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INFLUENCIA DE LA INOCULACIÓN MICORRÍCICA SOBRE LA COMPOSICIÓN FENÓLICA Y LA CAPACIDAD ANTIOXIDANTE DE LA VID (*VITIS VINIFERA* L.) CV. TEMPRANILLO EN DIFERENTES ESCENARIOS DE CAMBIO CLIMÁTICO

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Memoria presentada por Dª Nazareth Torres Molina para aspirar al grado de Doctor por la Universidad de Navarra

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El presente trabajo ha sido realizado bajo mi dirección en el Departamento de Biología Ambiental y autorizo su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, 17 de mayo de 2018

(firma de la Directora de la Tesis Doctoral)

Dra. Mª Carmen Antolín Bellver

(firma de la Co-directora de la Tesis Doctoral)

Dra. Nieves Goicoechea Preboste

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“Lock up your libraries if you like; but there is no gate, no lock, no bolt that you can set upon the freedom of my mind.” Virginia Woolf

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Abstract

The foreseen climate change is going to affect the agricultural suitability, viticulture being one of those sectors more sensible to environmental constraints. Projected warming combined with severe droughts in the growing season is expected to have detrimental impacts on the grape berry ripening affecting the berry quality. Thus, the demonstrated intra-varietal diversity of cv. Tempranillo has led to the search of new clones able to cope with the projected scenarios. On the other hand, new strategies such as deficit irrigation of vineyards have emerged as a promising tool to withstand water stress in this new challenging scenario. Also, the promotion of the symbiotic association of grapevines with arbuscular mycorrhizal fungi (AMF) which are known to benefit host plants by improving nutrient uptake, growth and water status of grapevines and helping them to cope with abiotic stresses. However, less is known about their effect on the phenolic content and antioxidant properties under climate change scenarios as well as, the role that plant hormones, which interact to regulate the establishment and functioning of symbiotic associations with AMF, play under abovementioned conditions. Besides, the cultivation of grapevines produced a huge amount of pruning wastes which could be reused in the pharmaceutical and nutritional industries due to their elevated content in nutraceuticals and antioxidant metabolites. Again, the role of AMF on the primary and secondary metabolism of grapevine leaves under elevated temperature remains unclear. Taking all into account the general objective of this PhD thesis was to assess the intra-varietal diversity of grapevine cv. Tempranillo to respond to different climate change scenarios (elevated air temperature and deficit irrigation) and to analyze if the potential benefits of mycorrhizal symbiosis on plant metabolism could be maintained under the predicted environmental conditions. The study was carried out on fruit-bearing cuttings clones of cv. Tempranillo with different agronomic traits and origins (CL-260, CL-1048, CL-1089, CL-8, CL-280 and CL-843) inoculated (+M) or not (−M) with AMF and grown under controlled conditions. Plants were subjected to two temperature regimes (24/14°C and 28/18°C (day/night)) from fruit set to berry maturity and different irrigation regimes (early season deficit irrigation, ED; late season deficit irrigation, LD; and full irrigation, FI) throughout berry development.

Berry skin metabolism of Tempranillo was clone-dependent resulting in different abilities to respond to deficit irrigation under warming. Under our experimental conditions, early deficit (ED) irrigation was worse than late deficit (LD) at 24/14°C concerning anthocyanin and flavonol levels and berry traits, however, such differences were attenuated at 28/18°C. Primary metabolism was mainly affected by temperature whereas deficit irrigation was the main factor affecting secondary metabolism. Both factors modified the amino acid, anthocyanin and flavonol profiles. Clonal diversity of Tempranillo also resulted in different abilities to respond to AMF inoculation under elevated

temperature. Thus, in CL-1048, AMF inoculation avoided warming effects on berry quality by improving antioxidant properties. This protective role of AMF under elevated temperatures was also evidenced when plants were subjected to deficit irrigation. AMF inoculation improved the potential benefits of LD at 28/18°C although this benefit was dependent on the intra-varietal differences of Tempranillo. Results underlined the importance of implementing measures to promote AMF-grapevines symbiosis in order to optimize the effect of irrigation strategy on berry properties under elevated temperature. In view of abscisic acid (ABA) involvement in the berry ripening regulation, we have investigated the potential role of AMF inoculation on the levels of free ABA and its catabolites throughout berry development and ripening. Results showed that ABA catabolism/conjugation was modified by AMF inoculation and by the climate change conditions evidencing that 7´OH-ABA has an important role in the berry quality of Tempranillo. In fact, the AMF inoculation modified berry ABA catabolism, leading to increased 7´OH-ABA content, providing an explanation to the ability of AMF to improve berry quality under global warming scenarios.

Tempranillo intra-varietal differences were also shown on the leaf phenolic composition and antioxidant properties responding to AMF inoculation and elevated temperature. Thereby, AMF inoculation at 28/18°C improved total soluble phenolics, flavonols and anthocyanins which contributed to a higher antioxidant power of grapevine leaves. Furthermore, the nutritional value of Tempranillo leaves was also enhanced under the predicted warming climate conditions by increasing several minerals, soluble sugars, photosynthetic pigments and soluble proteins, and by means of mycorrhizal inoculation which induced flavonol and hydroxycinnamic acid accumulation. Given this high nutritional value, Tempranillo leaves could be used for animal or human nutrition or as a food supplement. Finally, foliar extracts of Tempranillo exhibited high cytotoxic activity against some cancer cell lines, which strengthens the potential application of these agricultural wastes for biomedical purposes.

This dissertation provided evidence of the broad diversity within Tempranillo variety highlighting the importance of an appropriate clonal selection based on the plausible applications and/or the environmental or biotic modulator factors and demonstrates the importance of adopting measures to protect the indigenous cohorts of AMF in vineyards. Moreover, since each clone responded differently to each mycorrhizal inoculum and responses varied according to the environmental conditions, it may be profitable to identify the AMF inoculants most suitable for a given clone in a given environment.

Key words: Abscisic acid metabolism; Amino acids; Anthocyanins; Arbuscular mycorrhizal fungi (AMF); Clones; Global warming; Flavonols; Grapevines; Intra-varietal diversity; Phenolic compounds; Tempranillo; Total antioxidant capacity; Regulated deficit irrigation; Vegetative wastes.

Resumen

El cambio climático previsto va a afectar a la idoneidad de la agricultura, siendo la viticultura uno de los sectores más afectados por las condiciones medioambientales. Las predicciones de aumento de las temperaturas junto con períodos de sequía severa durante la estación de crecimiento de la vid van a tener un impacto muy negativo sobre la maduración de la baya afectando a la calidad de la misma. Todo ello sumado a la gran diversidad intra-varietal del cv. Tempranillo, ha impulsado la búsqueda de nuevos clones capaces de tolerar los futuros escenarios de cambio climático. Por otro lado, nuevas estrategias como el riego deficitario de viñedos han emergido como técnicas prometedoras para soportar el estrés hídrico asociado a las nuevas condiciones climáticas. También la promoción de la asociación simbiótica de vides con hongos micorrícicos arbusculares (HMA), los cuales benefician a la planta huésped mejorando la toma de nutrientes, el crecimiento y el estado hídrico de las vides, pudiendo ayudar a tolerar los estreses abióticos. Sin embargo, todavía se desconoce su efecto sobre el contenido fenólico y las propiedades antioxidantes en condiciones de cambio climático así como, el papel que las hormonas de la planta, las cuales interactúan para regular el establecimiento y funcionamiento de la asociación simbiótica con HMA, puedan desempeñar en dichas condiciones. Por otro lado, la viticultura genera una gran cantidad de deshechos de poda, los cuales podrían ser reutilizados en las industrias farmacéutica y nutricional debido a su alto contenido en nutracéuticos y metabolitos antioxidantes. A este respecto, todavía no se conoce claramente el papel que los HMA desempeñan en el metabolismo primario y secundario de las hojas de vides crecidas a temperaturas elevadas. Teniendo en cuenta todos estos antecedentes, el principal objetivo de esta tesis fue establecer la diversidad intra-varietal de vides cv. Tempranillo en respuesta a diferentes escenarios de cambio climático (temperatura elevada y déficit hídrico) y analizar si en las futuras condiciones medioambientales, los potenciales beneficios de la simbiosis micorrícica sobre el metabolismo de la planta se mantendrán. El estudio se llevó a cabo con esquejes fructíferos del cv. Tempranillo con diferentes características agronómicas y procedencias (CL-260, CL-1048, CL-1089, CL-8, CL-280 and CL-843) inoculados (+M) o no (−M) con HMA y crecidos en condiciones controladas. Las plantas fueron sometidas a dos regímenes de temperatura (24/14°C o 28/18°C (día/noche)) desde cuajado hasta la madurez de la baya y diferentes estrategias de riego (riego deficitario temprano, ED; riego deficitario tardío, LD; y riego completo, FI) aplicados durante el desarrollo del fruto.

El metabolismo del hollejo de las bayas de Tempranillo varió en función del clon analizado lo que se tradujo en diferentes habilidades para responder al déficit hídrico bajo temperaturas elevadas. En nuestras condiciones experimentales, con el riego deficitario temprano (ED) se obtuvieron peores resultados que con el riego deficitario tardío (LD) a 24/14°C con respecto a los niveles de

antocianinas y flavonoles y a los rasgos de la baya, sin embargo, estas diferencias se atenuaron a 28/18°C. El metabolismo primario de las bayas se vio principalmente afectado por el aumento de temperatura mientras que las diferentes estrategias de riego modificaron preferentemente el metabolismo secundario. Ambos factores modificaron los perfiles de aminoácidos, flavonoles y antocianinas. La diversidad clonal de Tempranillo también resultó en diferentes habilidades para responder a la inoculación micorrícica bajo altas temperaturas. Así, en el CL-1048, la inoculación con HMA evitó los efectos sobre la calidad de la baya asociados al calentamiento debido a la mejora de las propiedades antioxidantes. Este papel protector de los HMA a altas temperaturas también se evidenció cuando las plantas se sometieron a riego deficitario. De esta manera, la inoculación con HMA mejoró los potenciales beneficios del riego LD a 28/18°C, aunque este efecto también se dio en función de las diferencias intra-varietales de Tempranillo. Los resultados subrayan la importancia de la implementación de medidas para promover la simbiosis entre vides y HMA para así, optimizar los efectos de la estrategia de riego sobre las propiedades de la baya bajo temperatura elevada. Dado que el ácido abscísico (ABA) está implicado en la regulación de la maduración de la baya, hemos investigado el papel potencial de la inoculación micorrícica sobre los niveles de ABA libre y sus catabolitos a lo largo del desarrollo y la maduración de la baya. Los resultados demostraron que la conjugación/catabolismo del ABA se modificó por la inoculación micorrícica y por las condiciones de cambio climático evidenciando el papel importante que el 7´OH-ABA juega en la calidad de la baya de Tempranillo. De hecho, la inoculación micorrícica modifico el catabolismo del ABA en las bayas, aumentando el contenido de 7´OH-ABA lo que se pudo relacionar con la mejora de la calidad de la baya en respuesta a la inoculación micorrícica en escenarios de cambio climático. La diversidad intra-varietal de Tempranillo también se expresó en las variaciones de la composición fenólica de la hoja y de las propiedades antioxidantes en respuesta a la inoculación micorrícica y a la temperatura. Así, la inoculación micorrícica a 28/18°C mejoro los fenoles solubles totales, los flavonoles y las antocianinas lo cual contribuyo a una mayor capacidad antioxidante de las hojas de vid. Por otra parte, la temperatura elevada mejoró el valor nutricional de las hojas de Tempranillo debido al aumento de muchos minerales, azucares solubles, pigmentos fotosintéticos y proteínas solubles, y por medio de la inoculación micorrícica que indujo la acumulación de flavonoles y ácidos hidroxicinámicos. Dado el alto valor nutricional de las hojas de Tempranillo, se podrían utilizar para alimentación animal o humana o cómo suplemento alimentario. Finalmente, los extractos foliares de Tempranillo mostraron una alta actividad citotóxica frente a algunas líneas celulares de cáncer, lo cual reforzó la posible aplicación de estos residuos agrícolas para su aplicación biomédica.

Este trabajo evidenció la importancia de una adecuada selección clonal en el cv. Tempranillo debido a su amplia diversidad en función de las posibles aplicaciones y de los factores bióticos y ambientales moduladores y demostró la importancia de adoptar medidas para proteger las cohortes micorrícicas

en los viñedos. Además, ya que cada clon respondió de manera diferente a cada inóculo y que las respuestas variaron según las condiciones ambientales, sería aconsejable identificar los inóculos micorrícicos más adecuados para un determinado clon en un determinado ambiente.

Palabras clave: Metabolismo del ácido abscísico; Aminoácidos; Antocianinas; Hongos micorrícicos arbusculares (HMA); Clones; Calentamiento global; Flavonoles; Vid; Diversidad intra-varietal; Compuestos fenólicos; Tempranillo; Capacidad antioxidante total; Riego deficitario controlado; Residuos vegetativos.

ÍNDICE

INTRODUCCIÓN GENERAL

Adapted from: Torres, N., Antolín, M. C. Goicoechea, N. (2018). Arbuscular mycorrhizal symbiosis as a promising resource for improving berry quality in grapevines under changing environments. *Frontiers in Plant Science (doi: 10.3389/fpls.2018.00897)*

1. General Introduction:

Grapevine (*Vitis vinifera* L.) is an important perennial crop worldwide, consumed as fresh fruit or produced to make wine. Not only is the grapevine one of the most cultivated crops in Europe, with a production of 23.4 million tons (Eurostat Statistics Division, 2016), which represents the 39% of the world production but also, several studies have shown that the moderate consumption of wine is beneficial for human health (Georgiev et al., 2014; Artero et al., 2015). However, projected climate change is expected to have detrimental impacts on the grapes and wine quality and properties due to this crop is shown as highly dependent upon climatic conditions during the growing season.

Mutualistic plant-microbe interactions offer a novel approach to enhance agricultural productivity while reducing environmental costs (Hamilton et al., 2016). Numerous studies have demonstrated that (1) climate change may affect all types of beneficial plant-microorganism interactions and (2) plant-associated microorganisms are an important factor modulating the response of plants to climate change (Compant et al., 2010). Among these beneficial microorganisms, it is worth noting the arbuscular mycorrhizal fungi (AMF), because they can establish mutualistic association with most crops (Smith and Read, 2008) and have an increasingly important role in vineyard production systems (Trouvelot et al., 2015). Currently, in intensive agricultural production systems such as vineyards, new insights are essential to increase competitiveness and minimize the environmental impacts of agricultural practices (Nicolás et al., 2015). Although some aspects of effects of global climate change on grapevine cultivation are recently reviewed (Costa et al., 2016; Mosedale et al., 2016; Alonso et al., 2016b; Schultz, 2016; van Leeuwen and Darriet, 2016) so far, the mycorrhizal-mediated responses to grapevine under changing environments have not been updated. In this review, we summarize recent research progress on the effects of climate change on grapevine physiology and metabolism and the benefits that AMF can report for improving fruit quality and enhancing the adaptation of grapevines to the predicted environmental conditions in future climate change scenarios.

2. Climate change on a global basis for viticulture and for beneficial plant-microorganisms interactions

2.1. Climate change scenarios for viticulture and the expected consequences

The increasing research interest in the climate change effect on viticulture is not surprising due to the high socioeconomic relevance of the winemaking sector worldwide. Furthermore, climate change and their resulting impacts is becoming a concern for winegrowers (Fraga et al., 2013; Neethling et al., 2017). For most wine-production regions of the world, long-term climate records have shown rising temperatures (Webb et al., 2013; Barnuud et al., 2014; Fraga et al., 2016) together with shifting patterns in rainfall and extreme weather events (Andrade et al., 2012; IPCC, 2014).

Warming temperatures have been linked to anthropogenic climate change and are likely to continue. Thus, in most scenarios without additional mitigation efforts, atmospheric concentrations of CO_2 could reach about 1000 ppm by 2100 and, thus, air temperature is likely to exceed 4°C above pre-industrial levels (IPCC, 2014). This rise of temperature is expected to have detrimental impacts on grapevine physiology and quality (Mosedale et al., 2016) and to increase the risk of pests and diseases, especially with warmer winters (Caffarra et al., 2012). Consequently, heat waves during the growing season could impair vine productivity and cause a greater loss of water from the soil (Schultz, 2016). In addition, the absence of precipitation, considered as a major limiting factor for plant growth, is frequently accompanied by increased UV-B radiation levels (Bandurska et al., 2013).

This new scenario may allow future wine production in areas that are presently too cold for wine cultivation, while the actual grape growing regions may become unsuitable for premium wine production and will have to adapt to these changes (Hannah et al., 2013; Roy et al., 2017). The establishment of vineyards at higher altitudes (colder areas) and the election of exposures that lead to a lower interception of solar radiation would be some appropriated options facing these changes (Hannah et al., 2013; Palliotti et al., 2014). On the other hand, in south Mediterranean Europe region, climate may limit grapevine yield and berry quality because most of the berry growth and ripening period occurs under conditions of high air temperature and soil water deficit. Nevertheless, irrigation is expanded fast in this region to mitigate environmental stress and to guarantee stable grape yield and quality (Costa et al., 2016; Resco et al., 2016). Indeed, future strategies to optimize the environmental performance of the viticulture in the Mediterranean region must be focused on an adequate selection of

rootstocks and phenotypes resistant to drought and heat stress. However, currently, winegrowers identified them as the last resort strategies (Neethling et al., 2017) and, in Mediterranean countries (i.e. Spain) these strategies have been carried out in a non-climate-smart way, this causing that almost a third of the 1 million hectares of grapevines in the Spanish territory will face a different climate than the one they were planned for (Resco et al., 2016).

2.2. Climate change consequences for the beneficial plant- microorganism interactions

The plant growth-promoting attributes (AMF, ectomycorrhizal fungi, plant growth-promoting bacteria (PGPB) and other microbes) could play vital roles in the maintenance of plant fitness and soil health under stressed environments (Vimal et al., 2017). Climate change related factors may lead to increased C allocation to the root zone and potentially altered the composition of root exudates (i.e. chemoattractants or signal compounds) and the C/N ratio or nutrient availability affecting the composition, abundance or activity of plant-associated microbial communities (Compant et al., 2010). Rising temperatures will affect roots and their belowground associates will vary according to temperature optima of individual plant species (Pritchard, 2011). Indeed, it has been demonstrated that some distinct mycorrhizal strains, plant genotypes or specific associations might respond differently to altered environmental conditions (Compant et al., 2010). Thus, the same rise of temperature decreased and increased ectomycorrhizal fungal biomass in the rhizospheric soil of a natural forest and a plantation, respectively (Li et al., 2015a). Regarding the increased atmospheric CO_2 concentration, although it influences positively on the abundance of AMF and ectomycorrhizal fungi, the interactions between mycorrhizae and plants are not necessarily impacted (see Compant et al., 2010 for further details).

On the other hand, it was recently reported that PGPB increased plant productivity and decreased the microbial respiratory C loss under elevated temperature (Nie et al., 2015). Concerning the effect of water deficit on the beneficial plant-microorganism interactions, it was recently reviewed by Vimal et al. (2017) that the association between a PGPB and AMF positively affect plants subjected to drought stress by providing increased stomata conductance and photosynthesis, and improving plant growth and drought tolerance.

Warming, elevated CO_2 and drought affect plant-beneficial microorganisms in many ways, the effects being dependent on the climate change factor studied, plant species, ecosystem type, soil type and microbial genotype (Compant et al., 2010). However, a better understanding of the effect of climatic variability on the synchrony of plants and soil

microorganisms which play a key role in the cycle of nutrients and disease cycles is necessary (Pritchard, 2011). Additionally, further research about the different mechanisms involved in plant-microorganism interactions is required for developing new strategies to manage stressed agriculture (Vimal et al., 2017).

3. Grapevine physiology and metabolism under climate change

3.1. Grapevine physiology under climate change

There was a large body of evidences reporting the influence of climate change on grapevine physiology (Table 1, page 40). Regarding phenology, numerous studies showed that the increase in ambient temperature accelerated the succession of different phenological stages, in which, flowering, *véraison*, berry maturity and harvest happen sooner (Fraga et al., 2016; Hall et al., 2016; Ruml et al., 2016; Jarvis et al., 2017; Cola et al., 2017). Nonetheless, the combination of high temperature with other climate change-related factors (i.e., UV-B, water deficit or elevated CO_2) could perform distinctly due to differential response of plant development to each factor. For example, in Tempranillo, the UV-B treatment did not affect the time to reach berry maturity (Martínez-Lüscher et al., 2014) but berry maturity was delayed when UV-B was combined with water deficit (Martínez-Lüscher et al., 2015a), or with elevated CO_2 and warm temperature (Martínez-Lüscher et al., 2015b). Likewise, it has been reported early *véraison* and berry maturity in response to elevated temperature and high CO_2, but these effects diminished under water deficit conditions (Martínez-Lüscher et al., 2016; Leibar et al., 2017).

Global climate change is also affecting leaf gas exchange of grapevines, water deficit being the most extensively analysed factor (Table 1, page 41). In fact, different studies have reported reductions in net photosynthesis, stomatal conductance and transpiration that lead to improved water use efficiency (WUE) in water-stressed plants. Nevertheless, the photosynthetic performance could be different under simultaneous variations in CO_2, water availability and temperature. In this way, it has been reported that the effects of water deficit on leaf gas exchange were mitigated by increasing concentration of CO_2 (Salazar-Parra et al., 2015; da Silva et al., 2017) and the impact of UV-B radiation was overshadowed by the effects of water deficit (Martínez-Lüscher et al., 2015a). Moreover, the combination of elevated CO_2 and temperature with UV-B radiation or with water deficit resulted in higher photosynthetic rates (Martínez-Lüscher et al., 2015b, Leibar et al., 2015).

Physiological adjustments of grapevines to climate change usually result in reductions in plant growth and yield (Jones and Alves, 2012; van Leeuwen and Darriet, 2016; Santos et al., 2017) (Table 1, page 42). Thus, a decrease associated with the warming trends has been reported for berry mass, yield and wine production. Also, water deficit greatly reduced plant and berry growth while elevated CO_2 enhanced plant growth, mainly due to increased number of leaves (Leibar et al., 2017). In addition, the combination of water deficit and elevated temperature decreased berry fresh mass and proportion of pulp per berry and increased proportion of skin per berry (Bonada et al., 2015) but its impact on wine production was less relevant (Cunha and Richter, 2016).

3.2. Grapevine metabolism under climate change

3.2.1. Primary and secondary metabolites in berries under challenging environments

Primary and secondary metabolites in grapevine berries are directly involved in the organoleptic properties of grapes and wines (Conde et al., 2007). Climate change is particularly important for berry quality, in which heat, drought and light intensity are just some environmental stress factors that dramatically affect phenolic metabolism and berry chemical composition. In this regard, cultural practices, such as canopy management and irrigation may be optimized to adjust berry and wine quality (Teixeira et al., 2013).

Warming temperatures hasten sugar accumulation and delay colour development due to reduction of anthocyanin content (Table 2, page 44). This decoupling was explained by a relative shift in onset rather than rate of accumulation of these berry components leading to the elaboration of wines with higher alcohol contents (Sadras and Moran, 2012). Moreover, it has been reported that elevated temperatures resulted in higher proportion of acylated anthocyanins (De Rosas et al., 2017). These authors showed that colour development and pigment modifications under high temperature are regulated at transcriptional level by MYBA1 transcription factor, and by the UDP glucose:flavonoid-3-*O*-glucosyltransferase and anthocyanin acyltransferase genes. Berry acidity is another important quality trait dependent on the ratio of the concentration between free organic acids (mainly, malic and tartaric acids) and their potassium salt forms. Organic acid metabolism, and especially malic acid concentration, is highly responsive to warm temperatures during fruit ripening. High temperatures are known to induce the degradation of malic acid (Sweetman et al., 2014). Tartaric acid has been thought to be more stable than malic acid; however, several discrepancies were found when the impact of temperature on tartaric acid was studied (Table 2, page 44). Recent findings of Cholet et al. (2016) showed two groups of expression profiles

for the genes involved in the biosynthetic pathway of tartaric acid: those upstream of ascorbic acid, belonging to the Smirnoff-Wheeler pathway and those downstream of ascorbic acid. This study proposed that both groups of genes might be modulated by different environmental factors, which could aid to explain the above mentioned discrepancies on tartaric acid content.

Many studies have extensively reported the effects of irrigation practices on the accumulation of various grape secondary metabolites (Table 2, page 44). In addition to temperature, predicted reduction on the amount of rainfall implies that vines may require supplemental irrigation to limit water deficit stress during the grapevine growing season (Keller, 2010) and therefore, different irrigation programs have been implemented in viticulture areas (Chaves et al., 2010). Several studies have pointed out the role of water stress on berry quality enhancing total phenolics, particularly, anthocyanins (Table 2, page 44). Moreover, it has been reported that water deficit changed anthocyanin composition, as well as the composition and the accumulation of flavonols or proanthocyanidins. In general, water deficit enhanced phenylpropanoids, monoterpenes, and tocopherols, while carotenoids and flavonoid accumulations were differentially modulated by water stress according to the berry developmental stage (Savoi et al. 2016), grapevine variety (Niculcea et al., 2015; Kizildeniz et al., 2015) and/or deficit irrigation program applied (Table 2, page 44-45). Recent findings showed that the effects of deficit irrigation on berry composition were attenuated at high temperature and that both factors (temperature and deficit irrigation) contributed to modify metabolite profiles of amino acids, anthocyanins and flavonols in Tempranillo variety (Torres et al., 2017). Thus, the combination of elevated temperature and deficit irrigation resulted in high amino acid content mainly due to the accumulation of arginine, proline, threonine and glutamine. The high arginine and proline contents could be related to a transcriptional regulation of ornithine decarboxylase during water deficit (Berdeja et al., 2015), which could be exacerbated under warm temperatures. Torres et al. (2017) also showed that both temperature and irrigation modified anthocyanin profiles by increasing 3-acetyl-glucosides derivatives due to increased methoxylated forms (petunidin and malvidin). Berry skin flavonols were dominated by myricetin-3-O-glucoside but the changes in flavonol profiles were more pronounced at elevated temperatures when plants were subjected to the deficit irrigation. These changes on secondary metabolite profiles could be explained by the regulation at the transcriptional level of phenylpropanoid pathway genes that takes place during water deficit (Castellarin et al., 2007; Deluc et al. 2009) and under elevated temperatures (De Rosas et al., 2017).

On the other hand, exposure to visible and/or UV radiation is a key factor in the synthesis of phenols and their accumulation in berries. On this subject, several studies have reported increasing trends in flavonols, anthocyanins, flavanols, monoterpenes, and decreases in hydroxy-cinnamates and flavan-3-ols in response to visible, UV-A and/or UV-B radiations (see Table 2 for further details, page 45). However, under climate change scenarios (i.e., elevated CO_2 and temperature) the stimulation of UV-absorbing compound synthesis was reduced (Martínez-Lüscher et al., 2015b). Likewise, the combination of elevated CO_2, elevated temperature and drought significantly reduced the phenolic content in the same variety, but no effect was observed when the environmental factors were applied individually (Kizildeniz et al., 2015). These results highlight the importance of approach the combined effects of different environmental factors on berry composition.

