ns

Figure 4


A
PC2 (15.60 %)
PC1 (22.92 %)
CL-260 CL-1048 CL1089
-M24
+M24
-M28
+M28
B
PC2 (15.60 %)
PC1 (22.92 %)
Flavonols
Hydroxycinnamic acids
Flavan-3-ols
Flavonoids
TAC
Ca
Carotenoids
Mn
P
Proline
Chlorophylls
Mg
Fe
TSS
Starch
N
K
C
Cu
TSP
Zn