3.2.2. Hormonal status in berries under challenging environments

3.2.2.1. Hormonal signals during grape berry ripening

Grape and wine quality is extremely dependent on the fruit ripening process. Sensory and nutritional characteristics are crucial aspects for wine market, which are developed during berry ripening under a complex hormonal control (Figure 1, page 54). Grape berry development involves a complex series of changes, which can be divided into three major phases. Initial berry growth (Phase I) occurs along a sigmoid growth curve due to cell division and subsequent cell expansion. In this phase, the accumulation of organic acids, proanthocyanidins, and hydroxycinnamic acids starts to peak levels. In Phase II (lag phase), cell expansion ceases and sugars begin to accumulate. The beginning of Phase III is marked by the onset on ripening (*véraison*), in which berries undergo a second period of sigmoid growth due to mesocarp cell expansion and accumulate anthocyanin pigments. The accumulation of volatile compounds for aroma and sugars takes place in this phase, as well as the decline in organic acid content and the berry softening.

Grape berry is a non-climacteric fruit; therefore, the typical respiration peak of ethylene observed in the climacteric fruits, does not occur in grapevines. Several hormones participate in the control of grape berry development and ripening, such as auxin (IAA), ethylene, abscisic acid (ABA), gibberellins (GAs), cytokinins (CKs), and brassinosteroids (BRs) (Böttcher and Davies, 2012). In the early stages of berry development (from fertilization to fruit set), IAA, CKs, and GAs promote the cell division and expansion and in spite of having an essential role in berry development, they are mostly produced by the seeds. Then, from pre-

véraison to full ripening, changes are driven by sequential increases in ethylene, BRs and ABA (Fortes et al., 2015).

The accumulation of ABA during grape ripening has been widely reported. In fact, it was noticed that an increase in free ABA levels around *véraison* accompanied sugar accumulation, coloration, and softening, highlighting that ABA may play a major role in controlling several ripening-associated processes of grape berry (Kuhn et al., 2014; Pilati et al., 2017). In fleshy fruits as grape berries, ABA plays a crucial role not only in berry development and ripening, but also in adaptive responses to biotic and abiotic stresses. In these processes, the actions of ABA are under the control of complex regulatory mechanisms involving ABA metabolism, signal transduction, and transport. In fact, the endogenous ABA content is determined by the dynamic balance between biosynthesis and catabolism, so ABA conjugation by cytosolic UDP-glucosyltransferases, or release by β-glucosidases, constitute important ways for maintaining ABA homeostasis (see Leng et al., 2014 for further details).

3.2.2.2. Berry hormones as environmental mediators

In a context of climate change, some efforts are underway to better understand how endogenous and external signals are perceived by the grapevine plant, thus modulating ripening, metabolism and hence berry composition (Ferrandino and Lovisolo, 2014).

As mentioned above, ABA promotes grapevine berry ripening but is also a stress-related signal. For this reason, to date, ABA has been the hormone most widely studied in berries in relation to environmental stress factors (Table 3, page 49). Some studies have reported that berry ABA content diminished under warm temperatures. Likewise, Shinomiya et al. (2015) reported that temperatures exceeding 27°C during the ripening season lead to insufficient berry coloration as a result of low levels of ABA and anthocyanin biosynthetic gene expression levels. Carbonell-Bejerano et al., (2013) also showed that ABA concentration decreased throughout ripening under high temperature, but it was significantly higher at full ripeness. These authors suggest that when temperature exceeds 30°C, ABA could modulate thermotolerance responses in the berries.

Deficit irrigation during growing season also modifies the pattern of hormone accumulation in berries (Table 3, page 49). In this regard, Niculcea et al. (2013) showed that sustained deficit irrigation (SDI) caused a decrease in ABA and salicylic acid (SA) at *véraison* that affected the amount of anthocyanins at harvest. In the same study, authors concluded that the lower size of berries in SDI plants could be due to a decrease in IAA in pea-size and

suggested a role of jasmonic acid (JA) in the berry response to water stress. However, these modifications in berry hormonal patterns also depend on timing of deficit irrigation program applied. Thus, Niculcea et al. (2014) reported that both pre- and post-*véraison* water-deficit modified evolution of ABA, IAA, SA and JA in berries, which was related to changes in berry size, increases in phenolic substances and accumulation of amines in Tempranillo and Graciano varieties. With respect to ABA, it has been reported that water deficit increased transcript abundance of ABA signalling genes and ABA biosynthetic genes (Deluc et al., 2009), which indicates that ABA signal transduction pathways are modulated by water deficit during grape berry ripening (Savoi et al., 2017). In addition, changes on ABA catabolism could also account for berry responses to water deficit. Thus, Balint and Reynolds (2013a) reported that ABA was likely catabolized by conjugation to form abscisic acid glucose ester (ABA-GE) in treatments under high levels of water deficit, while in treatments with high water status, the oxidation pathway leading to dihydrophaseic acid (DPA) or phaseic acid (PA) predominated. Recent findings of Zarrouk et al. (2016) indicated that berry ABA homeostasis is achieved by degradation before *véraison*, while after *véraison* ABA homeostasis is realized by conjugation in Tempranillo. This study showed that changes in ABA catabolism/ conjugation along berry development were affected by water stress particularly under elevated temperatures, indicating that ABA-GE and ABA catabolites play an essential role in ABA homeostasis under environmental constraints.

4. Arbuscular mycorrhizal symbiosis in a changing climate

4.1. Effects of changing environments on the arbuscular mycorrhizal fungi

The data collected by Compant et al. (2010) in their review demonstrated that warming, elevated CO_2 and drought affect plant-beneficial microorganisms in many ways, the effects being dependent on the climate change factor studied, plant species, ecosystem type, soil type and microbial genotype. Thus, Mohan et al. (2014), summarizing the effect of rising temperatures on mycorrhizal communities, obtained that in 17% of such studies mycorrhizal abundance decreased, in 20% of them no significant change was observed and the 63% of the works concluded that the abundance of mycorrhizas can increase under elevated temperature. In subsequent studies, Augé et al. (2015) pointed out that AMF promotion was 10% higher when air temperatures were kept at or below 27°C than those that exceeded 27°C. Moreover, Wilson et al. (2016) concluded that the direct effect of increasing 3°C the temperature

decreases AMF colonization, and this appeared to be regionally consistent across the Mediterranean climate gradient. On the other hand, although the growth of external hyphae and the diversity of AMF species can increase at high temperatures (Hawkes et al., 2008; Zhang et al., 2016), the mycorrhizal activity generally decreases (Mohan et al., 2014). Furthermore, in a warmer world, the presumed enhanced growth of AMF hyphae is unlikely to balance the carbon losses to the atmosphere from the AMF respiration (Hawkes et al., 2008).

Elevated CO_2 can promote mycorrhizal colonization of plant roots as a consequence of the enhanced carbon allocation to roots (Zhu et al., 2016a; Asha et al., 2017), which may result in an increased mineral uptake from soil but not necessarily correlated with nutrient transfer to the host plant (Smith and Read, 2008). Regarding the community composition of AMF, elevated CO_2 increased the ratio of *Glomeraceae* to *Gigasporaceae* but this effect may be masked by the natural changes through time (Cotton et al., 2015) and also by the dependency of different fungi on water availability and precipitation (Veresoglou et al., 2016). The application of molecular tools revealed that, at present, *Glomeraceae* dominate the composition of the AMF communities in vineyards in Oregon (USA) (Schreiner and Mihara, 2009), Piedmont (Italy) (Balestrini et al., 2010) and Burgundy (France) (Bouffaud et al., 2016), the AMF diversity being relatively low, which contrasts with the high diversity of AMF in the rhizosphere of European wild grapevine *V. vinifera* L. ssp. *sylvestris* (Gmelin) Hegi found by Ocete et al. (2015). Projected droughts within the climate change are expected to cause AMF sporulation and reduction of the AMF activity (Guadarrama et al., 2014). Agricultural practices (high fertilizer inputs, tillage, weed control practices, and pest management practices, among others) may amplify the effect of environmental factors on the AMF communities present in vineyard soils (Trouvelot et al., 2015; Vukicevich et al., 2018). In semi-arid to arid regions, such as Mediterranean areas, soils of vineyards are periodically subjected to tillage or left totally bared in order to keep the soil free of weeds and grassland plant species, which influences on the development and reproduction of AMF. Oehl and Koch (2018) noted that AMF diversity decreased in Central European vineyards subjected to this cultivation management, which can in last instance affect yield and wine quality parameters.

A better understanding of the effect of climatic variability on the synchrony of plants and soil microorganisms which play a key role in the cycle of nutrients and disease cycles is necessary (Pritchard, 2011) and further research on the mechanisms involved in plant-microorganism interactions is required for developing new strategies to manage sustainable agriculture under stressful weather conditions (Vimal et al., 2017).

4.2. Growth and physiology of plants associated with AMF and undergoing changing environments

Expected climate change can create unfavourable conditions to plants. Drought, salinity, global warming and rising CO_2 in the atmosphere affect plant growth and yield and constitute a threat to sustainable agriculture and global food security. In this context, AMF are considered a promising tool for improving plant resilience to adverse environmental conditions and several mechanisms have been described to explain how and why mycorrhizal symbiosis can benefit metabolism and physiology of their host plants. In general, under water deficit plants associated with AMF show enhanced antioxidant activity, osmotic regulation and root hydraulic properties than non-mycorrhizal ones (Ruiz-Lozano et al., 2012a). Studies carried out by Yooyongwech et al. (2016) and Quiroga et al. (2017) showed that, when subjected to drought, mycorrhizal potato and maize plants had higher levels of photosynthetic pigments, improved chlorophyll fluorescence parameters, higher net photosynthetic rates, increased membrane stability and lower lipid peroxidation than those not colonized by AMF. Li et al. (2015b) found that mycorrhized *Populus cathayana* performed better under restricted irrigation by improving photosynthesis, intrinsic water use efficiency (WUE) and yield of photosystem II. Similarly, AMF increased the uptake of N and improved the photochemistry efficiency of photosystem II in the flag leaf of winter wheat subjected to water deficit (Garmendia et al., 2017). Armada et al. (2016) concluded that mycorrhizal inoculation was more efficient than chemical fertilization for improving growth, nutrition and enzymatic activities of *Retama sphaerocarpa* undergoing drought. The inoculation of *Zea mays* with *Rhizophagus intraradices* enhanced drought tolerance of plants by promoting nutrient uptake, adjusting C:N:P stoichiometry, improving WUE and water content in leaves, and accelerating the rehydration rate (Zhao et al., 2015). The role of some aquaporins in the drought tolerance induced by AMF in maize has been highlighted by several authors (Bárzana et al., 2014; Quiroga et al., 2017). The integrated physiological response of plants to salinity includes several coordinated mechanisms that may be affected or modulated by mycorrhizal symbiosis: the accumulation of compatible solutes, the control of ion homeostasis, the regulation of soil water uptake, the reduction of oxidative damage, and the maintenance of photosynthetic rates (Ruiz-Lozano et al. 2012b). Regarding global warming, Zhang et al. (2016) reported that mycorrhizal hyphae beneficed soil water absorption and nutrient uptake in grassland plants subjected to elevated temperatures. Similarly, Hu et al. (2015) found that AM association improved plant growth through increased root biomass, root to shoot ratio, and the concentrations of Zn in shoots and P in roots in *Medicago truncatula* undergoing night

warming. Under increased CO_2 concentration in the atmosphere, AMF favoured the allocation of carbon and nitrogen in roots of *Triticum aestivum*, leading to greater nitrogen use efficiency (Zhu et al., 2016b). However, there are conflicting results concerning the interaction between AMF and elevated CO_2 in the air on biomass production and allocation and uptake of nutrients within the family of legumes. While no significant interactions were found in pea (Gavito et al., 2000) and clover (Staddon et al., 1999), alfalfa plants associated with AMF and grown under elevated CO_2 enhanced root biomass and slightly increased the leaves to stems ratio in comparison with non-mycorrhizal plants just before flowering (Baslam et al., 2012a). Moreover, AMF increased the accumulation of sugars and proteins in leaves of *Medicago sativa*, which accelerated the photosynthetic acclimation under elevated CO_2 (Goicoechea et al., 2014).

The interaction of different factors related to the projected climate change can modify the effect of AMF on host plant physiology and sometimes benefits provided by the mycorrhizal symbiosis can diminish or even disappear. This is the case, for example, of winter wheat: elevated CO_2 in the atmosphere impaired the previously mentioned benefits exerted by mycorrhizal association to host plants undergoing water restriction (Garmendia et al., 2017). In contrast with these results, AMF improved growth, stomatal conductance, nitrogen use efficiency, and ion homeostasis of *T. aestivum* simultaneously subjected to elevated CO_2 and salinity (Zhu et al., 2016a).

4.3. Nutritional quality of crops and fruits of plants associated with AMF and undergoing changing environments

The application of mycorrhizal inocula has emerged as a reliable technique to enhance the agricultural productivity and nutritional value of edible vegetables whereas reducing environmental costs (Berruti et al., 2016; Goicoechea and Antolín, 2017). This is the case of strawberry fruits, whose levels of phenolic compounds and minerals increased when plants were inoculated with the arbuscular mycorrhizal fungus *Glomus intraradices*. Some colour parameters of strawberry fruits were also affected by AMF (Castellanos-Morales et al., 2010). Similarly, Hart et al. (2015) found that mycorrhizal inoculation enhanced the concentrations of several minerals (N, P, Cu), carotenoids, and some flavour compounds, as well as the antioxidant capacity in tomato fruits. This beneficial effect of AMF on the quality of tomatoes was corroborated by Bona et al. (2017) in a field study performed in a real industrial tomato farm. In another study carried out under nature conditions, Zeng et al. (2014) found that *Glomus versiforme* improved the quality of citrus fruits by increasing the ratio of sugar to acid,

and the amounts of vitamin C, flavonoids and minerals. Another woody plant beneficed by mycorrhization in field is *Libidibia ferrea*, a tree with medicinal properties whose bark accumulated higher amount of flavonoids and tannins when associated with AMF (dos Santos et al., 2017). Mycorrhizal fungi also improved the antioxidant potential of leaves from sweet basil, an aromatic plant widely used for medicinal and cooking purposes (Hristozkova et al., 2017). In lettuce, mycorrhizal symbiosis induced the accumulation of carotenoids, total soluble phenolics, anthocyanins, chlorophylls, tocopherol and some mineral nutrients in leaves (Baslam et al., 2011, 2013a), which makes the application of AMF a feasible tool for improving the nutritional quality of this horticultural crop. This improvement of the quality in lettuces associated with AMF was significant enough to allow extending cultivation of this crop to seasons in which non-mycorrhizal lettuces suffer relevant decreases in their levels of proteins, carotenoids and flavonoids (Baslam et al., 2013b).

However, since the different factors involved in the projected climate change can modify the effects of AMF on the metabolism and physiology of their host plants, they can also change the nutritional properties of crops associated with AMF. In fact, drought, salinity, global warming and rising CO_2 in the atmosphere constitute a threat to sustainable agriculture and global food security. In studies focused on the role of mycorrhizal symbiosis when plants are undergoing salt stress, some authors have found that AMF increase the plant salt-tolerance and improve fruit yield and quality. This is the case of cucumber cultivated under saline conditions: fruits produced by mycorrhizal plants had higher amounts of soluble proteins, sugars and vitamin C and lower levels of nitrate than those from non-mycorrhizal plants (Han et al., 2012). Likewise, Huang et al. (2013) measured higher contents of N, P, K and Ca in tomato fruits of hybrid cultivars associated with AMF than in those of non-inoculated ones. Beneficial effects of mycorrhizal symbiosis on yield and fruit quality of crops under salty conditions, however, can vary depending on plant cultivars and fungal strains, as demonstrated by results of Huang et al. (2013) in tomato and those obtained by Sinclair et al. (2014) working with strawberry. Water deficit is one of the most important factors affecting crop survival, growth, and productivity. Most times the beneficial effect of AMF on the host plant development is more evident when water supply is restricted than under plentiful water availability. For example, the beneficial effect of AMF on the growth and quality of chile ancho pepper fruits was especially clear when plants were undergoing drought conditions: fruits of mycorrhizal plants subjected to water deficit showed similar colour intensity and chlorophyll content and higher amount of carotenoids than those of non-mycorrhizal plants cultivated at optimal irrigation regime (Mena-Violante et al., 2006). In lettuce, while a moderate water

deficit prolonged in time reinforced the capacity of AMF for increasing the levels of antioxidant compounds in leaves (Baslam and Goicoechea, 2012), elevated CO_2 in the atmosphere impaired this beneficial effect probably due to the use of photoassimilates for enhancing growth of the host plant and spreading mycorrhizal colonization in detriment to the secondary metabolism (Baslam et al., 2012b). Similarly, Goicoechea et al. (2016) observed a general depletion of contents of micro- and macro-nutrients and gliadins in grains of durum wheat cultivated under elevated CO_2 in the air, regardless of mycorrhizal inoculation and water regime applied to plants, what contrasted with the higher accumulation of copper, iron, manganese, zinc and gliadins –wheat-seed storage proteins responsible together with glutenins for dough elasticity and extensibility that determine processing qualities in the production of end products- in grains of durum wheat inoculated with AMF and grown under water deficit at ambient CO_2. Notwithstanding the above, elevated CO_2 not always nullify the beneficial effect of mycorrhizal colonization on crop quality. For example, in alfalfa, the combination of AMF and elevated atmospheric CO_2 improved forage quality by increasing the amount of hemicellulose and decreasing that of lignin in leaves (Baslam et al., 2014). Moreover, the positive effect of the synergism between AMF and elevated atmospheric CO_2 may be reinforced by the simultaneous application of some cultural practices, such as the supply of humic substances to the soil. In this sense, Bettoni et al. (2014) concluded that the triple interaction between humic substances application, mycorrhizal inoculation and elevated CO_2 enhanced the accumulation of soluble sugars, proteins and proline in leaves of onion seedlings in a greater extent than the application of those factors separately, which increased the quality of onion shoots as source organs for posterior growth and quality of bulbs. These same authors found that the application of humic substances, AMF inoculum and elevated CO_2 in the air had an additive effect of increasing the content of soluble sugars, proteins, and phenolics in onion bulbs, thus reinforcing their energetic and antioxidant properties. This triple interaction also enhanced the ratio between soluble solids and total titratable acidity, which may favour the perception of sweetness and make onion more pleasant for consumption (Bettoni et al., 2017).

5. Arbuscular mycorrhizal fungi in viticulture

The ability for *V. vinifera* to tolerate unsuitable environmental conditions for most crops will partially be dependent on the functioning on its soil community (Holland et al., 2014). In viticulture, the concept of “terroir” relates the sensory attributes of wines to the environmental conditions of the grapes, and it therefore represents an important descriptor of

the connection between wines and their origins (Likar et al., 2015). For these reasons, considerable progress has been made in the last decade towards the use of soil microorganisms associations, such as AMF, to improve grapevine growth and yield (Table 4, page 51).

Mycorrhizal symbiosis has been associated with improved growth, increased tolerance against biotic and abiotic stresses and/or enhanced mineral uptake from soils (Trouvelot et al., 2015). Some studies evidenced that AMF colonization of grapevines improved the water status, induced an improvement in the photosynthetic performance that increased the WUE, promoted the uptake of P, potassium (K) and calcium (Ca) and led to a mobilization of starch reserves in the apex in winter, which was possibly responsible for enhancing root development (Table 4, page 51). Hence, Nicolás et al. (2015) concluded that AMF inoculation technique can be recommended for sustainable viticulture in arid and semi-arid areas.

Grapevine association with AMF also stimulates the synthesis of plant secondary metabolites (i.e., resveratrol, flavonols and anthocyanins), which are important for increased plant tolerance to environmental stresses and beneficial to berry quality (Table 4, page 52). In a recent study, Torres et al. (2016) have reported that inoculation of grapevines with AMF might play a relevant role in a future climate-change scenario to maintain or even improve berry quality by enhancing antioxidant properties. Other studies indicated that the positive effects due to AMF colonization of grapevines are mediated by the up-regulation of some genes (Table 4, page 53). Thus, Balestrini et al. (2017) showed that the expression of genes belonging to categories such as nutrient transport, transcription factors, and cell wall-related genes was significantly altered by AMF colonization. Moreover, the presence of AMF in roots of three grapevine varieties stimulated the transcription of phenylalanine ammonia-lyase, stilbene synthase, and a resveratrol O-methyltransferase, three genes involved in the phenylpropanoid pathway, as response to aerial pathogens *Plasmopara viticola* and *Botrytis cinerea* (Bruisson et al., 2016).

In addition to the induction of plant defences, many times through the activation of pathways belonging to the antioxidant and secondary metabolism, Thirkell et al. (2017) suggested that one of the strongest benefits of mycorrhizal symbiosis for crop plants is related to the improved soil properties mediated by AMF. It is known that fungal hyphae increase the mineralisation of soil organic matter (SOM) (Paterson et al., 2016). Moreover, mycorrhizal fungi can enhance the fixation of atmospheric CO_2 by their host plants, and then induce the transport of photoassimilates from the aerial part to the roots by exerting a sink effect. A

portion of the carbon present in the fungal biomass will remain in the soil as a part of the SOM after the AMF senescence (Treseder, 2016). These benefits exerted by AMF will become especially relevant in the context of the projected loss of soil organic carbon caused by the global warming, which in last instance will decrease agricultural productivity (Wiesmeier et al., 2016). Erosion and low organic matter stocks are common problems affecting soils of vineyards in Mediterranean areas as a consequence of the concurrence of environmental factors and some management practices such as the abovementioned continuous tillage (García-Díaz et al., 2018).

6. Concluding remarks

This review summarizes the current knowledge about the role of AMF on grapevine physiology, metabolism and hormonal status under changing environment. In the last decades, a growing concern about the potential consequences of climate change on viticulture and the detrimental impact on grape and wine quality has addressed several researches. Nevertheless, few studies have highlighted the role of AMF symbiosis in this scenario, in spite of the know benefits that mycorrhizas provide to host plants. The general utilization of fertilizers and/or phytohormones may damage or unbalance soil ecosystem of viticultural areas, so that its application need to be reduced. Thus, AMF has been presented as natural biofertilizers that can be the alternative to chemical fertilization without the concomitant loss of crop yield and quality (Berruti et al., 2016) and there is evidence that co-adaptation of the partners to a new environment may maximize benefits and minimize costs of the symbiosis (Johnson et al. 2013). On the other hand, exogenous phytohormone application (especially, ABA) to the vine has been used as a tool to improve the quality of the grapes (Balint and Reynolds, 2013b; Alonso et al., 2016b). Taking into account that under abiotic stress ABA concentration was enhanced by AMF (Wang et al., 2017), the symbiotic association can offer an alternative to phytohormone supply to improve grape quality. However, there is a need for more studies that deepen into the influence of AMF in the levels of the ABA when grapevines undergo challenging environments. In conclusion, the management of natural biotic interactions, such as AMF symbiosis, as well as the processes to retain carbon in the soil longer may be the key to maintain the resilience of viticulture to climate change. However, the fact that the responses of grapevines to the inoculation with AMF and/or to the environmental conditions may vary according to the plant variety or clone (Torres et al., 2015, 2016, 2018) indicates that it may be profitable to identify the AMF inoculants most suitable for a given cultivar in a given environment.

7. Future perspectives

Winegrowers are aware of reconsidering their viticultural practices in order to better manage climate-related risks and produce quality wines (Neethling et al., 2017). Given the global warming impact on berry quality traits, it is useful to reconsider the potential application of some new or traditional management techniques able to regulate sugar accumulation and/or to delay or balance berry ripening (Palliotti et al., 2014). Most wine-producing regions are subjected to seasonal drought but, based on the global climate models an increase in aridity is predicted in the future. Hence, an optimized irrigation schedule would still be one of the most desirable tools to improve WUE and crop productivity (Costa et al., 2016). Under low rainfall conditions, warm temperatures and high light intensity, spontaneous vegetation used as groundcover appears as an effective strategy to revert soil degradation in Mediterranean vineyards (García-Díaz et al., 2018) at the same time that it may benefit taste or quality of wines (Trigo-Córdoba et al., 2015). Although the impact of water deficit on berry ripening and quality has been extensively investigated during the last decades, the suitability of actual irrigation programs should be reviewed in the future climate conditions. Consequently, several researches have begun to address the combined effects of elevated temperature, UV-B radiation or high CO_2 with water deficit on grapevine quality (Bonada et al., 2015; Kizildeniz et al., 2015; Martínez-Lüscher et al., 2015a; Torres et al., 2016; Zarrouk et al., 2016). However, more research is needed to elucidate the potential effects of AMF symbiosis on the ability of grapevines to cope with water deficit in interaction with other environmental factors as well as to identify the mycorrhizal inoculants most appropriate for a given variety, cultivar or clone cultivated under a real and specific environmental scenario. In addition, the long-term site history and the previous management practices employed must be considered before introducing the AMF inocula in order to obtain benefits and ensure future food security (Thirkell et al., 2017).

The asexual propagation of the grapevine varieties allows the appearance and accumulation of somatic mutations, which are the basis for the clonal selection, which leads to differences in vigour, berry and cluster weight, yield production, resistance to plagues and diseases or oenological potential (Fernandes et al., 2015). One of the adaptive agronomic strategies to use in modern viticulture under the on-going climate change conditions is the selection of the best adapted rootstock and clones. Thus, the clonal selection could be oriented toward late-ripening clones to avoid alterations caused by high temperatures on fruit quality (van Leeuwen and Darriet, 2016), or to obtain clones with better balance between yield, acidity and alcoholic

degree (Gonçalves et al., 2016) and WUE characteristics (Tortosa et al., 2016). Recent findings provide evidence for this clonal diversity, which resulted in different abilities to respond to AMF inoculation (Torres et al., 2016). Therefore, the use of AMF for improving the fruit quality of grapevines needs to be included in an integrated management program of clonal selection.

Sustainable winemaking process, consisting of maximizing resources while decreasing emissions generated by the production process, is imperatively required. Mutualistic relationships between grapevines and AMF are being thought as a new tool to enhance agricultural productivity and nutritional quality of food crops whereas reducing environmental costs (Berruti et al., 2016; Goicoechea and Antolín, 2017). Indeed, AMF symbiosis involves a continuous cellular and molecular dialogue between AMF and host plant that stimulated the production of secondary compounds, which may be ascribed to the activation of host defence reactions and the accumulation of antioxidants (Rouphael et al., 2015; Goicoechea and Antolín, 2017). The viticulture industry generates large amounts of waste material because of extensive winter and summer pruning practices. Recent investigations confirmed the phenolic richness of pruning wastes such as grape pomace (Beres et al., 2017) and vegetative tissues (leaves and stems) (Eftekhari et al., 2017), which have been pointed out as a promising source of compounds with nutritional and nutraceutical properties and biological potential. Recent studies have reported that AMF colonization also can improve phenolic content and antioxidant power of grapevine leaves (Eftekhari et al., 2012a; Torres et al., 2015). Therefore, the use of AMF for improving composition and reutilizing the grape wastes from pruning and other viticultural activities as a potentially natural source of phenolic compounds will be a promising field for pharmacological, cosmetic and food industries.

8. References

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Table 1. Effects of changing climate on grapevine physiology.

Plant material	Region (Country)	Year	Experiment	Environmental factor	Trend	Reference
Phenology						
MTKA, OJAL, RKAT, and SAPE	Regions of Koshkebi and Saguramo (Georgia)	1974–2013	Data collection and modelling	T: + 1.4°C	Fruit set:~ 5.1-11.5 days earlier *Véraison* ~ 5.4-18.3 days earlier	Cola et al., 2017
Shiraz	65 wine regions of Australia	1975-2004	Modelling: Estimation of phenology dates under two projected temperature anomalies	T: + 1.26°C T: + 2.61°C	Budbreak: ~16 days earlier Harvest: ~30 days earlier Budbreak: ~8 days earlier Harvest: ~19 days earlier	Hall et al., 2016
16 red and white varieties	Douro, Lisbon and Vinhos Verdes (Portugal)	T (1981–2010), Phenological dates (1990-2012)	Model for 2006-2100 and validation in fields	T: + 0.01–0.02°C/year	1-day reduction between budbreak and flowering and 4-day reduction between flowering and *véraison* for both varieties	Fraga et al., 2016
16 red and white varieties	Douro, Lisbon and Vinhos Verdes (Portugal)	T (1981–2010), Phenological dates (1990-2012)	Model for 2006-2100 and validation in fields	T: + 0.03–0.05°C/year	2 and 3- day reduction between budbreak and flowering for red and white varieties, respectively and an 8-day reduction to reach *véraison* for both varieties	Fraga et al., 2016
Shiraz, Chardonnay, and Cabernet Sauvignon	Western Australia wine regions	1975-2005	Modelling and projections for 2030, 2050 and 2070	T: Low and high warming condition projected for Australia	Projected harvest between 3 and 7 weeks earlier in some viticultural regions of Australia	Barnuud et al., 2014
Tempranillo	Navarra (Spain)	n.m.	Fruit-bearing cuttings grown in greenhouses	UV-B: 0, 5.98 and 9.6 kJ m^{-2} d^{-1}	UV-B did not affect the time to reach berry maturity	Martínez-Lüscher et al., 2014
White and Red Tempranillo	Navarra (Spain)	2014-2015	Fruit-bearing cuttings grown in temperature-gradient-greenhouses	T: + 4°C; CO_2: 700 ppm	Berry maturity: 4.3 days earlier because of temperature and 4.7 days earlier because of CO_2. No interaction was found.	Martínez-Lüscher et al., 2016
Tempranillo	Navarra (Spain)	2011	Fruit-bearing cuttings grown in greenhouses	WD: 50% of field capacity; UV-B: 0, 5.98 and 9.66 kJ m^{-2} d^{-1}	Phenology was delayed 11 days after *véraison* by UV-B; WD shortened up to 3 days the onset of *véraison* and delayed berry maturity	Martínez-Lüscher et al., 2015a

Table 1 *(Continued)*

Plant material	Region (Country)	Year	Experiment	Environmental factor	Trend	Reference
Tempranillo	Navarra (Spain)	2012	Fruit-bearing cuttings grown in greenhouses	CC (CO_2: 700 ppm; T: 28/18°C); UV-B: 0, 5.98 and 9.66 kJ m^{-2} d^{-1}	UV-B delayed berry maturity 7 days under current situation of CO_2 and T, and 5 days under CC conditions	Martínez-Lüscher et al., 2015b
Tempranillo	Navarra (Spain)	2011	Fruit-bearing cuttings grown in greenhouses	CC (CO_2: 700 ppm; T: 28/18°C; 33-53% RH) WD (60% of controls) and type of soil	CC conditions caused early *véraison* (7 days) and early maturity (10 days), WD delayed 9 days berry maturity	Leibar et al., 2017
Gas exchange parameters						
Pseudo F1 off-spring of Syrah and Grenache	n.m.	2012 and 2013	Chamber experiments	WD	WD decreased evapotranspiration (decrease in night (37%) and daytime (50%) and transpiration efficiency	Coupel-Ledru et al., 2016
Shiraz and Cabernet Sauvignon	Negev Desert (Israel)	2011	Commercial vineyard	WD (50% of control)	WD reduced stomatal conductance	Hochberg et al., 2015
Malbec	Mendoza (Argentina)	2009, 2010, 2011	Commercial vineyard	UV-B	UV-B decreased photosynthesis, stomatal conductance, efficiency of PSII and Chlorophyll b	Regvar et al., 2013
Sangiovese	Florence (Italy)	2012	Pots in tunnels and outdoors	UV-B and UV-A: (exclusion and exposition again)	Exclusion reduced UV-absorbing compounds and delayed the development of the photosynthetic apparatus	Grifoni et al., 2016
Grenache	Balearic Islands (Spain)	2014	Experimental vineyard	WD (NI vs. WD 50%Ec) L exposition	Different responses in carbon and water fluxes at leaf or whole-plant level	Escalona et al., 2016
Tempranillo	Navarra (Spain)	2011	Fruit-bearing cuttings grown in greenhouses	WD (50% of field capacity) and UV-B (0, 5.98 and 9.66 kJ m^{-2} d^{-1})	WD decreased A, E, g_s and C_i. UV diminished A but this decrease was not accompanied by a reduction in C_i	Martínez-Lüscher et al., 2015a
Touriga nacional and Trincadeira	Pegoes , Setúbal (Portugal)	2007	Fruit-bearing cuttings grown under controlled conditions.	WS (4-5 days without irrigation), HS (42°C, 1 h) and LS (2000 µmol.quantam^{-2}s^{-1}, 1h)	WS alone or in combination (WSLS, WSHS, WSLSHS) decreased A. LS increased g_s. Treatments altered efficiency of PSII	Carvalho et al., 2015

Table 1 *(Continued)*

Plant material	Region (Country)	Year	Experiment	Environmental factor	Trend	Reference
Vitis lambrusca cv. Concord	n.m.	n.m.	Bearing cutting grown in growth chambers	CO_2 (800 ppm), PRI and NI	CO_2 down-regulated A. CO_2 and PRD increased the efficiency of RubisCo carboxylation, by increasing A, WUE and intrinsic WUE. CO_2 delayed the effect on A and RubisCo activity for four days, by reducing g_s, E and stomatal density in NI plants	da Silva et al., 2017
Tempranillo	Navarra (Spain)	2014	Fruit bearing cuttings in grown-chamber-greenhouses	CC (CO_2: 700 ppm; T: 28/18°C ; 33-53% RH) WD (60% of controls) and type of soil	CC increased C_i and intrinsic WUE. CC conditions only increased A in well irrigated vines (61% at *véraison* and 38% at maturity). WD decreased g_s, C_i and intrinsic WUE	Leibar et al., 2015
Tempranillo	Navarra (Spain)	2012	Fruit-bearing cuttings grown in greenhouses	CC (CO_2: 700 ppm; T: 28/18°C) and UV-B: 0, 5.98 and 9.66 kJ m^{-2} d^{-1}	UV-B decreased photosynthesis and chlorophylls while CC enhanced them	Martínez-Lüscher et al., 2015 b
Tempranillo	Navarra (Spain)	n.m.	Fruit-bearing cuttings grown in temperature-gradient-greenhouses	T: +4°C over ambient temperature; CO_2: 700 ppm; WD: 40% of field capacity	CO_2 induced photosynthetic acclimation of plants regardless the irrigation level and temperature. T, CO_2 and WD increased the photorespiration values.	Salazar-Parra et al., 2015
Plant growth and yield						
n.m.	Douro (Portugal)	1933-2013	Modelling	Spring T: +1°C,+3°C (immediate increase) and +3°C (gradual increase over 40 years)	T impacted on wine production	Cunha and Richter, 2016
n.m.	Douro (Portugal)	1933-2014	Modelling	WD (loss of water 20% by shock or gradually over 40 years)	WD caused no difference in wine production	Cunha and Richter, 2016
Pseudo F1 off-spring of Syrah and Grenache	n.m.	2012 and 2013	Chamber experiments	WD	WD plants showed lower shoot growth rate	Coupel-Ledru et al., 2016
Shiraz and Cabernet Sauvignon	Negev Desert (Israel)	2011	Commercial vineyard	WD (50% of control)	WD reduced berry weight	Hochberg et al., 2015

Table 1 *(Continued)*

Plant material	Region (Country)	Year	Experiment	Environmental factor	Trend	Reference
Tempranillo and Graciano	Navarra (Spain)	2011	Fruit-bearing cuttings grown in greenhouses	WD (50% of control)	WD decreased leaf area, yield and berry volume	Niculcea et al., 2015
Monastrell	Murcia (Spain)	2009-2012	Experimental station	NI, PRI and DI before *véraison*	NI vines had lower berry diameter and fresh berry weight	Romero et al., 2015
Malbec	Mendoza (Argentina)	2009, 2010, 2011	Commercial vineyard	UV-B	UV-B decreased leaves per shoot, length of shoots and foliar area	Berli et al., 2013
Tempranillo	Navarra (Spain)	n.m.	Fruit-bearing cuttings grown in greenhouses	UV-B: 0, 5.98 and 9.66 kJ m^{-2} d^{-1}	UV-B did not affect either berry weight and berry calibre, highest UV-B increased relative mass of skin	Martínez-Lüscher et al., 2014
Vitis vinifera cv. Shiraz	Barossa Valley (Australia)	2010-2012	Open-top chambers in vineyards	Heat and WD	Heat and WD accounted for decreasing berry fresh mass and proportion of pulp per berry and increasing proportion of skin per berry (no interaction found).	Bonada et al., 2015
White and Red Tempranillo	Navarra (Spain)	2013	Fruit-bearing cuttings grown in temperature-gradient-greenhouses	CC (T: +4°C over ambient temperature; CO_2: 700 ppm; CD)	CC decreased leaf area and bunch fresh weight, however, elevated CO2 mitigated the effect of elevated T in red Tempranillo with FI and in white Tempranillo with CD	Kizildeniz et al., 2015
Tempranillo	Navarra (Spain)	2011	Fruit-bearing cuttings grown in greenhouses	CC (CO_2: 700 ppm; T: 28/18°C ; 33-53% RH) WD (60% of controls) and type of soil	Elevated CO_2 increased number of leaves but did not change total leaf area, total dry weight and root fresh and dry weight. WD decreased total leaf area, total dry weight and root fresh and dry weight and increased skin/pulp ratio	Leibar et al., 2017
n.m.	North Coast American Viticultural Area (California-US)	1976-2012	Modelling with growth rings	CC	CC could decrease significantly winegrape yield by a 12.4% in 2099	Maxwell et al., 2016

A: net photosynthetic rate; CC: climate change; CD: Cyclic drought; C_i: sub-stomatal CO_2 concentration; CO_2: Increment of carbon dioxide; DI: deficit irrigation; E: transpiration rate; FI: full irrigation; g_s: stomatal conductance; n.m. not mentioned; NI: non irrigated; PRI: partial root irrigation; T: Temperature; UV-B: UV-B radiation; WD: Water deficit; WUE: water use efficiency.

Table 2. Berry metabolism response to environmental factors.

Plant material	Region (Country)	Year	Experiment	Environmental factor	Trend	Reference
Temperature (T)						
Shiraz, Chardonnay, and Cabernet Sauvignon	Western Australia wine regions	1975-2005	Modelling and projections for 2030, 2050 and 2070	T: Low and high warming condition projected for Australia	CC decreases tartaric acid content and earlier accumulation of sugar is expected. CC reduces anthocyanin accumulation depending on the projected scenario and the cultivar	Barnuud et al., 2014
Sangiovese	n.m.	2010	Potted vines grown in greenhouses	T: + 2°C over the average temperature and + 7°C over the maximum temperature	T decreased anthocyanin content although no changes in its composition were observed. T did not affect acidity	Movahed et al., 2016
n.m.	n.m.	n.m.	Microvines grown in greenhouses	Day and night HS in different ripening states	Night HS reduced total anthocyanin content. No effect on malate. Night HS up-regulated Pro metabolism related genes	Rienth et al., 2014
Malvec and Bonarda	Mendoza (Argentina)	2014 and 2015	Commercial vineyard	HT: increased mean diurnal temperatures	Day HT reduced total anthocyanins and shifted towards acylated derivatives due to the up-regulation of the acyltransferase gene	De Rosas et al., 2017
Kyoho	n.m.	n.m.	Potted vines in phytotron	T: 25, 27 and 30°C; Shade or sun-exposition	T (27 or 30°C) and shade decreased the anthocyanin content	Shinomiya et al., 2015
Water deficit (WD)						
Pinot noir	Geinseihem (Germany)	2009-2011	Field experiment	WS (no rainfall during the growing season) and rootstock	WD increased anthocyanin and other phenolic content in berries but the effect was dependent on the rootstock sensitiveness	Berdeja et al., 2014
Cabernet Sauvignon	Maipo Valley (Chile)	2014	Commercial vineyard	WD (3.6, 1.8 and 0.3 mm day^{-1})	WD increased total phenols and total proanthocyanidins and their polymerization but no differences in total anthocyanins were found	Cáceres-Mella et al., 2017
Chardonnay and Meski	n.m.	n.m.	Potted vines in growth chambers	WD (water privation for 8 days)	WD increased the concentration of Arg, Orn, Glu, Gln, GABA and Pro.	Hatmi et al., 2015
Shiraz and Cabernet Sauvignon	Israel	2011	Field experiment	WD (50% of control)	WD decreased resveratrol and TCA molecules and increased kaempferol and anthocyanins	Hochberg et al., 2015
Tempranillo and Graciano	Navarra (Spain)	2011	Fruit-bearing cuttings grown in greenhouses	WD (50% of control)	WD decreased anthocyanins due to decreasing glucoside derivatives and increasing acetil and coumaroryl derivatives. WD increased flavonols in Tempranillo and decreased flavonols and catechins in Graciano	Niculcea et al., 2015

Table 2 *(Continued)*

Plant material	Region (Country)	Year	Experiment	Environmental factor	Trend	Reference
Shiraz	Montpellier (France)	2004	Experimental vineyard	ED and LD	At maturity, LD increased total anthocyanins, trihydroxylated forms and the acetylated and coumaroylated derivatives. ED increased dihydroxylated forms. Both WD increased non-acylated anthocyanins	Ollè et al., 2011
Monastrell	Murcia (Spain)	2009-2012	Experimental station	NI, PRI and DI before *véraison*	NI decreased TSS, and increased malic acid, total anthocyanins and the acelylated derivatives. WD enhanced flavonol content until *véraison* and PRI induced amino acid accumulation regardless the amount of water	Romero et al., 2015
Sauvignon vert or Sauvignonasse	Udine (Italy)	2012	Experimental vineyard	NI	WD enhanced phenylpropanoids, monoterpenes, and tocopherols, while carotenoids and flavonoid accumulations were differentially modulated by WD according to the berry developmental stage. WD increased flavan-3-ols and proanthocyanidins before *véraison*, but decreased them after it	Savoi et al., 2016
Merlot	Udine (Italy)	2011 and 2012	Experimental vineyard	WD	WD increased Pro, Leu, Val and Ile accumulation and decreased the synthesis and concentration of stilbenoids	Savoi et al., 2017
Aglianico	Montegiordano Marina (Italy)	2008	Field experiment	NI	NI increased total anthocyanins and the ratio between acetylated and coumaroylated, flavonols were not affected	Sofo et al., 2012
Tempranillo	Estremoz (Portugal)	2007 and 2008	Field experiment	NI and DI	Sugar accumulation and acidity were not affected. NI increased total phenols but decreased total flavonols, anthocyanins and proanthocyanidins	Zarrouk et al., 2012
Light (L)						
Riesling	Geisenheim (Germany)	2012	Field experiment	L exposition	L exposition increased flavonol and monoterpene content of berries and their synthases	Friedel et al., 2016
Gamay Fréaux and Gamay	Bordeaux (France)	n.m.	Field experiments	L exposition and exclusion	L exclusion delayed the onset of sugar accumulation by 1 week, decreasing the final concentration of hexose in one cv. L exposition increased anthocyanin concentration after *véraison* whereas L exclusion decreased anthocyanin accumulation (mainly, Dp, Pt and Mv) in berry skin and flesh	Guan et al., 2016
Cabernet Sauvignon	Negev Desert (Israel)	2014-2015	Field experiment	L exposition (fully, 60% and 30% exposed)	L exposition decreased malic, Asp and fumaric acids while increased tartaric acid in the pulp and triggered the accumulation of Phe, narigenin-chalcone-4-O-glucoside, Cy-3-gluc and flavonols and decreased flavan-3-ols, hydroxy-cinnamates and Mv in the skin	Reshef et al., 2017

Table 2 *(Continued)*

Plant material	Region (Country)	Year	Experiment	Environmental factor	Trend	Reference
UV radiation (UV)						
Malbec	Mendoza (Argentina)	2008-2009	Commercial vineyard	UV-B	UV-B enhanced anthocyanins, gallic acid, proantocyanins, flavonols and flavanols and decreased TSS	Berli et al., 2011
Grenache and Carignan	Sardinia (Italy)	2009 and 2010	Fields	L exposition and visible R, and visible and UV-A exclusion. Two season, one of them warmer.	UV radiation induced accumulation of anthocyanins with a decrease in trihydroxylated and an increase in dihydroxylated anthocyanins. T caused a decrease in anthocyanin content regardless the L	Fernandes de Oliveira and Nieddu, 2016
Tempranillo	n.m.	n.m.	Fruit bearing cuttings grown under controlled conditions	UV-B: 0, 5.98 and 9.66 kJ m^{-2} d^{-1} applied during two ripening moments	UV-B did not affect sugars and acids. Medium UV-B increased extractable anthocyanins while high UV-B decreased them. UV-B increased flavonols and their mono- and di-substituted derivatives and decreased trisubstituted forms. UV-B did not affect total amino acid concentration although decreased Thr, Met, Ile, Ser and Gly and increased GABA	Martínez-Lüscher et al., 2014
Temperature (T) and Water deficit (WD)						
Tempranillo	n.m.	2014	Fruit-bearing cuttings grown under controlled conditions.	T: + 4°C; WD: ED and LD	T increased glucose and fructose and decreased tartaric acid in berries. Elevated T and LD enhanced amino acid content, mainly Pro, Arg, Thr and Gln. T increased methoxylated anthocyanins and flavonols.	Torres et al., 2017
Tempranillo	Alentejo (Portugal)	2013-2014	Field experiments	SDI and RDI; T: more hours of higher temperature depending on the cluster position	RDI enhanced sugars and decreased acidity, T decreased anthocyanins	Zarrouk et al., 2016
Temperature (T) and light (L)						
Gamay	n.m.	n.m.	Cell culture derived from red berry skins	HT: 40°C ; HL: 2500 $\mu molm^{-2}s^{-1}$	HL decreased anthocyanins although increased Pn and acetilglucoside derivatives and resveratrol. HT increased coumaroyl Pn and epigallocathechin. HT and HL and T increased Trp, Ala and Ser more than 3 fold	Ayenew et al., 2015
V. lambrusca (Pione)	Hiroshima (Japan)	n.m.	Research vineyard	L exclusion and T: 35°C	T or dark treatment decreased anthocyanin mainly, Mv and Pn derivatives, and enhanced flavonol content	Azuma et al., 2012
Pione (*Vitis* x lambruscana)	n.m.	n.m.	Berries incubated in a multi incubator for 10 days	HT: 35°C, LT:15°C and L (white and UV) or dark	HT or dark decreased anthocyanin accumulation. LT and light induced anthocyanin accumulation	Azuma et al., 2012

Table 2 *(Continued)*

Plant material	Region (Country)	Year	Experiment	Environmental factor	Trend	Reference
Muscat Hamburg	n.m.	2006	Fruit-bearing cuttings grown in greenhouses	HT: 30/25°C (day/night) and HL: (400 µmolm^{-2} s^{-1} PPFD)	T did not affect sugar content, decreased berry TA, malic acid and increased pH. Treatments did not affect amino acid proportion at maturity and total polyphenol content. T decreased anthocyanins and HL increased anthocyanins under elevated T	Carbonell-Bejerano et al., 2013
Pinot noir	Different location in Europe	2013	Field	Latitudinal gradient with changes in T and R	Higher values of solar R decreased phenolic compounds excepting anthocyanins, the ratio between trihydroxylated and dihydroxylated flavonols was strongly correlated with R related parameters. R increased total contents of phenolic groups, mainly flavonols and flavanonols	del Castillo-Alonso et al., 2016
Carignan and Grenache	Sardinia (Italy)	2009-2011	Field experiment	HT: + 1.5°C and 3°C over the average temperature(1971-2000) and attenuation of the PAR and UV radiation	HT decreased anthocyanin content although a positive effect of UV-A on acylation levels was observed increasing the content in Cy and Pn derivatives	Fernandes de Oliveira et al., 2015
Temperature (T) water deficit (WD) and light (L)						
Ugni blanc	Cognac region (France)	2011 and 2013	Field	Vintage effect (T, sun radiation and WD: less rainfall)	Warmer, sunnier and dryer vintage increased tartaric and ascorbic acids	Cholet et al., 2016
Touriga nacional and Trincadeira	Pegoes , Setúbal (Portugal)	2007	Fruit-bearing cuttings grown under controlled conditions.	NI (4-5 days without irrigation), HS (42°C, 1 h) and LS (2000 µmol.quanta $m^{-2}s^{-1}$, 1 h).	WD, HS and LS increased anthocyanins and carotenoids in leaves from Touriga Nacional	Carvalho et al., 2016
Light (L) and UV radiation (UV)						
Sauvignon Blanc	Elgin area of South Africa	2012, 2014 and 2015	Commercial vineyard	HL with UV-B attenuation, LL with UV-B attenuation.	UV-B attenuation in HL decreased quercetin-glucoside (responsive of polyphenolic compounds), UV-B exposition enhanced monoterpenes	Joubert et al., 2016

Table 2 *(Continued)*

Plant material	Region (Country)	Year	Experiment	Environmental factor	Trend	Reference
Water deficit (WD) and UV radiation (UV)						
Malbec	Mendoza (Argentina)	2011-2013	Commercial vineyard	UV-B, WD and ABA	UV-B increased quercetin and kaempferol	Alonso et al., 2016a
Climate Change conditions (CC)						
White and red Tempranillo	Navarra (Spain)	2013	Fruit-bearing cuttings grown in temperature-gradient-greenhouses	CC (T: +4°C over ambient temperature; CO_2: 700 ppm; CD)	CC decreased malic acid and tartaric acid in white Tempranillo while increased in red. CD and CO_2 increased sugars in must of both red and white Tempranillo. CC decreased TPI in white, no effect on red Tempranillo. CO_2 increased total anthocyanins	Kizildeniz et al., 2015
Tempranillo	Navarra (Spain)	2011	Fruit-bearing cuttings grown in greenhouses	CC (CO_2: 700ppm ; T: 28/18°C; 33-53% RH) WD (60% of controls) and type of soil	CC increased must pH, and decreased malic and tartaric acid concentrations. CC decreased total anthocyanins and colour intensity in the must	Leibar et al., 2017
Tempranillo	Navarra (Spain)	2012	Fruit-bearing cuttings grown in greenhouses	CC: (CO_2: 700ppm; T: 28/18°C) and UV-B: 0, 5,98 and 9,66 kJ m^{-2} d^{-1}	UV-B increased flavonols, anthocyanins and UV-absorbing compounds, CC conditions decreased them	Martínez-Lüscher et al., 2015b
Tempranillo	Navarra (Spain)	2008	fruit-bearing cuttings grown in greenhouses	CC: (T: +4°C over ambient temperature; CO_2: 700ppm) and WD: 40% of controls.	CC increased TSS, and pH; no effect on the TA or tartaric acid content. WD and CC decreased malic acid. WD decreased anthocyanins, CC mitigated this effect.	Salazar-Parra et al., 2010

Ala: alanine; Arg: arginine; Asp: aspartic acid; CC: climate change; CO_2: Increment of carbon dioxide; Cy: cyanidin; DI: deficit irrigation; Dp: delphidin; ED: early water deficit; GABA: γ-aminobutyric acid; Gln: glutamine; Glu: glutamic acid; Gly: glycine; HL: high light; HS: heat stress; HT: high temperature; Ile: isoleucine; L: light; LD: late water deficit; Leu: leucine; LL: low light; LS: light stress; LT: low temperature; Met: metionine; Mv: malvidin; n.m. not mentioned; NI: non irrigated; Orn: ornithine; Phe: phenylalanine; Pn: peonidin; PRI: partial root irrigation; Pro: proline; Pt: petunidin; R: radiation; Ser: serine; T: Temperature; TA: Total acidity; Thr: threonine; Trp: tryptophane; TSS: Total soluble solids; UV-B: UV-B radiation; Val: valine; WD: Water deficit.

Table 3. Berry hormones in response to environmental factors.

Plant material	Region (Country)	Year	Experiment	Environmental factor	Berry tissue	Trend	Reference
Water deficit (WD)							
Baco noir	Ontario, Canada	2006-2007	Field experiment	NI, and different levels of irrigation at different berry phases	Berry skin and pulp	WD increased ABA and ABA-GE. Irrigation enhanced DPA content	Balint and Reynolds, 2013a
Chardonnay	Ontario, Canada	2006-2007	Field experiment	NI, and different levels of irrigation at different berry phases	Berry skin and pulp	WD increased ABA and ABA-GE in both skin and pulp while decreased PA and DPA content in berry pulp	Balint and Reynolds, 2016
Pinot noir	Geinseingeim (Germany)	2009-2010	Field experiments	WS	Whole berry	WS induced genes related with the JA metabolism	Berdeja et al., 2015
Tempranillo	Navarra (Spain)	2010	Fruit-bearing cuttings grown under controlled conditions	SDI	Whole berry	At the pea-size stage, SDI berries had lower IAA and higher JA and SA than nonstressed berries. At *véraison* (onset of ripening), accumulation of ABA was less accentuated in SDI than in control berries	Niculcea et al., 2013
Tempranillo and Graciano	Navarra (Spain)	2011	Fruit-bearing cuttings grown under controlled conditions	ED and LD	Whole berry	ED caused an earlier ABA peak and LD postponed the peak. ED increased JA and SA concentrations in Tempranillo, and decreased IAA and JA and increased SA at pea size in Graciano	Niculcea et al., 2014
Tempranillo	Estremoz (Portugal)	2007 and 2008	Field experiment	NI vs. DI	Berry skin	NI increased ABA content at *véraison* (in 2007). DI increased ABA concentration in both years	Zarrouk et al., 2012

Table 3 *(Continued)*

Plant material	Region (Country)	Year	Experiment	Environmental factor	Berry tissue	Trend	Reference
Temperature (T) and water deficit (WD)							
Tempranillo	Alentejo (Portugal)	2013-2014	Field experiments	SDI and RDI, more hours of higher temperature depending on the cluster position	Berry skin	HT and RDI decreased free ABA content, the combination between both factors decreased ABA-GE and increased PA and DPA (in 2013) at full maturity	Zarrouk et al., 2016
Light (L) and UV radiation (UV)							
Gamay Fréaux and Gamay	Bordeaux (France)	n.m.	Field experiments	L exposition and L exclusion	Berry flesh and skin	Light exclusion reduced free ABA, ABA-GE, PA, and DPA	Guan et al., 2016
Temperature (T) and light (L)							
V. lambrusca (Pione)	Hiroshima (Japan)	n.m.	Research vineyard	L (white and UV) vs. Dark and elevated T	Berry skin	Elevated T decreased ABA content	Azuma et al., 2012
Muscat Hamburg	Navarra (Spain)	2006	Fruit bearing cuttings grown under controlled conditions	HT (35°C) and HL (400 µmol m^{-2} s^{-1})	De-seeded Berries	HT increased ABA content at different days during ripening	Carbonell-Bejerano et al., 2013
Kyoho	n.m.	n.m.	Potted vines in phytotron	T: 25, 27 and 30°C; and shade or sun-exposed	Berry skin	HT and sun exposure decreased ABA content	Shinomiya et al., 2015

ABA: abscisic acid; ABA-GE: ABA-glucosylester; CK: cytokinin; DI: deficit irrigation; DPA: dihydrophaseic acid; ED: early water deficit; ET: evapotranspiration; HS: heat stress; IAA: indol-3-acetic acid; JA: Jasmonic acid; L: light; LD: late water deficit; LS: light stress; LT: low temperature; n.m. not mentioned, NI: non irrigated; PRI: partial root irrigation; RDI: regulated deficit irrigation; SA: salicylic acid; SDI: sustained deficit irrigation; T: Temperature; UV: UV radiation; WD: water deficit; WS: water stress.

Table 4. Benefits of AMF for grapevines.

Plant material	Experiment	Mycorrhizal presence	Other factors	Effects	Reference
Plant water status and photosynthesis					
Asgari, Khalili, Keshmeshi and Shahroodi	Potted vines grown in greenhouses	*Glomus mosseae, Glomus fasciculatum, Glomus intraradices* and a mixture of species		AMF inoculation improved or maintain chlorophyll content	Eftekhari et al., 2012b
Crimson	Commercial vineyard	*Glomus iranicum* var. *tenuihypharum* sp. *nova*	Two years monitoring	AMF inoculation improved the photosynthetic performance, plant water status and increased WUE	Nicolás et al., 2015
Cabernet Sauvignon	Commercial vineyard	n.m.	RDI, ED and LD	AMF inoculation enhanced drought tolerance by compensating the reduced root length due to the more severe water deficits	Schreiner et al., 2007
Plant growth and nutrient uptake					
P1103 rootstock- *Vitis berlandierix Vitis rupestris*	Seedlings grown in greenhouses	*Dentiscutata heterogama, Gigaspora gigantea, Acaulospora morrowiae, A. colombiana, Rhizophagus clarus* and *R. irregularis*	Soil with high content in Cu	*R. clarus* and *R. irregularis* improved root dry mass although no effect on chlorophylls was observed	Ambrosini et al., 2015
Cabernet Sauvignon	Field experiment	*Glomus intraradices BEG 72*	Infection by *Armillaria mellea*	AMF inoculation increased plant shoot dry weight	Camprubí et al., 2008
Selection Oppenheim 4 (SO4) rootstock	Potted vines grown in greenhouses	*G. intraradices*	Infection by *Xiphinema index*	AMF increased shoot and root mass in both infected or not with the nematode	Hao et al., 2012
Razaki	Potted vines grown outdoors	*G. mosseae*	Different N fertilizers	AMF increased shoot dry weight and number of leaves	Karagiannidis et al., 2007
Crimson	Commercial vineyard	*G. iranicum* var. *tenuihypharum* sp. *nova*	Two years monitoring	AMF inoculation increased yield and improved quality of grapes	Nicolás et al., 2015
Cabernet Sauvignon	Field experiments	*G. intraradices*	Two rootstocks and infection by *A. mellea*	AMF increased total biomass	Nogales et al., 2009b
Pinot noir	Potted vines grown in greenhouses	*G. mosseae, G. intraradices* and *Scutellospora calospora*		AMF inoculation improved growth, native AMF were not necessary better than non-native ones	Schreiner, 2007

Table 4 *(Continued)*

Plant material	Experiment	Mycorrhizal presence	Other factors	Effects	Reference
SO4 and R110 rootstocks	Plants in a growth chamber	*R. irregularis*	Infection by *Fusarium oxysporum* f. sp. *herbemontis*	AMF inoculation increased growth as a defence mechanism	Vilvert et al., 2017
P1103 rootstock- *Vitis berlandierix Vitis rupestris*	Seedlings grown in greenhouses	*D. heterogama, G. gigantea, A. morrowiae, A. colombiana, R. clarus* and *R. irregularis*	Soil with high content in Cu	*R. clarus* and *R. irregularis* improved P absorption in contaminated soils	Ambrosini et al., 2015
Razaki	Potted vines grown outdoors	*G. mosseae*	Different N fertilizers	AMF modified the mineral concentration of leaves (increased P, K, and B and decreased Zn, Mn, Fe and Cu)	Karagiannidis et al., 2007
Crimson	Commercial vineyard	*G. iranicum* var. *tenuihypharum* sp. *nova*	Two years monitoring	AMF inoculation promoted the uptake of P, K and Ca and the mobilization of starch reserves for root development	Nicolás et al., 2015
Pinot noir	Potted vines grown in greenhouses	*G. mosseae, G. intraradices* and *S. calospora*		AMF inoculation improved P, K, Ca, Mg, Fe, and B uptake in some soil, and the nutrient content in stems, leaves, petioles and roots	Schreiner, 2007
Metabolism and phenolic content					
Pinot noir, Divico and Chasselas	Potted vines grown in greenhouses	*R. irregularis*	Leaf infection by *Plasmopara viticola* or *Botrytis cinerea*	AMF inoculation increased the active forms of resveratrol, viniferins and pterostilbene.	Bruisson et al., 2016
Asgari, Khalili, Keshmeshi and Shahroodi	Potted vines grown in greenhouses	*G. mosseae, G. fasciculatum, G. intraradices* and a mixture of species.		AMF inoculation enhanced total phenols and quercetin in leaves.	Eftekhari et al., 2012a
Asgari, Khalili, Keshmeshi and Shahroodi	Potted vines grown in greenhouses	*G. mosseae, G. fasciculatum, G. intraradices* and a mixture of species		AMF inoculation improved total sugars and phenol content in leaves	Eftekhari et al., 2012b
Tempranillo	Potted vines grown in greenhouses	*G. intraradices*	T: +4°C	AMF inoculation increased leaf total phenols and total antioxidant capacity, especially at elevated temperature	Torres et al., 2015
Tempranillo	Potted vines grown in greenhouses	*G. intraradices*	T: +4°C	AMF inoculation increased must phenolic compounds and total antioxidant capacity, under elevated temperature	Torres et al., 2016
Tempranillo	Potted vines grown in greenhouses	*G. intraradices*	T: +4°C, ED and LD	AMF inoculation improved the effects of LD irrigation on grape quality under elevated temperature	Torres et al., 2018

Table 4 *(Continued)*

Plant material	Experiment	Mycorrhizal presence	Other factors	Effects	Reference
Gene regulation					
Pinot noir	Potted vines grown in greenhouses	Two different inocula*: 1) Funneliformis mosseae 2)* 40% crude inoculum of AMF *(Glomus spp., Glomus mosseae,* and *G. viscosum),* and 21.6% bacteria and saprotrophic fungi		Mycorrhizal inoculation upregulated genes related with nutrient transport, TF, cell wall metabolism in relation with the arbuscular colonization, genes involved in the ABA level. Ethylene responsive factor genes were down regulated	Balestrini et al., 2017
Pinot noir, Divico and Chasselas	Potted vines grown in greenhouses	*R. irregularis*	Leaf infection by *P. viticola* or *B. cinerea*	AMF inoculation up-regulated stilbenoid biosynthesis genes related to defence mechanisms in leaves.	Bruisson et al., 2016
Selection Oppenheim 4 (SO4) rootstock	Potted vines grown under controled conditions	*Glomus irregulare* and *G. mosseae*	P starvation	AMF colonization increased genes and proteins involved in carbon metabolism due to P deficiency, P remobilisation, stress and defence, development and root architecture.	Cangahuala-Inocente et al., 2011
SO4 rootstock	Potted vines grown in greenhouses	*G. intraradices*	Infection by *X. index*	AMF up-regulated defence-related Vitis genes)	Hao et al., 2012
Pathogen resistance					
Cabernet Sauvignon	Field experiment	*G. intraradices BEG 72*	Infection by *A. mellea*	AMF inoculation decreased plant mortality	Camprubí et al., 2008
SO4 rootstock	Potted vines grown in greenhouses	*G. intraradices*	Infection by *X. index*	AMF induced protection against the parasitic nematode decreasing its presence in mycorrhizal roots	Hao et al., 2012
Richter 110 rootstock	Potted vines grown in greenhouses and shadowhouses	*G. intraradices*	Infection by *A. mellea*	AMF inoculation provided pathogen resistance	Nogales et al., 2009a
SO4 and R110 rootstocks	Plants in a growth chamber	*R. irregularis*	Infection by *F. oxysporum* f. sp. *herbemontis*	AMF inoculation provided pathogen resistance by increasing the expression of defence-proteins	Vilvert et al., 2017

Ca: calcium; Cu: copper; ED: early water deficit; Fe: iron; LD: late water deficit; N: nitrogen; P: phosphorus; RDI: regulated deficit irrigation; T: temperature; TF: transcription factor; WS: water stress; WUE: water use efficiency.

Figure 1.

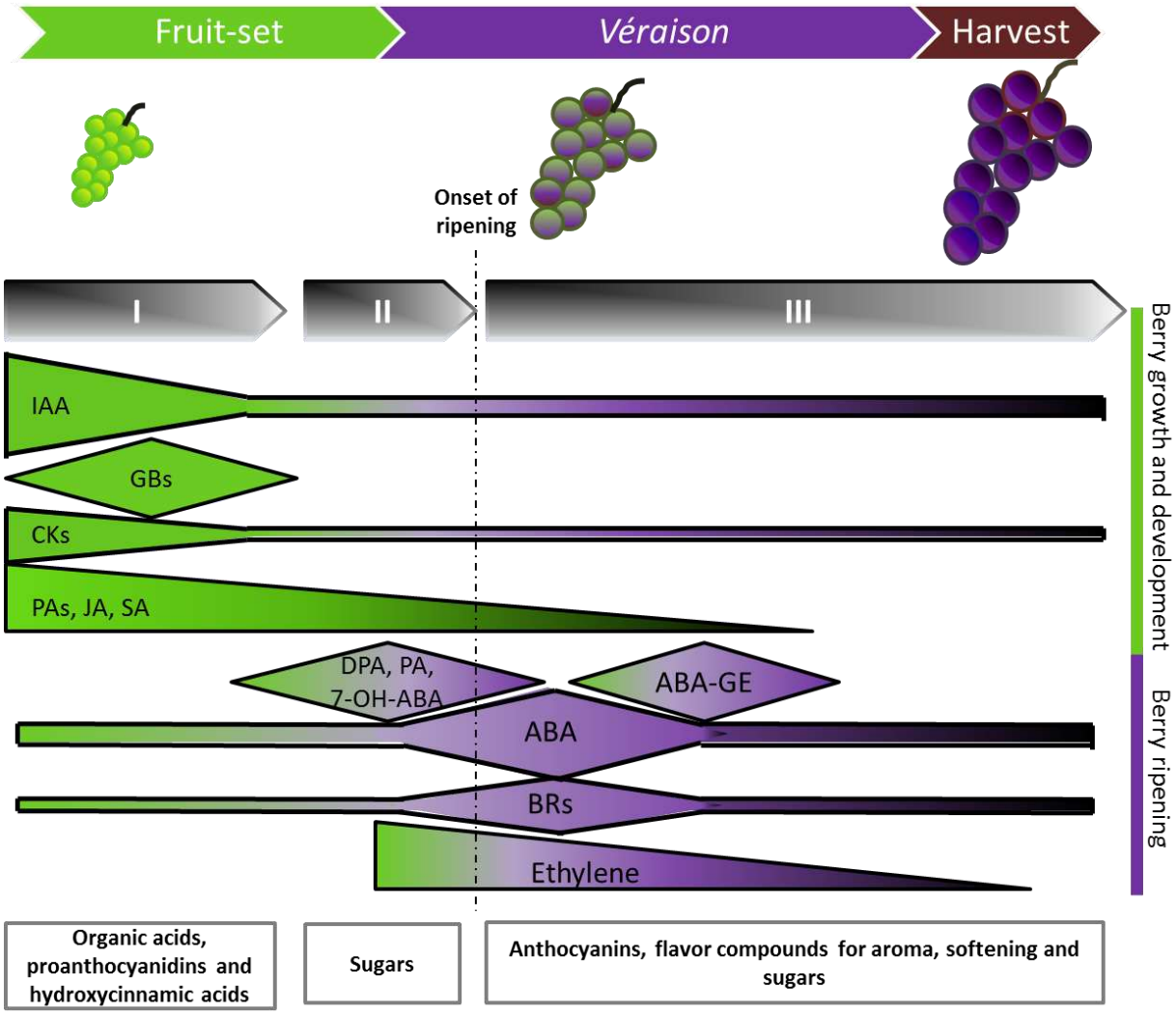

Figure 1. Berry hormonal content during its development and ripening (adapted from Conde et al. (2007) with the addition of the last findings). Phase I: early fruit development; Phase II: lag phase; Phase III: berry ripening. Hormonal content variations are indicated by arbitrary thicknesses volumes. ABA: abscisic acid; ABA-GE: abscisic acid glucosylester; *7-OH*-ABA: 7-hydroxy abscisic acid; BRs: brassinosteroids; CKs: cytokinins; DPA: dihydrophaseic acid; GBs: gibberellins; IAA: indol-3-acetic acid; JA: jasmonic acid; PAs: polyamines; PA: phaseic acid; SA: salicylic acid. The main compounds that are accumulated in the fruit are showed at the bottom.

OBJETIVOS

The general objective of this PhD thesis was to assess the intra-varietal diversity of grapevine cv. Tempranillo to respond to different climate change scenarios (elevated air temperature and deficit irrigation) and to analyze if the potential benefits of mycorrhizal symbiosis on plant metabolism could be maintained under the predicted environmental conditions.

The general objective was divided into the following objectives:

- **Objective 1.** Evaluate the impact of pre- and post-veraison deficit irrigation under elevated temperatures on berry metabolism of two Tempranillo clones.
- **Objective 2.** Evaluate the response of three Tempranillo clones to elevated temperature and to determine whether mycorrhizal inoculation can improve berry antioxidant properties under these conditions.
- **Objective 3.** Characterize the response of three clones of Tempranillo to the combination of different water deficit programs (pre- and post-veraison deficit irrigation) and mycorrhizal inoculation under elevated temperatures on fruit quality and antioxidant capacity.
- **Objective 4.** Determine if the ability of arbuscular mycorrhizal fungi for inducing the accumulation of anthocyanins in grapes under a climate change framework could be mediated by alterations in the metabolism of ABA during berry ripening.
- **Objective 5.** Evaluate the effect of mycorrhizal inoculation and elevated temperature on phenolic composition and antioxidant activity of leaf extracts of Tempranillo and to determine whether such effects differed among clones within this variety.
- **Objective 6.** Analyze the effects of mycorrhizal association and elevated temperature on the levels of some primary and secondary metabolites as well as on the concentrations of minerals in leaves of Tempranillo grapevines in order to assess the potential application of these vegetative wastes in the human diet or for pharmacological and biomedical purposes.

The abovementioned goals have been developed in the following chapters:

- **Chapter 1.** Flavonoid and amino acid profiling on *Vitis vinifera* L. cv. Tempranillo subjected to deficit irrigation under elevated temperatures. Published in *Journal of Food Composition and Analysis* (2017) 62: 51-62 (This chapter addresses objective 1).
- **Chapter 2.** Berry quality and antioxidant properties in *Vitis vinifera* cv. Tempranillo as affected by clonal variability, mycorrhizal inoculation and temperature. Published in *Crop & Pasture Science* (2016) 67: 961-977 (This chapter addresses objective 2).

- **Chapter 3.** Influence of irrigation strategy and mycorrhizal inoculation on fruit quality in different clones of Tempranillo grown under elevated temperatures. Published in *Agricultural Water Management* (2018) 202:285-298 (This chapter addresses objective 3).
- **Chapter 4.** Mycorrhizal symbiosis affects ABA metabolism during berry ripening in *Vitis vinifera* L. cv. Tempranillo grown under climate change scenarios. Published in *Plant Science* (doi: 10.1016/j.plantsci.2018.06.009; this chapter addresses objective 4).
- **Chapter 5.** Antioxidant properties of leaves from different accessions of grapevine (*Vitis vinifera* L.) cv. Tempranillo after applying biotic and/or environmental modulator factors. Published in *Industrial Crops and Products* (2015) 76: 77-85 (This chapter addresses objective 5).
- **Chapter 6.** Nutritional properties of Tempranillo grapevine leaves are affected by clonal diversity, mycorrhizal symbiosis and air temperature regime. Submitted to *Plant Physiology and Biochemistry* (under revision; this chapter addresses objective 6).
- **Chapter 7.** Potential biomedical reuse of vegetative residuals from mycorrhized grapevines subjected to warming. Submitted to *Archives of Agronomy and Soil Science* (under revision; this chapter addresses objective 6).

CAPÍTULO 1

Flavonoid and amino acid profiling on *Vitis vinifera* L. cv Tempranillo subjected to deficit irrigation under elevated temperatures.

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Flavonoid and amino acid profiling on *Vitis vinifera* L. cv Tempranillo subjected to deficit irrigation under elevated temperatures

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Abstract

Throughout the southern Mediterranean regions of Europe, projected climate warming combined with severe droughts during the growing season may alter grape metabolism, thus modifying the nutritional value of berries and the quality of wines. This study investigated the effects of pre- and post-veraison drought under elevated temperatures on berry skin metabolism of two Tempranillo clones (CL).

Experimental assays were performed on fruit-bearing cuttings from CL-1089 and CL-843 of *Vitis vinifera* (L.) cv. Tempranillo subjected to two temperature regimes (24/14°C or 28/18°C (day/night)) combined with three irrigation regimes during berry ripening: (i) water deficit from fruit set to veraison (early deficit, ED); (ii) water deficit from veraison to maturity (late deficit, LD); and (iii) full irrigation (FI). At 24/14°C, the LD treatment performed better than the ED treatment. Differences were attenuated at 28/18°C and responses were modulated by type of clone. Elevated temperatures induced the accumulation of hexoses and amino acids in berries. ED at 24/14°C reduced anthocyanins and flavonols, which may decrease the antioxidant properties of fruits. In contrast, the levels of these secondary metabolites did not decrease when LD was applied. Our results suggest that the adaptation of grapevines for climate change might be plausible with the optimization of timing of water deficit and the appropriate selection of clones.

***Keywords**: Amino acids; Anthocyanins; Berry skin metabolites; Climate change; Flavonols; Food analysis; Food composition; Grapevines; Regulated deficit irrigation*

1. Introduction

Grapes are one of the most important crops in Europe. According to the official dataset of the Statistics Division of Eurostat, in 2014 Europe produced 22.6 million tons, with Spain being one of the greatest producers (29.6% of European production) (Eurostat Statistics Division, 2014). The most distinctive characteristic of the South Mediterranean European climate is the concentration of rainfall in the winter half-year but future climate projections for the this region predict seasonal temperatures with higher rates of warming in summer and autumn (IPCC, 2014; Spinoni et al., 2015).

Abiotic stresses such as drought and high temperatures reduce grapevine yield due to their great impact on berry growth and ripening (Kuhn et al., 2014). Berry skin (exocarp) is the site for the synthesis of major compounds and defines grape berry quality, which mainly depends on sugars, organic acids, amino acids, phenolic compounds and aroma precursors (Castellarin et al., 2012; Darriet et al., 2012). This very active skin metabolism deeply influences the final characteristics of the berry and thus the understanding of metabolic responses to environmental constraints has both scientific and practical importance. The projected warming trends due to global climate change specially affect grapevine physiology. Harvest occurs sooner (Sadras and Petrie, 2011), berry sugar content (and alcohol in the wine) tends to increase (Petrie and Sadras, 2008) and phenolic and aromatic ripeness are delayed (Teixeira et al., 2013), which results in an imbalance between berry sugar accumulation and phenolic ripening (Sadras and Moran, 2012). Regarding drought, some studies have reported that application of regulated deficit irrigation during berry ripening has a significant impact on berry metabolism, which influences flavor and the quality characteristics of grapes and wines (Deluc et al., 2009). Indeed, water deficit improved accumulation of phenolic compounds, especially anthocyanins (Niculcea et al., 2014, 2015; Kyraleou et al., 2016) due to direct effects on flavonoid gene expression and metabolism (Castellarin et al., 2007). Thus, water deficit irrigation could enhance the nutraceutical value of berries since water restriction can induce the accumulation of anthocyanins (Kyraleou et al., 2016). Anthocyanins are the most important group of water-soluble pigments in plants, and they are regarded as important components in human nutrition due to their antioxidant capacities (Stintzing and Carle, 2004) and anti-carcinogenic effects against several types of cancer cells (You et al., 2011). However, expected benefits of water deficit may not be achieved under projected future warming conditions (Bonada et al., 2015).

The adaptation of grapevines grown in south Mediterranean Europe to a climate change scenario might require a selection of new grapevines varieties. Many studies have reported a

broad clonal diversity in grapevine varieties for precocity of the phenological cycle, yield, berry composition, skin phenolic compounds and disease resistance (Revilla et al., 2009; van Leeuwen et al., 2013), and for response to environmental changes (Berdeja et al., 2015; Torres et al., 2016). Therefore, the aim of this study was to investigate the impact of pre- and post-veraison deficit irrigation under elevated temperatures on berry metabolism of two Tempranillo clones.

2. Material and Methods

2.1. Plant material and growth conditions

Dormant 400-500 mm long *Vitis vinifera* (L.) cuttings from two clones of Tempranillo were collected during the winter of 2014 from an experimental vineyard of the Institute of Sciences of Vine and Wine (Logroño, Spain) (Denomination of Origin Rioja, North of Spain) (latitude: 42°28'12''N; longitude: 2°26'44''W, altitude: 384 mamsl). A brief description of the selected clones is presented in Table 1. Cuttings from each clone were selected to obtain fruit-bearing cuttings according to the steps originally outlined by Mullins (1966) with some modifications described in Ollat et al. (1998) and Morales et al. (2016). Briefly, cuttings were rooted in a heat-bed (27°C) and then kept in a cool room (4°C). One month later, the cuttings were planted 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 greenhouses, which were adapted to simulate climate change conditions (more details in Morales et al., 2014). Experiments were made with potted vines to ensure that both clones were subjected to the same temperature conditions and similar water stress patterns. Previous research has demonstrated that fruit-bearing cuttings are a meaningful and useful model system to study the response of berry ripening to environmental factors (Morales et al., 2016). Initial growth conditions were a 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} at the level of the inflorescence. Until fruit set, plants were watered twice per day with the nutrient solution reported by Ollat et al. (1998) alternated with water to maintain the soil water content at 80% of pot capacity.

2.2. Experimental design

We established a two-factorial experiment where two temperature regimes were combined with three water regimes. At fruit set (Eichhorn and Lorenz (E-L) growth stage 27) (Coombe, 1995) fruit-bearing cuttings (36 plants per clone) were divided into two groups that were exposed to different temperature regimes: 24/14°C (day/night) and 28/18°C (day/night). At this stage, plants have 4-5 fully expanded leaves. The 24/14°C temperature regime was selected according average temperatures recorded in La Rioja (1981-2010) (AEMET, Spain) during the growing season. The 28/18°C temperature regime was selected according to predictions of a rise of 4.0°C at the end of the present century (IPCC, 2014). Both temperature regimes were maintained until berries ripened (21-23°Brix) (E-L 38 stage).

Within each temperature regime, plants were divided into three groups that were subjected to different irrigation programs. Two deficit irrigation strategies were compared with full irrigation (FI). In the FI treatment, pots were maintained at 80% of pot capacity from fruit set to harvest. In the deficit irrigation treatments, plants received 50% of the water given to FI plants from fruit set (E-L 27 stage) to onset of veraison (E-L 35 stage) (early deficit, ED) or from onset of veraison (E-L 35 stage) to maturity (E-L 38 stage) (late deficit, LD). Volumetric soil water content was monitored with an EC 5 water sensor (Decagon Devices, Inc., Pullman, WA, USA) placed within each pot. There were three replicates for each treatment.

Non-destructive determinations were made at four stages of berry development: 1) when berries began to soften (E-L 34 stage, green berries); 2) when berries began to colour and enlarge (E-L 35 stage, veraison); 3) seven days after veraison (E-L 36 stage); and 4) fourteen days after veraison (E-L 37 stage). At fruit maturity (E-L 38 stage), plants were harvested separately based on sugar level (21-23°Brix) from berry subsamples (2-3 berries) taken weekly. Length of phenological phases was recorded as the number of days from fruit set (E-L 27 stage) to veraison (E-L 35 stage), and from veraison (E-L 35 stage) to maturity (E-L38 stage).

2.3. Plant measurements

Predawn leaf water potential (Ψ_{pd}) was measured with a SKYE SKPM 1400 pressure chamber (Skye Instruments Ltd, Llandrindod, Wales, UK) on three fully expanded leaves per treatment at each sampling date just prior to irrigation.

The evolution of epidermal levels of flavonols and anthocyanins was estimated *in situ* by using a handheld, non-destructive fluorescence-based proximal Multiplex3^{TM} sensor (Force-A, Orsay, France) as described by Agati et al. (2013). At maturity, ten berries from each treatment were collected and weighed. Mean fresh berry mass was determined and then, berries were separated into skin and flesh (including seeds). Subsequently, each berry fraction was oven-dried at 80°C until constant mass was reached. Berry water content was calculated as 100*(FM-DM)/ FM, where FM is fresh mass and DM is dry mass. The relative skin mass was calculated as the quotient between skin FM and total berry FM expressed as a percentage. The rest of the berries were counted, weighed and frozen at -80°C for further analysis.

2.4. Sugars, organic acids and amino acids profiles in berry skins

Samples of 5-10 berries per plant were separated into skin and flesh. Skins were powdered separately in an MM200 ball grinder (Retsch, Haan, Germany) and then, they were freeze dried in a Vir Tis Bench Top K lyophilizer (SP Scientific, Warminster, Philadelphia, PA, USA). Skins from each plant were used to analyse primary and secondary metabolites. Primary metabolites were extracted according to Bobeica et al. (2015) with minor modification. Subsamples of 50 mg fine powder of skins were extracted with 80% ethanol (v/v) at 80°C for 15 min followed by two extractions with 50% ethanol (v/v) and ultrapure water, respectively, dried in Speed-Vac, and re-dissolved in ultrapure water. The resultant extracts were used for determinations of sugars, organic acids and amino acids.

Sugars were measured enzymatically with an automated micro-plate reader (Elx800UV, Biotek Instruments Inc., Winooski, VT, USA) using the Glucose/Fructose kit from BioSenTec (Toulouse, France). Malic acid was determined using an enzyme-coupled spectrophotometric method that measures the change in absorbance at 340 nm from the reduction of NAD^+ to NADH. Tartaric acid was assessed by using the colorimetric method based on ammonium vanadate reactions (Pereira et al., 2006). Both compounds were quantified with a Bran and Luebbe TRAACS 800 autoanalyzer (Bran & Luebbe, Plaisir, France).

After derivation with 6-aminoquinolyl-N-hydroxy-succinimidyl-carbamate (AccQ-Tag derivatization reagent, Waters, Milford, MA, USA) according to Hilbert et al. (2003), free amino acids were measured according to Habran et al. (2016). Briefly, amino acids were analysed using an UltiMate 3000 UHPLC system (Thermo Electron SAS, Waltham, MA USA) equipped with an FLD-3000 Fluorescence Detector (Thermo Electron SAS, Waltham, MA USA). Separation was performed on a AccQ•Tag Ultra column, 2.1 x 100 mm, 1.7 µm (Waters,

Milford, MA, USA) at 37°C with elution at 0.5 ml min^{-1} (eluent A, sodium acetate buffer, 140mM at pH 5.7 ; eluent B, acetonitrile ; eluent C, water) according to the gradient described by Habran et al. (2016). Chromatograms corresponding to excitation at 250 nm and emission at 395 nm were recorded (Figure S1). To maintain consistent retention time and a stable baseline, a control was performed before each run of 18 samples. Chromeleon software, version 7.1 (Thermo Electron SAS, Waltham, MA USA) was used to calculate peak area. A standard of 20 amino acids (Alanine, Arginine, Aspartic acid, Asparagine, Cysteine, GABA, Glycine, Glutamic acid, Glutamine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tyrosine, Valine) purchased from Sigma (St Louis, Missouri, USA) was used after the control and in the middle of each run to calibrate amino acid quantification. Seventeen amino acids were identified and quantified in skin extracts as described by Pereira et al. (2006). Results were expressed in µmol g^{-1} dry matter (DM) of skin.

2.5. Anthocyanin and flavonol profiles in berry skins

Another subsample of powdered berry skins was used in order to analyse individual anthocyanins and flavonols. Samples were extracted according to Acevedo de la Cruz et al. (2012) then analysed as described in Martínez-Lüscher et al. (2014) with some modifications. Extracts were analysed using an UltiMate 3000 UHPLC system (Thermo Electron SAS, Waltham, MA USA) equipped with DAD-3000 diode array detector operating at 520 nm and at 360 nm (Thermo Electron SAS, Waltham, MA USA). Separation was performed on a Syncronis C18, 2.1 x 100 mm, 1.7 µm Column (Thermo Fisher Scientific, Waltham, MA USA) at 25°C with elution at 0.368 ml min^{-1} according to the following gradient (v/v): 0 min 92.2% A 7.8% B, 9.6 min 73% A 27% B, 14.1 min 70 % A 30% B, 14.8 min 92.2% A 7.8% B (eluent A, water and formic acid, 90/10 v/v; eluent B, acetonitrile). Identification and peak assignment of phenolic compounds were based on comparison of their retention times and UV-vis spectrometric data with that of pure standards. Formal identification of flavonoids was performed by liquid chromatography coupled to mass spectrometry and nuclear magnetic resonance spectrometry as in previous studies (Acevedo de la Cruz et al., 2012; Hilbert et al., 2015). Chromeleon software, version 7.1 (Thermo Electron SAS, Waltham, MA USA) was used to calculate peak area. The concentration of individual flavonoids was calculated in milligrams per gram (mg g^{-1}) of dry skin weight (DM) using malvidin-3-O-glucoside was used as external standard for all the quantified anthocyanins (at 520 nm), and quercetin-3-O-glucoside was used for all the quantified flavonols (at 360 nm) (Extrasynthese, Genay, France). A chromatographic profile of anthocyanins is shown in Figure S2.

2.6. Statistical analysis

Statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS) (SPSS Inc., Chicago, IL, USA) version 21.0 for Windows. Data were subjected to Kolmogorov-Smirnov normality tests due to the small sample size (3 plants per treatment). Data appeared to follow a normal distribution; therefore an analysis of variance (ANOVA) was conducted within each clone. Percentage data were transformed into arcsine root square before ANOVA. The test was performed to assess the main effect of the factors temperature (T) (24/14°C, 24 and 28/18°C, 28) and irrigation program (I) (FI, ED and LD) and the interaction between them (T×I). Means ± standard errors (SE) were calculated and when the F ratio was significant ($P \leq 0.05$), a Duncan test was applied. A two-way ANOVA was performed to determine significant differences in all parameters measured. Berry characteristics and the main primary and secondary metabolites were analysed by principal component analysis (PCA) with the same software.

3. Results and discussion

3.1. Phenology and physiological characteristics during berry ripening

Clones of grapevine cv. Tempranillo tested in this study show significant diversity for some agronomic traits such as length of cycle, yield, bunch mass and berry mass (Table 1). With regard to phenology, vineyard-grown plants of CL-1089 were characterized by a shorter reproductive cycle than CL-843. Temperature was the main factor influencing time from fruit set to veraison which was shortened by increasing temperature to 28/18°C (Table 2) as has been shown other studies (Petrie and Sadras, 2008; Hall et al., 2016). Moreover, interaction between ED irrigation and 24/14°C lengthened time from veraison to maturity.

Some studies have reported that the application of deficit irrigation to grapevines caused different effects on berry ripening due to differences in the timing of water stress, its severity and duration, and cultivar (Intrigliolo and Castel, 2010; Niculcea et al., 2014). The present study has attempted to eliminate such variations by subjecting two clones of Tempranillo to a similar water stress level at either pre- or post-veraison, for comparison (Figure 1). Results showed that temperature and irrigation treatments caused significant differences in grapevine water status throughout fruit ripening, as indicated by the decrease in predawn Ψ_{pd} measured in plants subjected to ED or LD compared with FI plants (Figure 2).

The FERARI and FLAV indices measured with the Multiplex3^{TM} sensor are good indicators of anthocyanin and flavonol contents of tissues (Agati et al., 2013). Both types of compounds belong to flavonoids, the most widely studied group of polyphenols. From the nutritional point of view, polyphenol rich diets provide important protection against the development and progression of many chronic pathological conditions, such as cancer, diabetes, cardio-vascular problems and aging in part due to their antioxidant activity (Pandey and Rizvi, 2009). Anthocyanins are the main agents responsible for the color of red grapes and the wines produced from them. Our data revealed that irrigation was the main factor modulating the FERARI index in CL-1089, but in CL-843 both temperature and irrigation significantly accounted for diminished anthocyanin content (Figure 1). Flavonols serve as UV-protecting agents but also, contribute to both bitterness and to wine colour as copigments with anthocyanins (Castellarin et al., 2012; Hilbert et al., 2015). During berry ripening, temperature and irrigation modulated the FLAV index in both clones, resulting in significant interaction between both factors for most stages. At the E-L 37 stage the combination of LD and elevated temperature (28LD) significantly improved the FLAV index, especially in CL-1089, which may have counteracted the expected loss of antioxidant capacity associated with the decreased level of anthocyanins in berries from the 28LD treatment.

3.2. Berry characteristics and berry skin primary and secondary metabolites at maturity

Irrigation was the main factor modifying berry traits in both clones, resulting in small berries with high relative skin mass in the treatment ED (Table 2). In CL-843, increasing temperature to 28/18°C also contributed to decreased relative skin mass and berry water content.

In CL-1089, temperature was the main factor modulating primary metabolites such as sugars and organic acids. In fact, warm temperatures resulted in increased concentrations of glucose and fructose, and reduced tartaric acid (Figure 2). Increased levels of sugars may enhance the alcohol content in wine after the must fermentation carried out by yeasts. Total amino acid content was modulated by both temperature and irrigation, thus 28LD led to the highest concentration. The accumulation of amino acids is a common phenomenon in plant stress response related with nitrogen-reallocation following growth inhibition and/or with enhanced protein catabolism (Less and Galili, 2008). Free amino acids in must are of great importance, because they constitute a source of nitrogen for yeast in alcoholic fermentation, for lactic acid bacteria in malolactic fermentation and can also be a source of aromatic compounds (Moreno-Arribas and Polo, 2009) that contribute to wine aroma, taste and appearance (Garde-Cerdán et al., 2009; Darriet et al., 2012). However, in certain cases, some

amino acids can produce undesirable compounds in wines, such as ethyl carbamate (from urea produced in the metabolism of arginine), biogenic amines (from tyrosine, arginine, histidine, phenylalanine and lysine), ochratoxin A (from 2-phenylalanine, when grapes are contaminated by some fungi), and β-carbolines (from tryptophan) (Moreno-Arribas and Polo, 2009).

Regarding secondary metabolites, in CL-1089 irrigation was the main factor modulating skin anthocyanin and flavonol concentrations, with the 24ED being the treatment most severely affected (Figure 2). Flavonol content was also modulated by temperature, since 28LD have improved flavonol content. Similarly, other studies have shown that the flavonoid pathway responds to water deficit at the transcript and metabolite level thus determining increased phenolic concentrations (Savoi et al., 2016). The enhancement of flavonol content was more evident in CL-1089 grown at 28/18°C, which supports our previous observations showing that this clone seemed quite tolerant of warm temperatures (Torres et al., 2016).

In CL-843, temperature was the main factor modulating primary metabolites (i.e., glucose, fructose and tartaric acid) whereas both, temperature and irrigation contributed to increased amino acid content, resulting in a highly significant interaction between both factors (T×I, $P \leq 0.001$) (Figure 3). Regarding the secondary metabolites of CL-843, temperature and irrigation accounted for diminished anthocyanins, but no significant changes were recorded in flavonol concentrations (Figure 3). Overall, for both Tempranillo clones, differences in secondary metabolites between the three irrigation procedures assayed were attenuated in plants grown at 28/18°C (Figures 2 and 3).

3.3. Berry skin amino acid and flavonoid profiles at maturity

Amino acid profiles are known to change in response to environmental constraints such as water deficit (Niculcea et al., 2014; Berdeja et al., 2015). LD under warm temperatures (28) enhanced the amount of most amino acids in grape skins of both clones, which was mainly due to the accumulation of arginine, proline, threonine and glutamine (Table 3). The marked abundance of arginine reflects its role as a precursor of the remaining amino acids and is also an important yeast nitrogen source (Garde-Cerdán et al., 2009). Increased levels of arginine, however, can decrease wine safety when this amino acid serves as a precursor for the synthesis of putrescine, a biogenic amine frequently found in wine. Excessive intake of biogenic amines can cause health problems, such as headache, flushing, itching, skin irritation, hypotension, vomiting or tachycardia (Guo et al., 2015). Proline may contribute to berry taste but it can also act as an osmoprotectant in response to deficit irrigation (Castellarin et al., 2007) and threonine is strongly correlated with the accumulation of odorants related to fatty

acid synthesis (Hernández-Orte et al., 2002). The high arginine and proline contents in berry skins subjected to 28LD could be related to increased amount and/or activity of ornithine decarboxylase during water deficit (Berdeja et al., 2015), which in our case, could be exacerbated at 28/18°C (Table 3). Phenylalanine was significantly enhanced in berry skins from both clones in the 24ED treatment (Table 3), and such an increase coincided with decreased contents of anthocyanins (Figures 2 and 3), suggesting reductions in the amount and/or activity of phenylalanine ammonia lyase under ED (Deluc et al., 2009). As mentioned above, 2-phenylalanine may be the precursor of ochratoxin A, a dangerous mycotoxin produced as a secondary metabolite by several fungi of the *Aspergillus* or *Penicillium* families that may be contaminating grapes. This mycotoxin has been shown to be nephrotoxic, hepatotoxic, teratogenic and carcinogenic in animals and has been classified as a possible carcinogen in humans (Mateo et al., 2007). Overall, changes observed in amino acid profiles due to temperature and/or irrigation could be relevant because they can affect the aromatic characteristics of wines (Hernández-Orte et al., 2002).

The berry skin anthocyanins of both clones were dominated by malvidin-3-glucosides regardless of the temperature and water irrigation applied (Table 4). According to Huang et al. (2014), malvidin-3-glucosides are promising molecules for the development of nutraceuticals to prevent chronic inflammation. In CL-1089, irrigation was the main factor accounting for the diminished contribution of 3-monoglucosides and increased 3-*p*-coumaroyl-glucosides to total anthocyanins. However, both temperature and irrigation accounted for increased 3-acetyl-glucosides due mainly to increased derivatives of petunidin and malvidin. Other studies have indicated that water deficit stimulated anthocyanin hydroxylation, which converts hydroxylated anthocyanins (cyanidin and delphinidin) into their methoxylated derivates (peonidin, petunidin and malvidin) through the differential regulation of flavonoid 3'-hydroxylase, flavonoid 3', 5'-hydroxylase and O-methyltransferase (Castellarin et al., 2007; Deluc et al., 2009). Our results are in accordance with these observations in the 24ED treatment, in which the proportion of malvidin increased (Table 4). However, in CL-1089 this effect disappeared under warming conditions whereas in CL-843, temperature was the main factor accounting for increased proportion of methoxylated derivatives (i.e., malvidin) indicating that the anthocyanin hydroxylation pathway confers stability of anthocyanins under warm conditions (Movahed et al., 2016). In CL-843, temperature was the main factor modulating the distribution of the three anthocyanin fractions (Table 4). Thus, elevated temperature reduced the contribution of 3-monoglucosides to total anthocyanins increasing that of 3-acetyl-glucosides (malvidin and petunidin) and 3-*p*-coumaroyl-glucosides (malvidin), a finding in line with previous studies (Tarara et al., 2008). In this clone, the ED treatment also

contributed to changes in 3-acetyl-glucosides profiles showing reduced petunidin and increased peonidin derivatives. Overall, changes in anthocyanin profiles were more evident at 24/14°C than at 28/18°C and were limited to the ED treatment. By contrast, for both temperature regimes, anthocyanin profiles of LD were almost the same as those analyzed in FI plants.

Berry skin flavonols were dominated by myricetin-3-O-glucoside regardless of the temperature and irrigation applied (Table 5). In CL-1089, temperature was the main factor modifying flavonol composition. Thus, the high concentration of flavonols observed at 28/18°C (Figure 2) was mainly due to increased myricetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-glucoside, laricitrin-3-O-glucoside and isorhamnetin-3-O-glucoside (Table 5). Taking into account that flavonols provide photoprotection (Martínez-Lüscher et al., 2014), the enhanced flavonol synthesis of the 28LD treatment could contribute to high tolerance of this clone under warm temperatures (Torres et al., 2016). In contrast to this trend, temperature and/or irrigation did not affect the total flavonol concentration of CL-843 but influenced its individual composition in different ways (Figure 3). Thus, raising temperature enhanced quercetin-3-O-galactoside, laricitrin-3-O-glucoside and isorhamnetin-3-O-glucoside but deficit irrigation reduced myricetin-3-O-glucoside, quercetin-3-O-glucoside and kaempferol-3-O-glucoside (Table 5). In general, changes in flavonol profiles were more pronounced at 28/18°C than at 24/14°C, especially when plants were subjected to the LD treatment. Environmental factors (elevated temperature in our study) that induce the accumulation of quercetin can enhance the healthy properties of berry grapes because this flavonoid has often been linked to beneficial effects on the cardiovascular system (Bondonno et al., 2016). In addition, increases in the levels of laricitrin-3-O-glucoside and isorhamnetin-3-O-glucoside may have improved the antioxidant capacity of berries (Jiang and Zhang, 2012).

PCA was conducted in the present study to determine general trends in the different samples. Figure 4A shows the scores plot obtained by PCA where samples of each clone are grouped in the plot of PC1 versus PC2. PC1 accounted for about 35.23% of the total variance while PC2 covered about 24.01%. Different Tempranillo clones could not be clearly distinguished (Figure 4A) but irrigation treatments were separated by PC2 for both clones (Figure 4A). The discrimination was mainly based on total anthocyanins and the distribution of the three anthocyanin fractions, total flavonols, relative skin mass and berry mass (Figure 4B). Otherwise, temperature treatments were distinguished by PC1 (Figure 4A) and were mainly based on differences in sugars, main amino acids and tartaric acid (Figure 4B). This analysis also showed that LD and FI appeared together under both temperature conditions, reinforcing

the idea that LD irrigation could continue being valid under a projected warming scenario in order to maintain berry characteristics and metabolite profiles.

4. Conclusions

This research provides evidence for clonal-specific changes in berry skin metabolism of Tempranillo resulting in different abilities to respond to deficit irrigation under warm temperatures. In our experimental conditions, at 24/14°C LD performed better than ED in terms of anthocyanins, flavonols and berry traits but such differences were clearly attenuated at 28/18°C. The extent of alteration in primary metabolism due to temperature was higher than in secondary metabolism, which was mainly affected by deficit irrigation. While the increased levels of hexoses in berries developing at 28/18°C may enhance the alcohol content in wine, the decreased levels of anthocyanins and flavonols in berries from plants subjected to ED at 24/14°C may be associated with decreased antioxidant properties. Temperature and irrigation modified the metabolite profiles of amino acids, anthocyanins and flavonols in both clones, with CL-843 appearing to be more sensitive than CL-1089 to high temperature. These results indicate that the adaptation to climate change in south Mediterranean Europe might be plausible with the optimization of timing of water deficit and the appropriate selection of clones. Nevertheless, the limitations of controlled environments with regard to grapevine growth require that extrapolations to field-grown grapevines should be made with caution.

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Table 1. Summary of the agronomic characteristics of the Tempranillo clones used in this study. Data provided by Institute of Sciences of Vine and Wine (Logroño, Spain) were collected and averaged over the 2009-2012 period from plants grown in field.

	CL-1089	CL-843
City of origin (region)	Bargota (Navarra) (Latitude: 42°33´40´´N; longitude: 2°18´43´´W; altitude: 587 mamsl).	Oyón (Álava) (Latitude: 42°30´21´´N; longitude: 2°26´11´´W; altitude: 435 mamsl).
Agronomic classification		
Reproductive cycle	Short	Long
Yield	High	High
Reproductive cycle		
Fruit set-veraison (days)	52	61
Veraison-maturity (days)	33	56
Yield components		
Yield (kg vine^{-1})	21.91	12.65
Bunch mass (g bunch^{-1})	154	199
Berry mass (g)	2.05	1.50

Table 2. Effect of three irrigation treatments on main phenological phases of berry ripening and main berry characteristics recorded at harvest from fruit-bearing cuttings of Tempranillo clones grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation.

		CL-1089													
		Treatments						*Main effects*					*ANOVA*		
		24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)			T	I	T×I
								24	28	FI	ED	LD			
Phenology	Fruit set-veraison (days)	50 a	50 a	53 a	38 b	54 a	35 b	51	42	54	52	44	*	ns	*
	Veraison-maturity (days)	46 b	74 a	45 b	31 b	39 b	36 b	55	35	39	57	41	***	**	*
Berry traits	Berry mass (g berry^{-1})	1.08 ab	0.47 c	1.19 a	1.12 a	0.46 c	0.93 b	0.91	0.84	1.10	0.47	0.98	ns	***	*
	Relative skin mass (% berry FM)	20.84	36.56	34.76	26.61	36.83	26.70	30.72	30.05	23.73 b	36.70 a	30.73 a	ns	**	ns
	Berry water content (%)	76.72	82.30	75.07	77.44	79.64	71.14	78.03	76.07	77.08	80.97	73.11	ns	ns	ns

		CL-843													
		Treatments						*Main effects*					*ANOVA*		
		24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)			T	I	T×I
								24	28	FI	ED	LD			
Phenology	Fruit set-veraison (days)	53 b	55 b	64 a	39 c	48 bc	40 c	57	42	46	52	52	***	ns	*
	Veraison-maturity (days)	38 c	77 a	38 c	44 bc	56 b	34 c	51	45	41	67	36	ns	***	*
Berry traits	Berry mass (g berry^{-1})	1.06	0.63	1.01	1.00	0.65	1.46	0.90	1.04	1.03 a	0.64 b	1.24 a	ns	***	ns
	Relative skin mass (% berry FM)	33.16	43.85	25.08	21.49	34.64	21.56	34.03 a	25.89 b	23.77 b	39.25 a	23.32 b	**	***	ns
	Berry water content (%)	78.38	75.29	79.11	75.77	69.97	75.19	77.59 a	73.64 b	77.08	72.63	77.15	ns	ns	ns

Values represent means (n=3) separated by Duncan test (P≤0.05). Different letters within line, not file or column, indicate significant differences as affected by the main factors 'temperature, T', 'irrigation, I' and their interaction (T×I). ns, *, ** and *** indicate non-significance or significance at 5%, 1% and 0.1% probability levels, respectively. FM indicates fresh matter.

Table 3. Effect of irrigation treatments on amino acid profiles measured at harvest in grape skins of fruit-bearing cuttings of 'Tempranillo' grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation.

		Concentration (mmol g^{-1} DM)													
CL-1089		Treatments						*Main effects*					*ANOVA*		
Precursor	Amino acid	24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)			T	I	T×I
								24	28	FI	ED	LD			
3-Phosphoglycerate	Glycine	0.34 b	0.33 b	0.29 b	0.26 b	0.28 b	0.55 a	0.32	0.37	0.30	0.31	0.42	ns	**	***
	Serine	1.61	1.81	2.44	3.25	2.99	4.61	1.96 b	3.62 a	2.43 b	2.40 b	3.53 a	***	**	ns
Phosphoenolpyruvate	Tyrosine	0.37 bc	0.16 c	0.48 b	0.79 a	0.26 bc	0.42 bc	0.49	0.34	0.59	0.21	0.45	ns	**	*
	Phenylalanine	0.33 bc	0.67 a	0.38 bc	0.51 ab	0.24 c	0.35 bc	0.46	0.37	0.42	0.45	0.37	ns	ns	***
Oxaloacetate	Aspartic acid	1.78	1.61	3.00	3.95	1.46	4.89	2.13 b	3.43 a	2.86 ab	1.54 b	3.94 a	*	**	ns
	Asparagine	2.70	2.17	5.78	1.24	0.62	2.20	3.55 a	1.35 b	1.97 b	1.39 b	4.00 a	*	*	ns
	Threonine	5.31	5.60	6.76	9.10	7.68	14.09	5.89 b	10.29 a	7.21 ab	6.64 b	10.42 a	**	*	ns
	Methionine	0.02 d	0.03 d	0.01 d	0.22 b	0.12 c	0.79 a	0.02	0.38	0.12	0.07	0.40	***	***	***
	Isoleucine	0.37	0.32	1.42	0.40	0.34	2.71	0.70	1.15	0.38 a	0.33 a	0.21 b	ns	***	ns
α-ketoglutarate	Glutamic acid	2.27	1.55	3.82	3.80	3.05	6.24	2.55 b	4.36 a	3.03 b	2.30 b	5.03 a	***	***	ns
	Glutamine	6.43 b	4.09 b	7.50 a	7.25 b	6.67 b	12.96 a	6.01	8.96	6.84	5.38	10.23	ns	**	***
	Histidine	1.01 c	0.48 d	2.11 b	3.16 a	2.28 b	3.34 a	1.20	2.93	2.09	1.38	2.73	***	***	*
	Arginine	31.48	40.32	36.93	59.01	49.71	63.60	36.24 b	57.44 a	45.24	45.01	50.27	**	ns	ns
	γ-aminobutyric acid	3.21c	7.34 a	4.31 bc	5.88 ab	5.03 b	5.58 ab	4.95	5.50	4.54	6.18	4.94	ns	*	**
	Proline	19.38	17.11	34.98	33.75	25.53	45.47	23.82 b	35.01 a	26.57 b	21.45 b	40.23 a	*	**	ns
Pyruvate	Alanine	3.73	6.16	6.71	6.67	6.41	8.73	5.53	7.27	5.20	6.28	7.72	ns	ns	ns
	Valine	0.65 b	0.67 b	2.09 a	1.25 ab	1.13 ab	0.88 b	1.14	1.09	0.95	0.90	1.49	ns	ns	*

Table 3 *(Continued)*

CL-843 Precursor	Amino acid	Concentration (mmol g^{-1} DM) Treatments 24FI	24ED	24LD	28FI	28ED	28LD	*Main effects* Temperature (T) 24	28	Irrigation (I) FI	ED	LD	*ANOVA* T	I	T×I
3-Phosphoglycerate	Glycine	0.31 b	0.24 b	0.27 b	0.37 b	0.24 b	0.90 a	0.27	0.50	0.34	0.24	0.58	***	***	***
	Serine	2.21 b	2.19 b	1.97 b	3.19 b	2.19 b	6.20 a	2.12	3.86	2.70	2.19	4.09	***	**	**
Phosphoenolpyruvate	Tyrosine	0.28 bc	0.40 bc	0.21c	0.51 b	0.16 c	0.89 a	0.30	0.52	0.40	0.28	0.55	**	*	***
	Phenylalanine	0.46 bc	0.60 ab	0.27 c	0.46 bc	0.24 c	0.82 a	0.44	0.51	0.46	0.42	0.54	ns	ns	***
Oxaloacetate	Aspartic acid	2.65 b	1.26 b	2.42 b	3.08 b	2.11 b	7.88 a	2.11	4.36	2.87	1.68	5.15	***	***	**
	Asparagine	4.19	2.73	6.09	0.86	0.53	4.73	4.34 a	2.04 b	2.53 b	1.63 b	5.41 a	***	***	ns
	Threonine	6.25 b	6.49 b	6.41 b	10.97 b	6.65 b	19.86 a	6.38	12.50	6.81	6.57	13.14	**	*	*
	Methionine	0.03 b	0.06 a	0.01 c	0.05 ab	0.01 c	0.04 b	0.03	0.03	0.04	0.03	0.03	ns	ns	***
	Isoleucine	0.33 c	0.59 c	1.28 b	0.42 c	0.27 c	4.08 a	0.73	1.59	0.37	0.43	2.68	***	***	***
α-ketoglutarate	Glutamic acid	3.16 bc	1.62 c	2.57 c	5.13b	1.95 c	10.19 a	2.45	5.75	4.14	1.78	6.38	***	***	***
	Glutamine	8.61 b	3.60 b	8.13 b	5.45 b	2.54 b	46.01 a	6.78	18.02	7.03	3.07	27.09	***	***	***
	Histidine	0.34 c	0.23 c	0.22 c	1.24 b	0.79 bc	2.73 a	0.27	1.59	0.79	0.51	1.48	***	**	**
	Arginine	29.94 c	41.40bc	30.99 c	55.27b	38.47 bc	83.18 a	34.11	58.97	42.60	39.94	57.08	***	*	***
	γ-aminobutyric acid	3.32	3.92	3.44	4.50	4.86	7.09	3.56 b	5.48 a	3.91	4.39	5.26	***	ns	ns
	Proline	27.19 b	30.70 b	32.33 b	27.92 b	19.41 b	66.55 a	30.07	37.96	27.56	25.06	49.43	ns	***	**
Pyruvate	Alanine	4.46 b	5.45 b	4.91 b	6.60 b	5.13 b	18.07 a	4.94	9.93	5.53	5.29	11.49	***	***	***
	Valine	0.74 b	0.95 b	1.98 b	1.26 b	0.76 b	7.85 a	1.23	3.29	1.00	0.86	4.92	***	***	***

Values represent means (n=3) separated by Duncan test (P≤0.05). Different letters within line, not file or column, indicate significant differences as affected by the main factors 'temperature, T', 'irrigation, I' and their interaction (T×I). ns, *, ** and *** indicate non-significance or significance at 5%, 1% and 0.1% probability levels, respectively. DM indicates dry matter.

Table 4. Effect of irrigation treatments on anthocyanin derivatives and their distribution into different fractions measured at harvest in grape skins of fruit-bearing cuttings of 'Tempranillo' grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation.

CL-1089		Concentration (mg·g-1 DM)													
		Treatments						*Main effects*					*ANOVA*		
	Compound	24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)			T	I	T×I
								24	28	FI	ED	LD			
3-Monoglucosides	Delphinidin	8.34 a	0.65 d	7.36 a	3.38 bc	2.25 cd	4.43 b	5.45	3.36	5.86	1.45	5.89	**	***	***
	Cyanidin	2.74 a	0.10 b	2.73 a	0.67 b	0.65 b	0.81 b	1.86	0.71	1.71	0.37	1.77	***	***	***
	Petunidin	4.93	0.78	4.89	2.98	2.08	3.83	3.53	2.96	3.96 a	1.43 b	4.36 a	ns	***	ns
	Peonidin	3.78 ab	0.55 d	4.26 a	1.88 cd	1.76 cd	2.33 bc	2.86	1.99	2.83	1.16	3.29	ns	**	*
	Malvidin	10.45	4.16	10.72	9.42	7.52	12.08	8.44	9.67	9.93 a	5.84 b	11.40 a	ns	**	ns
	% of total	90.9 a	71.9 c	92.0 a	83.4 b	79.7 b	84.2 b	84.9	82.4	87.2	75.8	88.1	ns	***	***
3-Acetyl-glucosides	Delphinidin	0.23	0.03	0.22	0.20	0.19	0.29	0.16	0.23	0.22 a	0.11 b	0.26 a	ns	**	ns
	Petunidin	0.15	0.05	0.14	0.24	0.18	0.31	0.11 b	0.24 a	0.20 a	0.11 b	0.22 a	**	**	ns
	Peonidin	ND	0.23 a	ND	0.05 b	0.08 b	0.06 b	0.23	0.06	0.02	0.15	0.03	*	***	***
	Malvidin	0.32	0.25	0.30	0.55	0.72	0.72	0.29 b	0.66 a	0.44	0.49	0.51	***	ns	ns
	% of total	2.3 c	6.7 a	2.0 c	4.7 b	6.5 a	5.1 b	3.7	5.4	3.5	6.6	5.6	***	***	**
3 p-Coumaroyl-glucosides	Peonidin	0.32	0.19	0.33	0.30	0.21	0.27	0.28	0.26	0.31 a	0.20 b	0.30 a	ns	**	ns
	Malvidin	1.60	1.64	1.56	2.32	2.25	2.56	1.60 b	2.38 a	1.96	1.95	2.06	**	ns	ns
	% of total	7.8 cd	21.4 a	5.9 d	11.9 bc	13.8 b	10.7 bc	11.7	12.1	9.9	17.6	8.3	ns	***	**
% of anthocyanin family	Delphinidin	28.97 a	7.85 c	23.29 a	16.22 b	13.47 bc	16.65 b	20.04	15.45	22.6	10.66	19.97	*	***	**
	Cyanidin	9.21 a	1.08 b	8.38 a	3.04 b	3.77 b	2.99 b	6.23	3.27	6.13	2.43	5.69	**	**	***
	Petunidin	16.08 a	9.64 c	15.42 a	14.64 ab	12.5 b	14.7 ab	13.71	13.95	15.36	11.07	15.06	ns	***	*
	Peonidin	12.83	11.04	14.11	10.14	12.16	9.49	12.66	10.6	11.48	11.6	11.8	ns	ns	ns
	Malvidin	41.14 c	70.12 a	38.79 c	55.96 b	57.98 b	55.73 b	50.02	56.56	48.55	64.05	47.26	*	***	**

Table 4 *(Continued)*

CL-843		Concentration (mg·g^{-1} DM)													
		Treatments						*Main effects*					*ANOVA*		
	Compound	24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)			T	I	T×I
								24	28	FI	ED	LD			
3-Monoglucosides	Delphinidin	8.84 a	1.78 b	7.97 a	2.58 b	3.02 b	2.03 b	5.75	2.44	5.43	1.92	4.94	***	***	***
	Cyanidin	3.26 a	0.68 b	3.30 a	0.33 b	0.23 b	0.13 b	2.06	0.16	1.60	0.36	1.37	***	**	**
	Petunidin	5.83 a	1.45 b	5.45 a	1.87 b	2.65 b	0.67 b	3.94	1.27	3.34	1.56	2.92	***	*	***
	Peonidin	4.32 a	1.58 b	5.76 a	1.22 b	1.54 b	0.99 b	3.42	1.28	2.66	1.22	3.18	***	*	***
	Malvidin	11.83 ab	5.16 d	12.76 a	7.64 cd	9.21 bc	9.34 bc	9.29	8.04	9.07	6.29	10.64	ns	**	**
	% of total	92.3 a	78.9 b	91.5 a	81.2 b	80.6 b	74.4 b	87.6	78.7	86.7	79.8	82.9	***	*	**
3-Acetyl-glucosides	Delphinidin	0.27 a	0.10 b	0.27 a	0.20 ab	0.19 ab	0.10 b	0.21	0.17	0.22	0.14	0.21	ns	ns	**
	Petunidin	0.18	0.08	0.18	0.28	0.27	0.26	0.14 b	0.24 a	0.21 a	0.14 b	0.23 a	***	**	ns
	Peonidin	ND	0.11	ND	0.01	0.04	0.03	0.02	0.04	0.01b	0.05 a	0.02b	ns	***	ns
	Malvidin	0.36	0.27	0.42	0.48	0.69	0.68	0.34 b	0.62 a	0.43	0.46	0.55	***	ns	ns
	% of total	2.3	4.7	2.3	5.8	5.9	6.4	3.1 b	6.0 a	4.0	5.3	4.3	***	ns	ns
3 p-Coumaroyl-glucosides	Peonidin	0.35	0.27	0.44	0.26	0.24	0.31	0.32 a	0.25 b	0.28 b	0.22 b	0.35 a	***	***	ns
	Malvidin	1.59	1.67	1.96	1.80	2.51	2.96	1.63 b	2.46 a	1.73 b	2.02 a	2.38 a	***	**	ns
	% of total	5.5 c	16.4 ab	6.2 c	13.1 b	13.5 b	19.2 a	9.4	15.2	9.2	15.0	12.7	**	*	**
% of anthocyanin family	Delphinidin	24.81	15.32	21.4	19.7	15.44	12.61	20.51 a	15.92 b	22.26 a	15.38 b	17.01 ab	*	*	ns
	Cyanidin	8.97 a	5.57 a	8.48 a	1.91 b	1.05 b	0.75 b	7.68 a	1.23 b	5.44	3.31	4.61	***	ns	ns
	Petunidin	16.52	12.55	14.65	13.65	14.1	5.42	14.57	11.06	15.09	13.33	10.04	ns	ns	ns
	Peonidin	12.67	15.85	16.06	9.57	8.74	7.91	14.86 a	8.74 b	11.12	12.3	11.99	*	ns	ns
	Malvidin	38.56 c	57.42 b	39.42 c	68.75 ab	60.65 ab	76.28 a	45.13	68.56	53.66	59.04	57.85	***	ns	*

Values represent means (n=3) separated by Duncan test (P≤0.05). Different letters within line, not file or column, indicate significant differences as affected by the main factors 'temperature, T', 'irrigation, I' and their interaction (T×I). ns, *, ** and *** indicate non-significance or significance at 5%, 1% and 0.1% probability levels, respectively. Within each anthocyanin, total derivatives were calculated as the sum of 3-Monoglucosides, 3-Acetyl-glucosides and 3-*p*-Coumaroyl glucosides. DM indicates dry matter. ND: not detected.

Table 5. Effect of irrigation treatments on individual composition of flavonols determined at harvest in grape skins of fruit-bearing cuttings of 'Tempranillo' grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation.

CL-1089	Concentration (mg·g^{-1} DM)													
	Treatments						*Main effects*					*ANOVA*		
	24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)			T	I	T×I
							24	28	FI	ED	LD			
Myricetin-3-O-glucoside	0.47 bc	0.10 d	0.61 ab	0.47 bc	0.36 c	0.64 a	0.39	0.49	0.47	0.23	0.62	*	***	*
Quercetin-3-O-galactoside	ND	0.04	ND	0.05	0.10	0.06	0.04 b	0.07 a	0.05 b	0.07 a	0.06 ab	***	*	ns
Quercetin-3-O-glucoside	0.10 b	0.06 b	0.13 ab	0.12 ab	0.18 a	0.13 ab	0.10	0.14	0.11	0.12	0.13	*	ns	*
Laricitrin-3-O-glucoside	0.06	0.06	0.07	0.11	0.11	0.15	0.07 b	0.12 a	0.09	0.08	0.11	***	ns	ns
Kaempferol-3-O-glucoside	0.10	ND	0.20	0.11	0.09	0.12	0.15	0.10	0.10 ab	0.04 b	0.16 a	ns	*	ns
Isorhamnetin-3-O-glucoside	0.07	0.07	0.11	0.14	0.11	0.18	0.08 b	0.14 a	0.10	0.49	0.14	*	ns	ns

CL-843	Concentration (mg·g^{-1} DM)													
	Treatments						*Main effects*					*ANOVA*		
	24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)			T	I	T×I
							24	28	FI	ED	LD			
Myricetin-3-O-glucoside	0.43 a	0.18 b	0.43 a	0.48 a	0.24 b	0.17 b	0.43	0.45	0.57	0.27	0.49	ns	**	*
Quercetin-3-O-galactoside	0.01 c	0.09 ab	0.04 bc	0.10 a	0.07ab	0.04 bc	0.01	0.05	0.02	0.03	0.02	***	ns	*
Quercetin-3-O-glucoside	0.11	0.10	0.08	0.14	0.10	0.05	0.10	0.09	0.12 a	0.09 b	0.08 b	ns	*	ns
Laricitrin-3-O-glucoside	0.06	0.06	0.07	0.09	0.09	0.08	0.06 b	0.12 a	0.10	0.08	0.09	**	ns	ns
Kaempferol-3-O-glucoside	0.22 a	0.05 c	0.16 ab	0.14 b	0.05 c	0.04 c	0.10	0.08	0.10	0.08	0.09	**	***	*
Isorhamnetin-3-O-glucoside	0.10	0.05	0.08	0.09	0.09	0.08	0.07 b	0.09 a	0.11	0.09	0.09	**	ns	ns

Values represent means (n=3) separated by Duncan test (P≤0.05). Different letters within line, not file or column, indicate significant differences as affected by the main factors 'temperature, T', 'irrigation, I' and their interaction (T×I). ns, *, ** and *** indicate non-significance or significance at 5%, 1% and 0.1% probability levels, respectively. . DM indicates dry matter. ND: not detected.

Figure legends

Figure 1. Effect of irrigation treatments on anthocyanin content (FERARI index) and flavonol content (FLAV index) measured with the Multiplex^{3TM} sensor a different stages of berry ripening in fruit-bearing cuttings of 'Tempranillo' clones grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation. Values represent means ± SE (n = 9). Two-way ANOVA analysis to evaluate temperature (T), irrigation (I) and interaction (T×I) effects was performed. ns, *, **, and *** indicate non-significance or significance at 5%, 1%, and 0.1% probability levels, respectively.

Figure 2. Effect of irrigation treatments on main primary (glucose, fructose, malic and tartaric acids and amino acids) and secondary (anthocyanins and flavonols) metabolites quantified at harvest in grape skins of fruit-bearing cuttings of 'Tempranillo' CL-1089 grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation. Values represent means ± SE (n = 3). Two-way ANOVA analysis to evaluate temperature (T), irrigation (I) and interaction (T×I) effects was performed. ns, *, **, and *** indicate non-significance or significance at 5%, 1%, and 0.1% probability levels, respectively. Within each parameter, when interaction between the main factors 'temperature, T' and 'irrigation, I' was significant, histograms with different letter indicate that values differed significantly ($P \leq 0.05$). Values of amino acids, anthocyanins and flavonols refer to sum of individual compounds quantified.

Figure 3. Effect of irrigation treatments on main primary (glucose, fructose, malic acid and tartaric acids and amino acids) and secondary (anthocyanins and flavonols) metabolites quantified at harvest in grape skins of fruit-bearing cuttings of 'Tempranillo' CL-843 grown

either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation. Values represent means ± SE (n = 3). Two-way ANOVA analysis to evaluate temperature (T), irrigation (I) and interaction (T×I) effects was performed. ns, *, **, and *** indicate non-significance or significance at 5%, 1%, and 0.1% probability levels, respectively. Within each parameter, when interaction between the main factors 'temperature, T' and 'irrigation, I' was significant, histograms with different letter indicate that values differed significantly (P≤0.05). Values of amino acids, anthocyanins and flavonols refer to sum of individual compounds quantified.

Figure 4. Principal component analysis score (A) and loading plot (B) obtained from the statistical analysis of the berry characteristic and primary and secondary metabolites data of 36 samples from fruit-bearing of 'Tempranillo' clones grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation.

Figure 1.

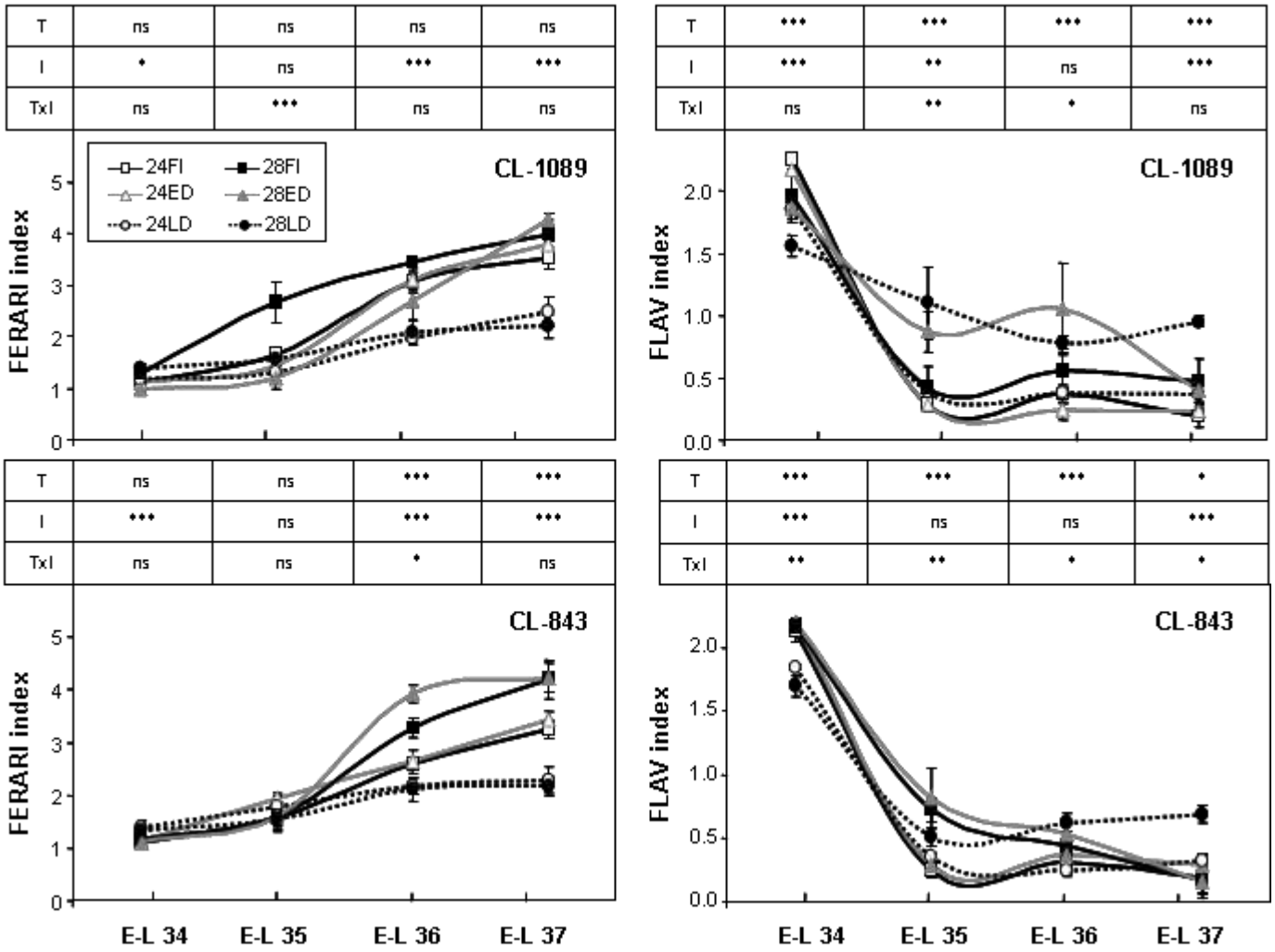

T ns ns ns ns
I * ns *** ***
TxI ns *** ns ns
24FI
28FI
24ED
28ED
24LD
28LD
CL-1089
FERARI index
T *** *** *** ***
I *** ** ns ***
TxI ns ** * ns
CL-1089
FLAV index
T ns ns *** ***
I *** ns *** ***
TxI ns ns * ns
CL-843
FERARI index
T *** *** *** *
I *** ns ns ***
TxI ** ** * *
CL-843
FLAV index
E-L 34
E-L 35
E-L 36
E-L 37

Figure 2.

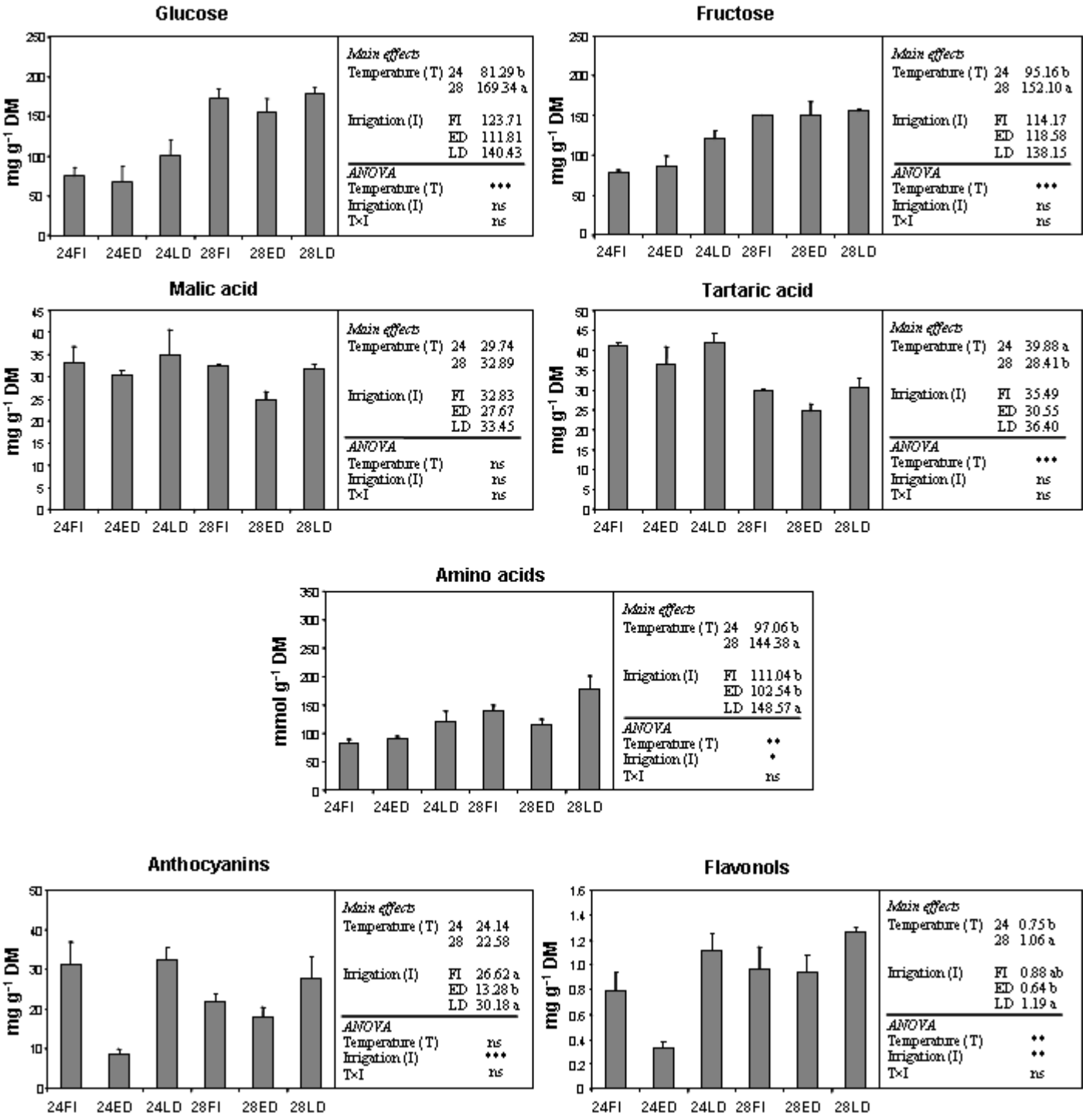

Glucose
mg g-1 DM
Main effects
Temperature (T) 24 81.29 b
28 169.34 a
Irrigation (I) FI 123.71
ED 111.81
LD 140.43
ANOVA
Temperature (T) ***
Irrigation (I) ns
T×I ns
24FI 24ED 24LD 28FI 28ED 28LD
Fructose
mg g-1 DM
Main effects
Temperature (T) 24 95.16 b
28 152.10 a
Irrigation (I) FI 114.17
ED 118.58
LD 138.15
ANOVA
Temperature (T) ***
Irrigation (I) ns
T×I ns
24FI 24ED 24LD 28FI 28ED 28LD
Malic acid
mg g-1 DM
Main effects
Temperature (T) 24 29.74
28 32.89
Irrigation (I) FI 32.83
ED 27.67
LD 33.45
ANOVA
Temperature (T) ns
Irrigation (I) ns
T×I ns
24FI 24ED 24LD 28FI 28ED 28LD
Tartaric acid
mg g-1 DM
Main effects
Temperature (T) 24 39.88 a
28 28.41 b
Irrigation (I) FI 35.49
ED 30.55
LD 36.40
ANOVA
Temperature (T) ***
Irrigation (I) ns
T×I ns
24FI 24ED 24LD 28FI 28ED 28LD
Amino acids
mmol g-1 DM
Main effects
Temperature (T) 24 97.06 b
28 144.38 a
Irrigation (I) FI 111.04 b
ED 102.54 b
LD 148.57 a
ANOVA
Temperature (T) **
Irrigation (I) *
T×I ns
24FI 24ED 24LD 28FI 28ED 28LD
Anthocyanins
mg g-1 DM
Main effects
Temperature (T) 24 24.14
28 22.58
Irrigation (I) FI 26.62 a
ED 13.28 b
LD 30.18 a
ANOVA
Temperature (T) ns
Irrigation (I) ***
T×I ns
24FI 24ED 24LD 28FI 28ED 28LD
Flavonols
mg g-1 DM
Main effects
Temperature (T) 24 0.75 b
28 1.06 a
Irrigation (I) FI 0.88 ab
ED 0.64 b
LD 1.19 a
ANOVA
Temperature (T) **
Irrigation (I) **
T×I ns
24FI 24ED 24LD 28FI 28ED 28LD

Figure 3.

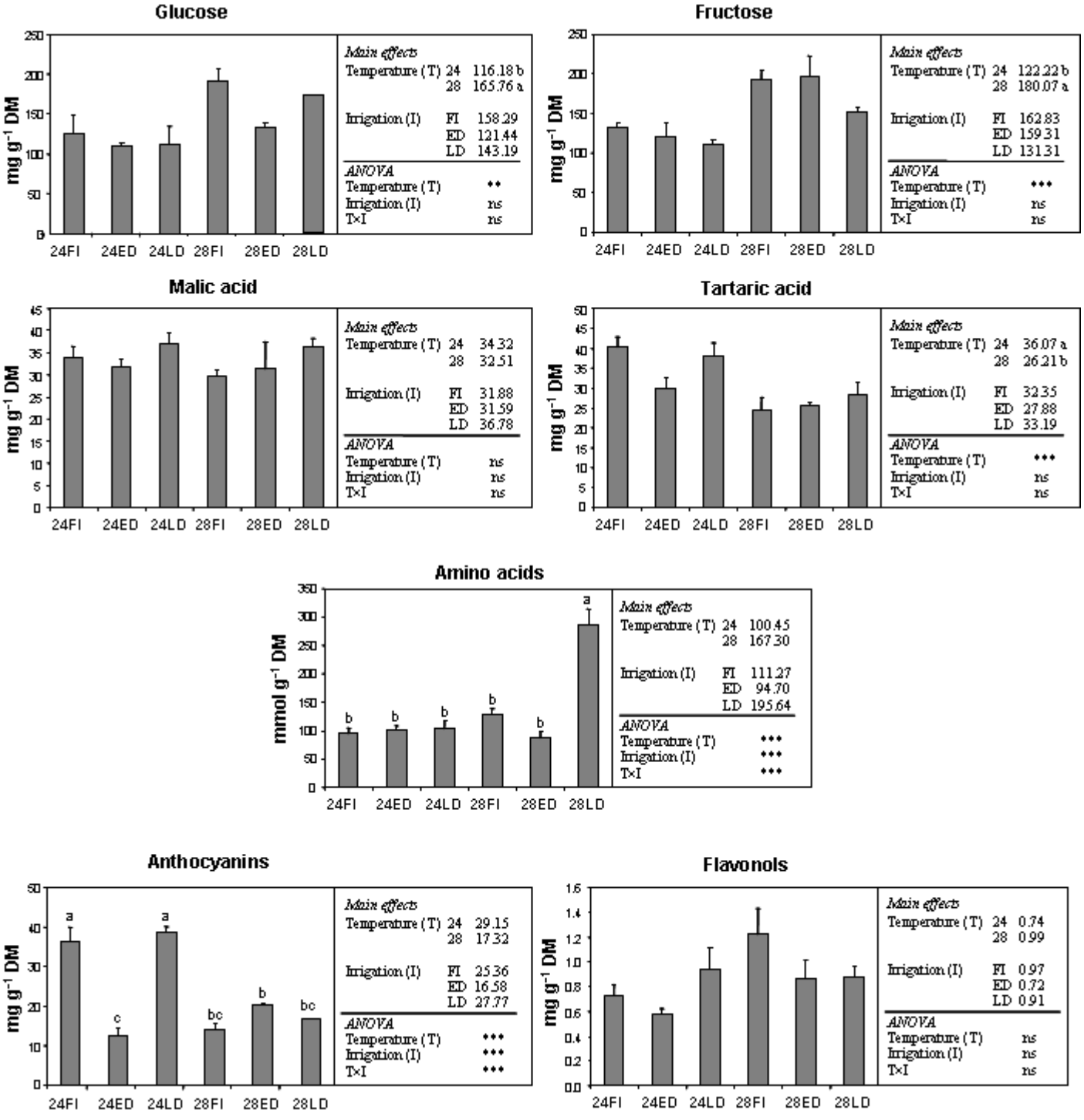
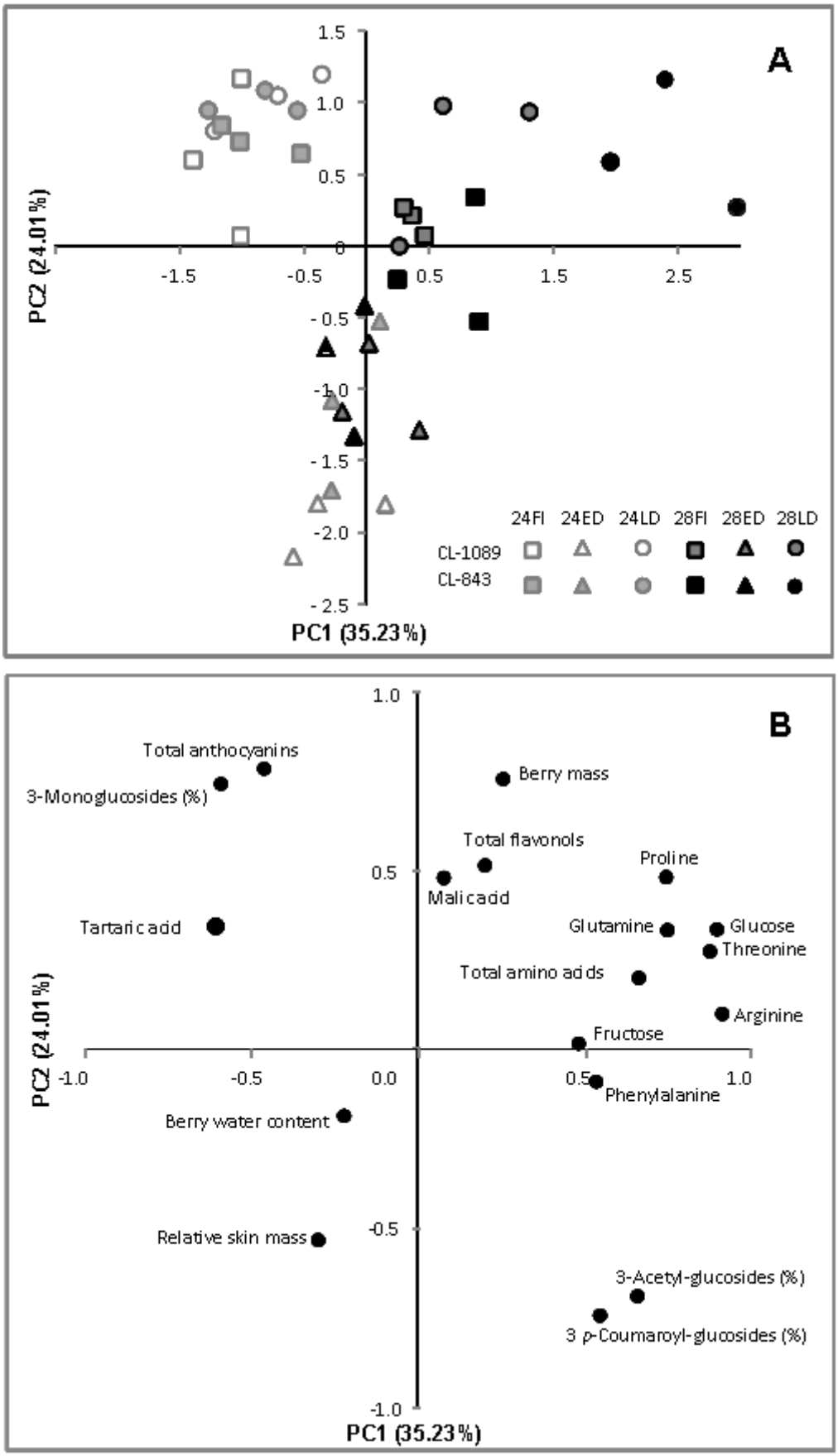

Glucose
mg g-1 DM
24FI 24ED 24LD 28FI 28ED 28LD
Main effects
Temperature (T) 24 116.18 b
28 165.76 a
Irrigation (I) FI 158.29
ED 121.44
LD 143.19
ANOVA
Temperature (T) **
Irrigation (I) ns
T×I ns
Fructose
mg g-1 DM
24FI 24ED 24LD 28FI 28ED 28LD
Main effects
Temperature (T) 24 122.22 b
28 180.07 a
Irrigation (I) FI 162.83
ED 159.31
LD 131.31
ANOVA
Temperature (T) ***
Irrigation (I) ns
T×I ns
Malic acid
mg g-1 DM
24FI 24ED 24LD 28FI 28ED 28LD
Main effects
Temperature (T) 24 34.32
28 32.51
Irrigation (I) FI 31.88
ED 31.59
LD 36.78
ANOVA
Temperature (T) ns
Irrigation (I) ns
T×I ns
Tartaric acid
mg g-1 DM
24FI 24ED 24LD 28FI 28ED 28LD
Main effects
Temperature (T) 24 36.07 a
28 26.21 b
Irrigation (I) FI 32.35
ED 27.88
LD 33.19
ANOVA
Temperature (T) ***
Irrigation (I) ns
T×I ns
Amino acids
mmol g-1 DM
24FI 24ED 24LD 28FI 28ED 28LD
Main effects
Temperature (T) 24 100.45
28 167.30
Irrigation (I) FI 111.27
ED 94.70
LD 195.64
ANOVA
Temperature (T) ***
Irrigation (I) ***
T×I ***
Anthocyanins
mg g-1 DM
24FI 24ED 24LD 28FI 28ED 28LD
Main effects
Temperature (T) 24 29.15
28 17.32
Irrigation (I) FI 25.36
ED 16.58
LD 27.77
ANOVA
Temperature (T) ***
Irrigation (I) ***
T×I ***
Flavonols
mg g-1 DM
24FI 24ED 24LD 28FI 28ED 28LD
Main effects
Temperature (T) 24 0.74
28 0.99
Irrigation (I) FI 0.97
ED 0.72
LD 0.91
ANOVA
Temperature (T) ns
Irrigation (I) ns
T×I ns

Figure 4.


A
PC2 (24.01%)
PC1 (35.23%)
1.5
1.0
0.5
0
-0.5
-1.0
-1.5
-2.0
-2.5
-1.5
-0.5
0.5
1.5
2.5
24FI 24ED 24LD 28FI 28ED 28LD
CL-1089
CL-843
B
PC2 (24.01%)
PC1 (35.23%)
1.0
0.5
0.0
-0.5
-1.0
-1.0
-0.5
0.5
1.0
Total anthocyanins
3-Monoglucosides (%)
Berry mass
Total flavonols
Proline
Malic acid
Tartaric acid
Glutamine
Glucose
Threonine
Total amino acids
Arginine
Fructose
Phenylalanine
Berry water content
Relative skin mass
3-Acetyl-glucosides (%)
3 p-Coumaroyl-glucosides (%)

Supplementary material

Original research article

Flavonoid and amino acid profiling on *Vitis vinifera* L. cv Tempranillo subjected to deficit irrigation under elevated temperatures

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Table S1. Standard errors (SE) of means from main phenological phases of berry ripening and berry characteristics recorded at harvest from fruit-bearing cuttings of 'Tempranillo'.

Table S2. Standard errors (SE) of means from amino acids measured at harvest in grape skins of fruit-bearing cuttings of 'Tempranillo'.

Table S3. Standard errors (SE) of means from anthocyanin derivatives and their distribution into different fractions measured at harvest in grape skins of fruit-bearing cuttings of 'Tempranillo'.

Table S4. Standard errors (SE) of means from individual composition of flavonols determined at harvest in grape skins of fruit-bearing cuttings of 'Tempranillo'.

Figure S1. Chromatographic profile of amino acids.

Figure S2. Chromatographic profile of anthocyanins.

Figure S3. Soil water content measured from fruit set to harvest in pots subjected to different water treatments.

Figure S4. Effect of irrigation treatments on pre-dawn leaf water potential (Ψ_{pd}) recorded at different stages of berry ripening.

Table S1. Standard errors (SE) of means from main phenological phases of berry ripening and berry characteristics recorded at harvest from fruit-bearing cuttings of 'Tempranillo' clones grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation.

		CL-1089										
		Treatments						*Main effects*				
		24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)		
								24	28	FI	ED	LD
Phenology	Fruit set-veraison (days)	4	2	2	2	7	1	1	4	3	4	4
	Veraison-maturity (days)	1	7	6	2	4	4	6	2	3	9	4
Berry traits	Berry mass (g berry^{-1})	0.05	0.05	0.03	0.11	0.03	0.03	0.11	0.10	0.06	0.03	0.06
	Relative skin mass (% berry FM)	1.00	2.40	4.33	2.30	4.48	4.56	2.88	2.59	1.71	2.27	3.34
	Berry water content (%)	0.82	6.64	0.68	0.32	5.81	6.30	2.23	2.78	0.43	3.99	2.97

		CL-843										
		Treatments						*Main effects*				
		24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)		
								24	28	FI	ED	LD
Phenology	Fruit set-veraison (days)	2	3	2	1	5	3	2	2	3	3	6
	Veraison-maturity (days)	1	3	6	1	8	2	4	7	2	6	3
Berry traits	Berry mass (g berry^{-1})	0.06	0.02	0.02	0.12	0.05	0.24	0.07	0.14	0.06	0.03	0.15
	Relative skin mass (% berry FM)	3.33	0.83	2.53	2.61	5.44	0.87	2.81	2.98	3.22	3.21	1.43
	Berry water content (%)	1.18	0.26	1.67	0.51	4.65	0.42	1.64	0.84	0.82	2.40	1.17

FM indicates fresh matter.

Table S2. Standard errors (SE) of means from amino acids measured at harvest in grape skins of fruit-bearing cuttings of 'Tempranillo' grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation.

		Concentration (mmol g-1 DM)										
CL-1089		Treatments						*Main effects*				
Precursor	Amino acid	24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)		
								24	28	FI	ED	LD
3-Phosphoglycerate	Glycine	0.03	0.03	0.06	0.02	0.03	0.02	0.02	0.05	0.03	0.02	0.06
	Serine	0.33	0.23	0.40	0.33	0.11	0.53	0.21	0.31	0.42	0.29	0.57
Phosphoenolpyruvate	Tyrosine	0.13	0.09	0.09	0.11	0.05	0.07	0.07	0.09	0.12	0.03	0.05
	Phenylalanine	0.03	0.06	0.04	0.09	0.04	0.08	0.06	0.05	0.06	0.10	0.04
Oxaloacetate	Aspartic acid	0.19	0.10	0.45	0.49	0.68	1.09	0.26	0.63	0.54	0.18	0.68
	Asparagine	0.37	0.41	0.67	0.27	0.07	0.24	0.97	0.25	0.39	0.39	0.44
	Threonine	0.53	0.45	1.44	0.36	1.29	2.75	0.51	1.31	0.90	0.77	2.15
	Methionine	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.06	0.01	0.01	0.04
	Isoleucine	0.01	0.01	0.10	0.01	0.01	0.04	0.18	0.20	0.01	0.01	0.20
α-ketoglutarate	Glutamic acid	0.32	0.14	0.51	0.62	0.37	0.73	0.38	0.57	0.46	0.38	0.67
	Glutamine	0.65	0.10	0.18	1.53	0.71	2.50	0.75	1.71	0.77	0.66	2.49
	Histidine	0.14	0.02	0.20	0.19	0.21	0.14	0.25	0.19	0.49	0.41	0.30
	Arginine	4.52	2.86	4.74	2.50	9.63	5.33	2.43	3.84	6.58	4.96	6.76
	γ-aminobutyric acid	0.32	0.66	0.85	0.54	0.47	0.55	0.70	0.26	0.64	0.63	0.53
	Proline	2.74	1.66	5.36	3.86	1.07	9.53	3.34	4.13	3.85	2.13	5.42
Pyruvate	Alanine	0.58	0.78	1.11	0.57	0.42	1.64	0.81	0.63	0.75	0.40	1.08
	Valine	0.14	0.09	0.62	0.10	0.11	0.26	0.30	0.15	0.15	0.12	0.44

Table S2 *(Continued)*

CL-843 Precursor	Amino acid	Concentration (mmol g^{-1} DM)										
		Treatments						*Main effects*				
		24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)		
								24	28	FI	ED	LD
3-Phosphoglycerate	Glycine	0.06	0.05	0.03	0.07	0.04	0.06	0.03	0.11	0.04	0.03	0.14
	Serine	0.29	0.24	0.27	0.59	0.25	0.83	0.14	0.67	0.37	0.16	0.10
Phosphoenolpyruvate	Tyrosine	0.04	0.08	0.03	0.04	0.03	0.19	0.04	0.12	0.06	0.07	0.17
	Phenylalanine	0.05	0.12	0.04	0.07	0.04	0.15	0.06	0.10	0.04	0.10	0.14
Oxaloacetate	Aspartic acid	0.25	0.12	0.34	0.11	0.29	0.76	0.25	0.98	0.52	0.23	1.28
	Asparagine	0.35	0.25	0.54	0.22	0.05	0.92	0.52	0.73	0.77	0.51	0.56
	Threonine	0.56	0.45	0.64	2.00	1.19	3.95	2.80	2.35	1.41	0.57	3.50
	Methionine	0.01	0.01	0.01	0.01	0.62	0.01	0.01	0.01	0.01	0.01	0.01
	Isoleucine	0.06	0.11	0.18	0.06	0.02	0.43	0.150	0.64	0.04	0.09	0.66
α-ketoglutarate	Glutamic acid	0.36	0.32	0.55	0.65	0.27	1.33	0.31	1.28	0.55	0.20	1.82
	Glutamine	1.49	0.66	1.53	1.19	0.65	3.27	1.01	7.12	1.34	0.48	8.84
	Histidine	0.58	0.04	0.07	0.13	0.12	0.23	0.04	0.33	0.18	0.16	0.57
	Arginine	1.94	3.05	2.60	6.68	6.57	8.91	2.24	7.51	6.46	3.31	12.39
	γ-aminobutyric acid	0.08	0.61	0.22	0.54	0.65	0.84	0.21	0.53	0.36	0.45	0.90
	Proline	3.93	3.30	4.54	4.85	2.95	9.73	2.12	7.95	2.80	3.21	9.03
Pyruvate	Alanine	0.40	1.09	0.69	0.73	0.63	1.72	0.42	2.12	0.60	0.57	3.10
	Valine	0.03	0.08	0.29	0.07	0.08	1.03	0.21	1.18	0.12	0.07	1.40

DM indicates dry matter.

Table S3. Standard errors (SE) of means from anthocyanin derivatives and their distribution into different fractions measured at harvest in grape skins of fruit-bearing cuttings of 'Tempranillo' grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation.

CL-1089		Concentration (mg·g-1 DM)										
		Treatments						*Main effects*				
	Compound	24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)		
								24	28	FI	ED	LD
3-Monoglucosides	Delphinidin	0.58	0.13	0.74	0.44	0.46	0.68	0.24	0.48	1.16	0.42	0.88
	Cyanidin	0.44	0.02	0.54	0.08	0.01	0.10	0.48	0.04	0.50	0.13	0.49
	Petunidin	1.03	0.15	0.53	0.29	0.39	0.86	0.77	0.38	0.65	0.34	0.51
	Peonidin	0.15	0.07	0.43	0.14	0.26	0.52	0.66	0.22	0.60	0.36	0.53
	Malvidin	1.51	0.57	1.11	0.72	1.44	2.35	1.21	1.06	0.78	1.02	1.20
	% of total	2.19	1.33	0.93	0.31	2.74	1.60	3.36	1.15	1.94	2.22	1.93
3-Acetyl-glucosides	Delphinidin	0.04	0.01	0.04	0.03	0.03	0.02	0.04	0.03	0.02	0.04	0.04
	Petunidin	0.02	0.01	0.02	0.04	0.01	0.04	0.02	0.02	0.03	0.03	0.04
	Peonidin	ND	0.04	ND	0.01	0.01	0.01	0.04	0.01	0.01	0.04	0.02
	Malvidin	0.05	0.03	0.01	0.04	0.12	0.08	0.02	0.05	0.06	0.12	0.10
	% of total	0.24	0.72	0.05	0.10	0.47	0.26	0.79	0.32	0.54	0.39	0.68
3 p-Coumaroyl-glucosides	Peonidin	0.04	0.03	0.04	0.03	0.01	0.03	0.03	0.02	0.02	0.01	0.03
	Malvidin	0.21	0.18	0.19	0.20	0.30	0.14	0.12	0.17	0.23	0.27	0.24
	% of total	1.26	1.71	0.90	0.40	2.29	1.38	2.53	0.90	1.08	2.14	1.30
Proportion of each anthocyanin family	Delphinidin	4.13	1.12	0.65	1.14	0.73	0.76	3.39	0.67	3.44	1.39	1.55
	Cyanidin	1.55	0.57	1.46	0.19	0.49	0.22	1.44	0.21	1.55	0.69	1.38
	Petunidin	0.50	1.65	0.34	0.54	0.38	0.37	1.14	0.42	0.46	0.99	0.28
	Peonidin	1.17	3.56	0.43	0.18	3.94	0.23	1.18	1.21	0.80	2.39	1.06
	Malvidin	5.07	1.59	2.33	1.68	5.42	1.48	5.31	1.73	4.08	3.71	3.98

Table S3 *(Continued)*

CL-843		Concentration (mg·g^{-1} DM)										
		Treatments						*Main effects*				
	Compound	24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)		
								24	28	FI	ED	LD
3-Monoglucosides	Delphinidin	1.37	0.18	0.45	0.34	0.44	0.27	0.72	0.24	0.92	0.28	0.71
	Cyanidin	0.34	0.06	0.73	0.03	0.02	0.01	0.30	0.07	0.04	0.09	0.04
	Petunidin	0.61	0.13	0.26	0.06	0.31	0.07	0.04	0.03	0.29	0.16	0.08
	Peonidin	0.74	0.21	0.83	0.30	0.26	0.25	0.42	0.17	0.47	0.15	0.60
	Malvidin	0.57	0.82	0.73	1.42	0.18	1.41	0.69	0.60	0.64	0.65	0.55
	% of total	1.20	3.43	0.37	2.95	2.60	1.90	2.40	1.67	2.85	1.96	3.91
3-Acetyl-glucosides	Delphinidin	0.02	0.01	0.04	0.03	0.01	0.02	0.02	0.02	0.02	0.01	0.02
	Petunidin	0.01	0.01	0.04	0.03	0.02	0.04	0.01	0.02	0.02	0.02	0.02
	Peonidin	ND	0.04	ND	0.01	0.02	0.01	0.02	0.01	0.01	0.03	0.01
	Malvidin	0.03	0.01	0.02	0.05	0.07	0.10	0.01	0.03	0.04	0.05	0.06
	% of total	0.37	0.51	0.29	0.83	0.69	0.64	0.45	0.38	0.88	0.46	0.97
3 p-Coumaroyl-glucosides	Peonidin	0.03	0.01	0.03	0.03	0.02	0.02	0.02	0.02	0.02	0.01	0.02
	Malvidin	0.16	0.09	0.04	0.15	0.26	0.20	0.07	0.13	0.15	0.13	0.19
	% of total	0.86	3.11	0.17	2.13	1.93	1.27	1.99	1.35	1.98	1.76	2.96
Proportion of each anthocyanin family	Delphinidin	1.69	1.46	0.72	2.99	2.03	2.02	1.54	1.58	1.92	1.12	2.19
	Cyanidin	0.26	0.56	1.66	1.91	1.05	0.75	0.74	0.69	1.80	1.14	1.91
	Petunidin	0.15	1.43	0.71	6.24	0.81	3.71	0.74	2.54	2.87	0.81	2.67
	Peonidin	0.90	0.83	2.00	3.86	0.79	2.86	0.87	1.43	1.90	1.67	2.40
	Malvidin	4.01	3.11	2.27	5.63	4.04	9.92	3.47	4.16	1.48	1.40	1.32

DM indicates dry matter. ND: not detected.

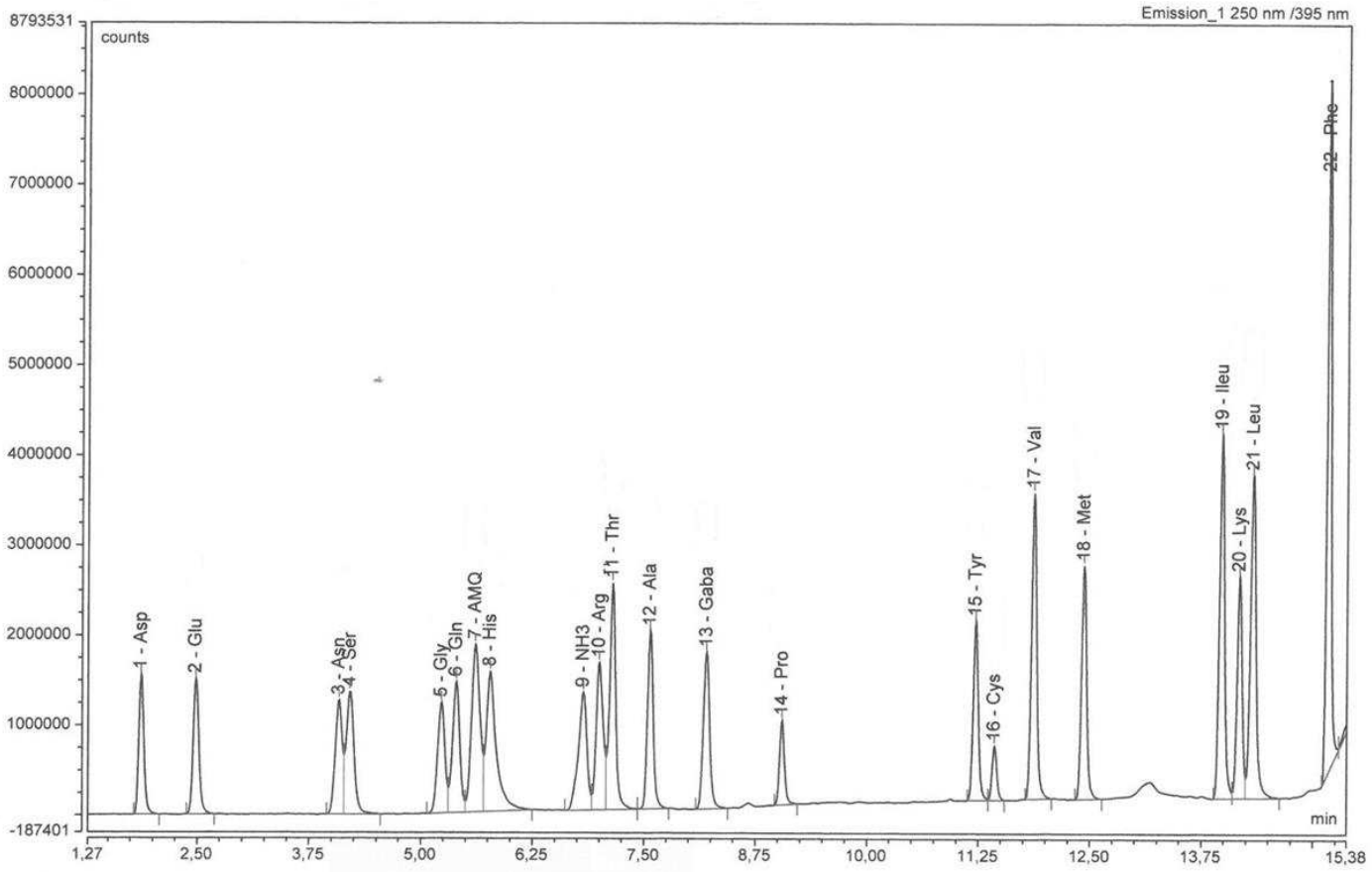
Table S4. Standard errors (SE) of means from individual composition of flavonols determined at harvest in grape skins of fruit-bearing cuttings of 'Tempranillo' grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation.

CL-1089	Concentration (mg·g^{-1} DM)										
	Treatments						*Main effects*				
	24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)		
							24	28	FI	ED	LD
Myricetin-3-O-glucoside	0.04	0.02	0.06	0.07	0.05	0.02	0.08	0.05	0.04	0.06	0.03
Quercetin-3-O-galactoside	ND	0.01	ND	0.01	0.02	0.01	0.01	0.01	0.01	0.02	0.01
Quercetin-3-O-glucoside	0.01	0.01	0.01	0.02	0.03	0.01	0.01	0.02	0.02	0.03	0.01
Laricitrin-3-O-glucoside	0.01	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.02	0.02	0.02
Kaempferol-3-O-glucoside	0.06	ND	0.04	0.01	0.02	0.01	0.01	0.03	0.03	0.02	0.03
Isorhamnetin-3-O-glucoside	0.02	0.01	0.02	0.03	0.02	0.02	0.01	0.02	0.03	0.01	0.02

CL-843	Concentration (mg·g^{-1} DM)										
	Treatments						*Main effects*				
	24FI	24ED	24LD	28FI	28ED	28LD	Temperature (T)		Irrigation (I)		
							24	28	FI	ED	LD
Myricetin-3-O-glucoside	0.13	0.02	0.05	0.12	0.07	0.06	0.05	0.04	0.05	0.03	0.03
Quercetin-3-O-galactoside	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Quercetin-3-O-glucoside	0.03	0.02	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Laricitrin-3-O-glucoside	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.02	0.01	0.01
Kaempferol-3-O-glucoside	0.01	0.02	0.07	0.02	0.02	0.02	0.02	0.01	0.03	0.01	0.02
Isorhamnetin-3-O-glucoside	0.05	0.02	0.04	0.01	0.01	0.02	0.01	0.01	0.03	0.01	0.02

DM indicates dry matter. ND: not detected.

Figure S1. HPLC chromatograms of amino acid profile from berry skins. Excitation and emission wavelengths were 250 nm and 395 nm, respectively.



Asp: Aspartic acid; Glu: Glutamic acid; Asn: Asparagine; Ser: Serine; Gly: Glycine; Gln: Glutamine; His: Histidine; Arg: Arginine; Thr: Threonine; Ala: Alanine; GABA: γ-aminobutyric acid; Pro: Proline; Tyr: Tyrosine; Cys: Cysteine; Val: Valine; Met: Methionine; Ileu: Isoleucine; Lys: Lysine; Leu: Leucine; Phe: Phenylalanine.

Figure S2. HPLC chromatograms showing the anthocyanin profile from berry skins. A detection wavelength of 520 nm was used.

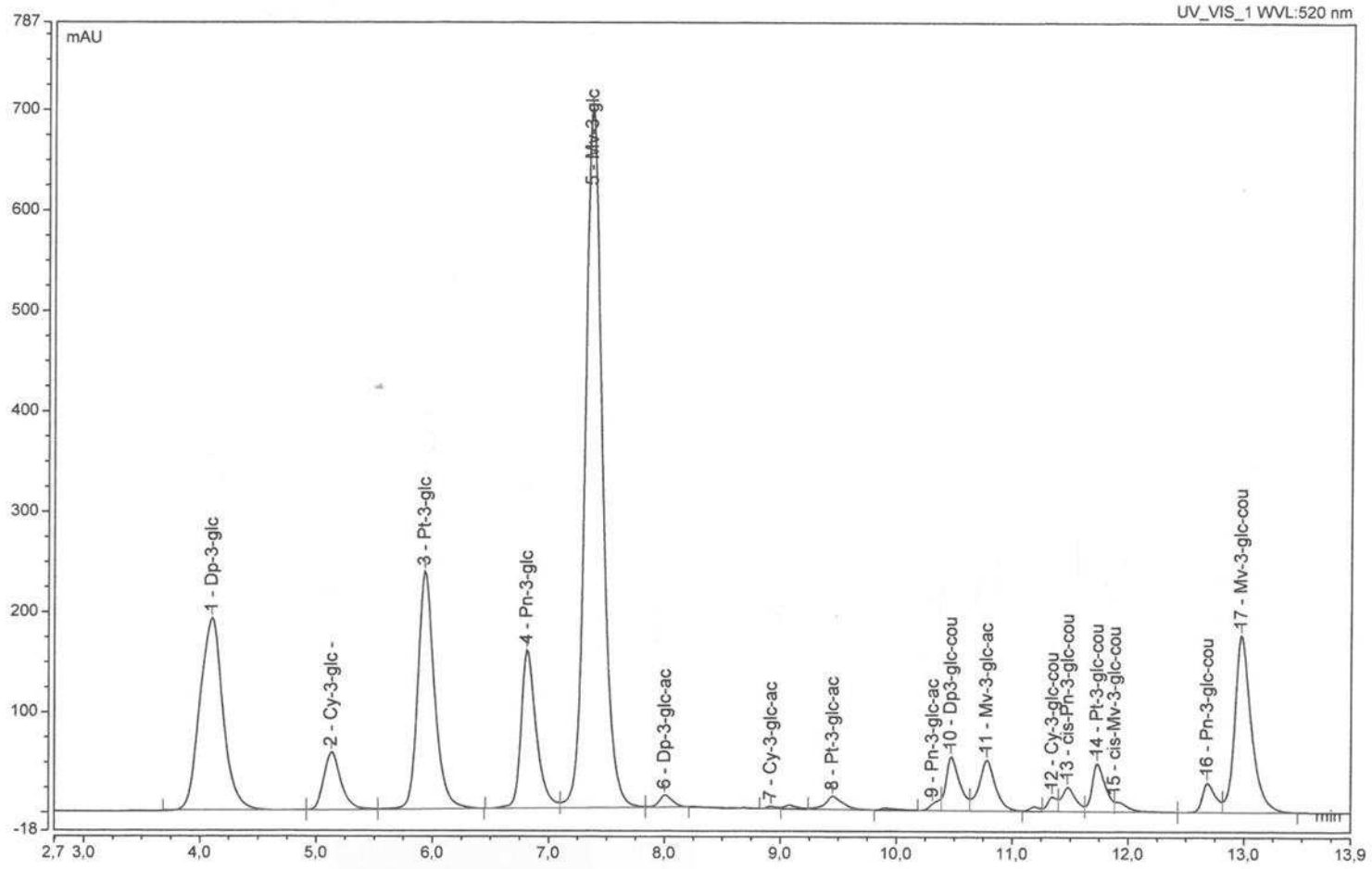


Figure S3. Soil water content measured from fruit set to harvest in pots subjected to different water treatments: full irrigation (FI), early season deficit irrigation (ED) or late season deficit irrigation (LD) either at 24/14°C or 28/18°C (day/ night) temperature regimes. Values represent means ± SE (n = 3). Vertical dotted lines mark the boundary for veraison for all treatments.

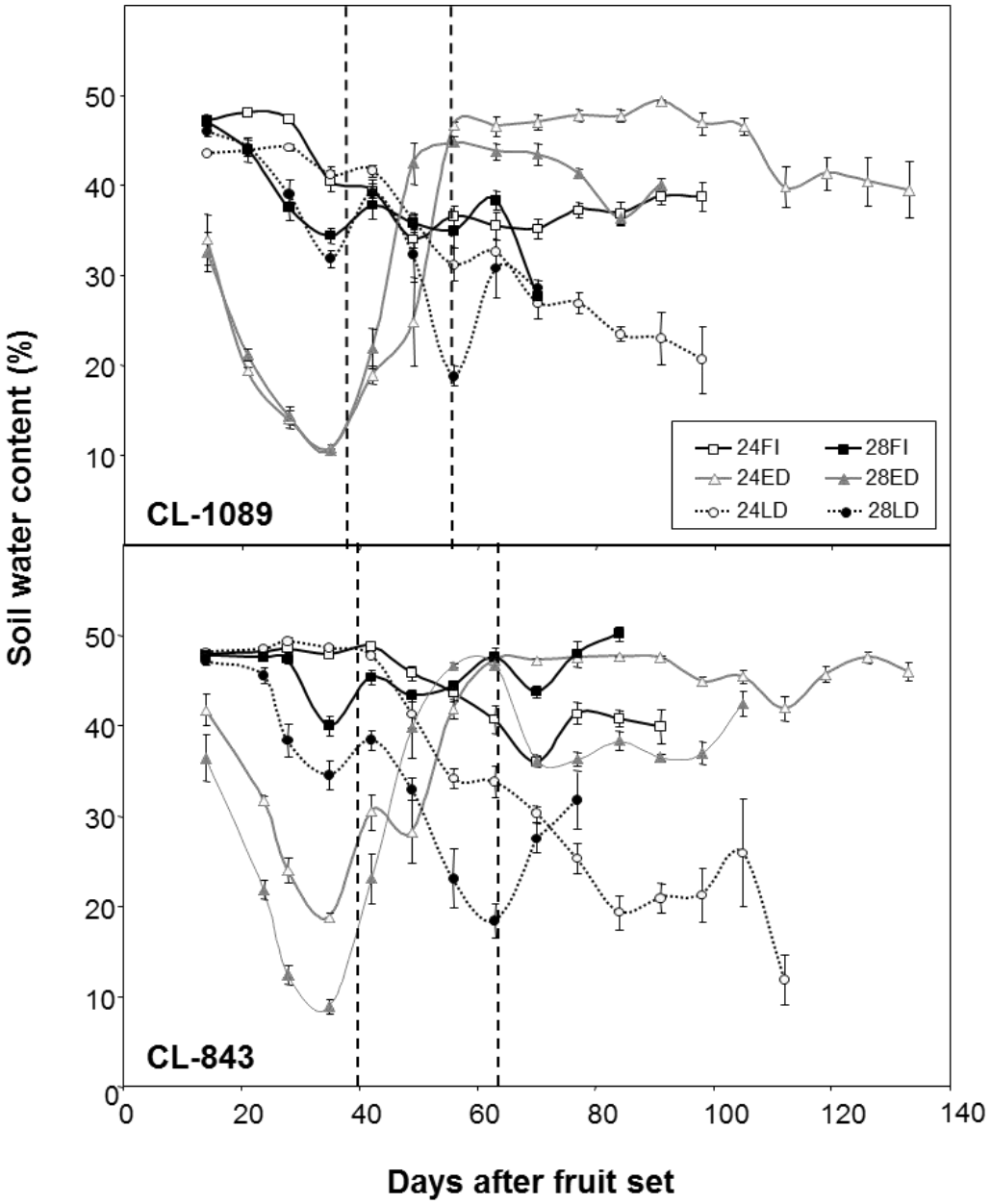
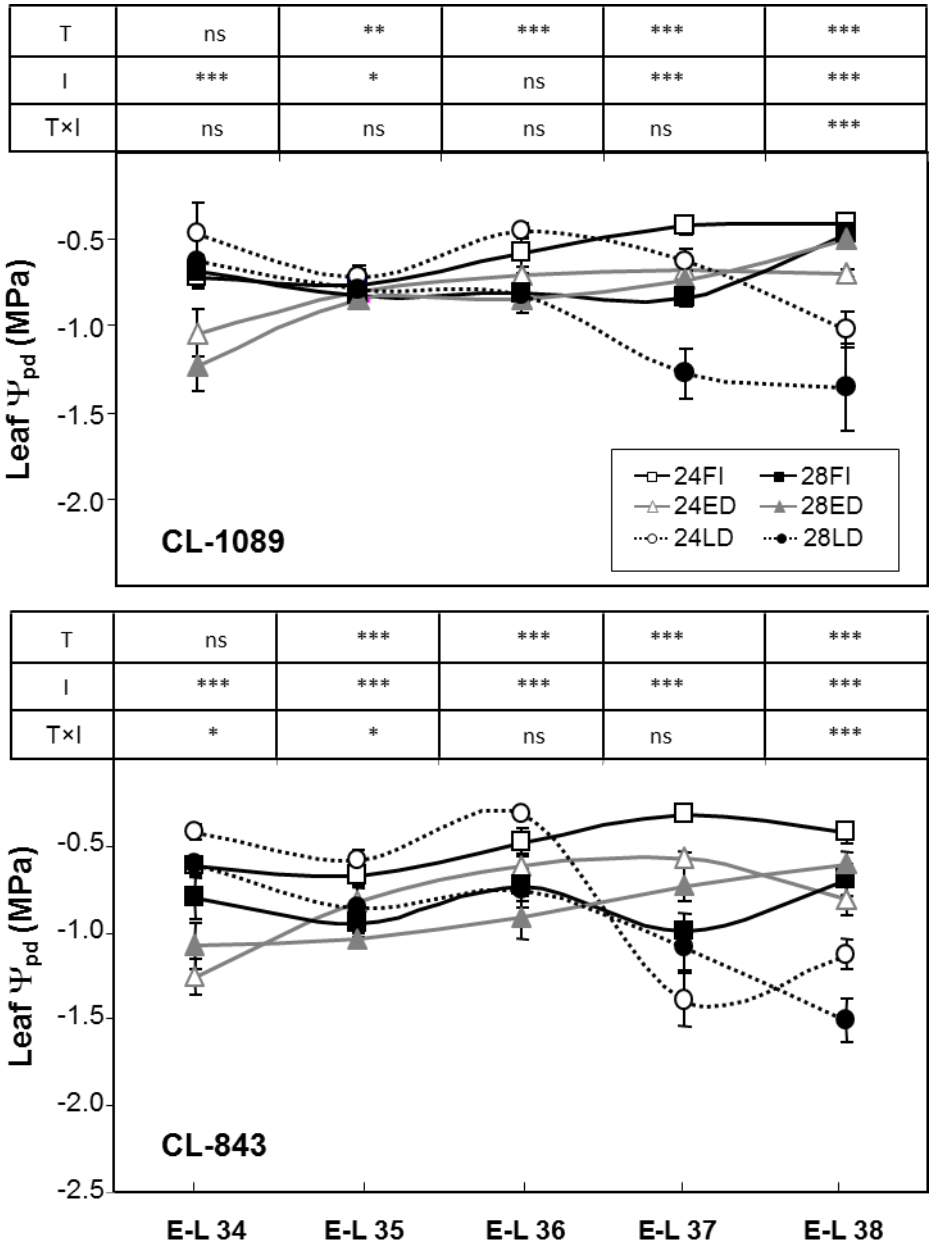


Figure S4. Effect of irrigation treatments on pre-dawn leaf water potential (Ψ_{pd}) recorded at different stages of berry ripening in fruit-bearing cuttings of Tempranillo clones grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation. Values represent means ± SE (n = 3). Two-way ANOVA analysis to evaluate temperature (T), irrigation (I) and interaction (T×I) effects was performed. ns, *, **, and *** indicate non-significance or significance at 5%, 1%, and 0.1% probability levels, respectively.



CAPÍTULO 2

Berry quality and antioxidant properties in *Vitis vinifera* cv. Tempranillo as affected by clonal variability, mycorrhizal inoculation and temperature.

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Berry quality and antioxidant properties in *Vitis vinifera* cv. Tempranillo as affected by clonal variability, mycorrhizal inoculation and temperature

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Abstract

The projected increase in mean temperatures caused by climate change is expected to have detrimental impacts on berry quality. Microorganisms as arbuscular mycorrhizal fungi (AMF) produce numerous benefits to host plants and can help plants to cope with abiotic stresses such as high temperature. The aims of this research were to characterise the response of three clones of *Vitis vinifera* L. cv. Tempranillo to elevated temperatures and to determine whether AMF inoculation can improve berry antioxidant properties under these conditions. The study was carried out on fruit-bearing cuttings three clones of cv. Tempranillo (CL-260, CL-1048 and CL-1089) inoculated with AMF or uninoculated and subjected to two temperature regimes (day–night: 24°C–14°C and 28°C–18°C) during berry ripening. Results showed that clonal diversity of Tempranillo resulted in different abilities to respond to elevated temperature and AMF inoculation. In CL-1048, AMF inoculation improved parameters related to phenolic maturity such as anthocyanin content and increased antioxidant activity under elevated temperature, demonstrating a protective role of AMF inoculation against warming effects on berry quality. The results therefore suggest that selection of new clones and/or the implementation of measures to promote the association of grapevines with AMF could be strategies to improve berry antioxidant properties under future warming conditions.

ToC Abstract: The projected increase in mean temperatures caused by climate change is expected to have detrimental impacts on berry quality but inoculation with arbuscular mycorrhizal fungi (AMF) could help grapevines to cope with abiotic stresses such as high temperature. Different fruit-bearing cuttings clones of cv. Tempranillo were inoculated with AMF or uninoculated and subjected to two temperature regimes (day–night: 24°C–14°C and 28°C–18°C) during berry ripening. In some clones the association of grapevines with AMF may play a relevant role in a future climate change scenario to maintain o even improve fruit quality by enhancing antioxidant properties.

Running head: Mycorrhizas and temperature on Tempranillo clones

Additional keywords: DPPH assay, global warming, mycorrhizal efficiency index (MEI), polyphenols, technological maturity.

1. Introduction

Human activities, especially during the last 50 years, have contributed to increased atmospheric greenhouse gas concentration, which is responsible for the Earth's surface warming (Webb *et al.* 2013). Climate change particularly affects winemaking regions in southern Europe, where from 1950 to 1999, growing-season average temperatures have increased by 1.26°C (Jones *et al.* 2005). Moreover, according to the Intergovernmental Panel on Climate Change (IPCC 2014), global average temperature could increase by 4°C in the next 100 years. Associated with warming trends over the last few decades, early maturity of grapevines has been reported for Europe, North America and Australia (Duchêne and Schneider 2005; Petrie and Sadras 2008; Sadras and Petrie 2011) and these shifts in the timing of wine-grape maturity can have implications for the viticultural sector. Understanding the effects of elevated temperature on different grapevine varieties is important for the implementation of appropriate agronomic practices and relevant for the knowledge and attribution of trends in the context of climate change (Teixeira *et al.* 2013).

High temperature impairs processes associated with grapevine ripening (Kuhn *et al.* 2014). Characteristic warming effects on berry composition include reduction of anthocyanin content (Mori *et al.* 2007; Azuma *et al.* 2012; Carbonell-Bejerano *et al.* 2013), rapid fall in acidity and malate content (Conde *et al.* 2007; Keller 2010; Sweetman *et al.* 2014), and changes in the composition of phenolic compounds (Tarara *et al.* 2008; Cohen *et al.* 2012). This may have significant implications for wine quality due to changes in the hue and intensity of the grape colour (Mori *et al.* 2007). In addition, elevated temperatures hasten sugar accumulation at the expense of other relevant compounds, leading to the elaboration of wines with higher alcohol contents (Greer and Weston 2010). However, the magnitude of all described impacts on berry quality differs among varieties (Barnuud *et al.* 2014). Clonal diversity within grapevine cultivars has been studied for a broad range of characteristics. Thus, precocity of the phenological cycle, yield, sugar concentration, skin phenolic compounds, disease resistance or response to environmental conditions varied widely among clones (Anderson *et al.* 2008; van Leeuwen *et al.* 2013). Because projected warming during the growing season over southern Europe is expected to have detrimental effects on grapevine development and wine quality, different possibilities are emerging. One possibility is the adaptation of vineyards to projected future warming through varieties being able to maintain berry characteristics under the new conditions and through the development of breeding programs selecting the most adaptable grapevine clones in each country (Duchêne *et al.* 2010; Fraga *et al.* 2013; Webb *et al.* 2013).

Recent studies indicate that microorganisms may help plants to cope with abiotic stresses such as high temperature (Grover *et al.* 2011; Maya and Matsubara 2013). Among these, arbuscular mycorrhizal fungi (AMF) have received increasing attention because of their numerous benefits to host plants. Under field conditions, grapevine roots are normally colonised by AMF, with the general AMF structure and composition in vineyards more influenced by soil type than by host plant features or management practices (Balestrini *et al.* 2010). The association between AMF and grapevine roots has been related to increased plant growth (Linderman and Davis 2001), drought tolerance (Nikolaou *et al.* 2003), nutrient uptake (Karagiannidis *et al.* 2007; Schreiner 2007) and protection against pathogens (Nogales *et al.* 2009), without changes to berry composition (Karagiannidis *et al.* 2007). Associations with AMF can stimulate the synthesis of plant secondary metabolites, which are important for increased plant tolerance to environmental stresses and beneficial to human health through their antioxidant activity (Baslam *et al.* 2013; Bettoni *et al.* 2014).

Nevertheless, little is known about the potential benefits of AMF colonisation on grape quality under conditions of climate change. Therefore, the objectives of the present research were: (i) to characterise the response of three *Vitis vinifera* L. cv. Tempranillo clones to elevated temperatures, focusing on technologic and phenolic maturity; and (ii) to determine whether AMF inoculation can improve berry antioxidant properties under these conditions. Tempranillo is a Spanish variety widely cultivated in northern and central Spain where it is the main variety in half of the Denominations of Origin. This variety exhibits a broad clonal diversity (Cervera *et al.* 2002), and we hypothesise that this could result in different abilities to respond to warm climates and AMF inoculation. Thus, the goal of this study was to identify some clones of Tempranillo that maintain fruit quality as well as antioxidant capacity under changing environmental conditions. Potted vines were used to ensure that all clones experienced the same temperature conditions, and to control mycorrhizal inoculation and to have comparable non-inoculated plants. Previous researchers have demonstrated that fruit-bearing cuttings are a meaningful and useful model system to study grape berry metabolism (Dai *et al.* 2013) and evaluate the response of berry ripening to environmental factors (Antolín *et al.* 2010; Niculcea *et al.* 2014; Martínez-Lüscher *et al.* 2015). In addition, the chronology of flowering and fructification of the fruit-bearing cuttings is similar to that of the vineyard-grown grapevines (Ollat *et al.* 1998; Lebon *et al.* 2008). However, experiments with greenhouse experiments and potted plants to assess the effect of temperature and/or AMF inoculation have some limitations such as abrupt changes in temperature cycles, lack of wind, small soil volume or warming of roots above air temperature (Passioura 2006; Poorter *et al.* 2012; Bonada and Sadras 2015).

2. Material and methods

2.1. Biological material and growth conditions

Dormant 400–500-mm-long cuttings of different clones of Tempranillo with were obtained in winter 2013 from an experimental vineyard of the Institute of Sciences of Vine and Wine, Logroño, Spain (Denomination of Origin Rioja, North of Spain). Three clones (CL-260, CL-1048 and CL-1089) were selected on basis of previous research showing that phenolic content and antioxidant activity of leaves were stimulated by the combination of elevated temperature and mycorrhizal inoculation (Torres *et al.* 2015). A brief description of the clones is presented in Table 1. Cuttings of each clone were selected for fruit-bearing according to steps originally outlined by Mullins (1966) with some modifications described in Ollat *et al.* (1998) and Antolín *et al.* (2010). Research has demonstrated that the fruit-bearing cutting technique is a useful model for grapevine physiology studies that allows development of vegetative and reproductive organs that is similar to vineyard grapevines but under fully controlled environmental conditions (Antolín *et al.* 2010; Dai *et al.* 2013). Briefly, rooting was made in a heat bed (27°C) kept in a cool room (4°C). One month later, the cuttings were planted 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 glasshouses. Properties of the peat (Floragard; Vilassar de Mar, Barcelona, Spain) were: pH 5.2–6.0, nitrogen 70–150 mg L–1, P2O5 80–180 mg L–1, and K2O 140–220 mg L–1. The peat was previously sterilised at 100°C for 1 h on 3 consecutive days.

At transplanting, half of the plants were inoculated with the mycorrhizal inoculum GLOMYGEL Vid, Olivo, Frutales (Mycovitro S.L., Pinos Puente, Spain) (+M plants). The concentrated commercial inoculum derived from an in-vitro culture of AMF Rhizophagus intraradices (Schenck & Smith) Walker & Schüßler comb. nov. (Krüger *et al.* 2012). It contained ~2000 mycorrhizal propagules (inert pieces of roots colonised by AMF, spores and vegetative mycelium) per mL inoculum. In order to facilitate its application, the concentrated commercial inoculum was diluted with distilled water to a mycorrhizal inoculum of ~250 propagules mL–1. Each +M plant received 8 mL diluted mycorrhizal inoculum close to the roots, thus making 2000 propagules in total. A filtrate was added to plants that did not receive the mycorrhizal inoculum (–M plants) in order to restore other soil free-living microorganisms accompanying AMF. The filtrate was obtained by passing diluted mycorrhizal inoculum through a layer of 15–20-µm filter paper with particle retention of 2.5 µm (Whatman 42; GE Healthcare, Little Chalfont, UK), and each –M plant received 8 mL filtrate close to the roots. The selection of in-vitro-produced inoculum of R. intraradices was based on two expected benefits: (i) easy

application of the product; and (ii) low colonisation of roots by contaminant fungi (Vimard *et al.* 1999).

Plants were transferred to greenhouses, which were adapted to simulate climate change conditions as described by Morales *et al.* (2014). Initial growth conditions were 25°C–15°C and 50%–90% relative humidity (day–night). Natural daylight (photosynthetic photon flux density, PPFD, on average 850 µmol m^{-2} s^{-1} at midday) was 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} at the level of the inflorescence. Humidity and temperature were controlled by using M22W2HT4X transmitters (Rotronic Instrument Corp., Hauppauge, NY, USA). PPFD was monitored with a LI-190SZ quantum sensor (LI-COR Biosciences, Lincoln, NE, USA). Under these conditions, bud-break took place after 1 week. Careful control of vegetative growth before flowering improves the partitioning of stored carbon towards the roots and the reproductive structures. Thus, only a single flowering stem was allowed to develop on each plant during growth. Plants were irrigated 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 Instruments SA, Alella, Spain). Plants were watered twice daily with a nutrient solution (140 mL day^{-1}) with phosphorus (P) level 0.30 mM. This was lower than the amount of P applied by Petit and Glubler (2006) to Vitis inoculated with R. intraradices under controlled conditions.

2.2. Experimental design

Fruit-bearing cuttings (20 plants $clone^{-1}$) were exposed to two day–night temperature regimes at fruit set (Eichhorn and Lorenz (E-L) fruit stage 27; Coombe 1995): 24°C–14°C and 28°C–18°C. At this stage, plants have 4–5 fully expanded leaves. The 24°C–14°C temperature regime was selected according to the average temperatures registered in La Rioja (1971–2000) (AEMET, Spain) during growing season. The 28°C–18°C temperature regime was selected according to predictions of a rise of 4.0°C by the end the present century (IPCC 2014). Both temperature regimes were maintained until ripeness (21°–23°Brix) (E-L 38). To avoid excessive soil warming, which can negatively affect AMF infection, sides of pots were shaded by covering with a reflecting material (Passioura 2006; Poorter *et al.* 2012). Soil temperature was measured at 5 cm soil depth by using temperature probes (PT100; Coreterm, Valencia, Spain) and reached 24°C ± 0.5°C and 27°C ± 0.5°C for 24°C–14°C and 28°C–18°C air temperature regimes, respectively.

Length of phenological phases was recorded as the number of days from fruit set (E-L 27) to veraison (E-L 35), and from veraison to harvest ripe (E-L 38). Thermal time was calculated on a daily basis by using a base temperature of 10°C (Sadras and Morán 2013). Plants were harvested separately based on sugar level from berry subsamples (two or three berries) taken weekly.

2.3. Mycorrhizal colonisation

Root samples were cleared and stained (Phillips and Hayman 1970), and mycorrhizal colonisation was determined by examining 1-cm root segments (45 per pot) under the microscope. Then, parameters of mycorrhizal colonisation were calculated for each pot. First, the extension (*E*) of mycorrhizal colonisation was first determined for every root segment and was calculated as the product of value of mycorrhizal colonisation in width (*W*) and value of mycorrhizal colonisation in length (*L*). Values of mycorrhizal colonisation in *W* and length *L* were ascribed according a 0–10 scale where 0 is complete absence of fungal structures and 10 is fungal structures occupying the full length or width of the root segment. Total *E* per pot was calculated as $E = \sum(W \times L)/n$, where *n* is total number of root segments observed under the microscope (*n* = 45 per pot), and was expressed as a percentage. Second, the incidence (*I*) of mycorrhizal colonisation per pot was calculated as the ratio between number of root segments with fungal structures (arbuscules, vesicles and/or hyphae) and total number of root segments observed under the microscope (45 per pot). Third, the intensity of mycorrhizal colonisation per pot was calculated as $E \times I$, and results were expressed as percentage of infection (Hayman *et al.* 1976). The mycorrhizal efficiency index (MEI) was estimated from fresh matter (FM) according to Bagyaraj (1994) as: MEI = (bunch FM of +M plant – bunch FM of –M plant) × 100/(bunch FM of +M plant). Determination of MEI allows assessment of the improvement provided by inoculation of plants with a mycorrhizal fungus.

2.4. Plant growth, yield and leaf nutrients

Leaf area was measured with a portable area meter (model LI-3000; LI-COR). A good correlation (r = 0.97) was obtained between the length of the main vein of the leaf and leaf area by using several leaves of each clone. Total leaf area of each plant was calculated after measuring the length of the main vein in all leaves and applying the formula: leaf area = –4.98 + (2.54 × vein length) + (0.90 × vein length^2). Then, all leaves were removed and counted.

To obtain yield, bunches were weighed and then 10 berries from each plant were collected and weighed individually. Mean berry mass was determined and berries were separated into skin and flesh. The remaining berries were counted, weighed and frozen at –80°C for further analysis. The relative skin mass was calculated as the quotient between skin FM and total berry FM expressed as percentage.

For mineral analyses, leaf samples (0.5 g dry matter (DM)) were dry-ashed and dissolved in HCl according to Duque (1971). Phosphorus was determined by using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Optima 4300; PerkinElmer, Waltham, MA, USA). The operating parameters for 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 N was quantified after combustion (950°C) of leaf DM with pure oxygen by an elemental analyser (TruSpec CN; LECO Corp., St. Joseph, MI, USA).

2.5. Technological maturity

A subsample of 25 berries was crushed and then extracts were centrifuged at 4300g at 4°C for 10 min. The supernatant was used for the following determinations: total soluble solids (mainly sugars) measured with a temperature-compensating refractometer (Zuzi model 315; Auxilab, Beriáin, Spain) and expressed as °Brix; must pH measured with a pH meter (Crison Instruments, Barcelona, Spain) standardised to pH 7.0 and 4.0; titratable acidity measured by titration with NaOH according to International Organisation of Vine and Wine methods (OIV 2014), and expressed as g tartaric acid L^{-1}; L-malic acid measured by an enzymatic method (Enzytec L-Malic Acid; R-Biopharm, Darmstadt, Germany); and tartaric acid by using the modified method of Rebelein (1973).

2.6. Phenolic maturity

Another 25-berry subsample per plant was weighed and taken for the analysis of anthocyanins and total phenols. Total and extractable anthocyanins were calculated according to the procedure described by Saint-Cricq *et al.* (1998). Two samples of the non-filtered, crushed grape homogenate were macerated for 4 h at pH 1 (hydrogen chloride) and pH 3.2 (tartaric acid), respectively. Once maceration was over, the macerated samples were centrifuged at 4300g at 4°C for 10 min. Total and extractable anthocyanins were determined in both supernatants (macerated at pH 1 and pH 3.2) according to Ribéreau-Gayon and Stonestreet

(1965) by using absorbance at 520 nm. Both data were used to calculate the cellular extractability of anthocyanins as described in Nadal (2010). The seed maturity index estimates the contribution of tannins of seeds to the ripeness evaluation and it was obtained by the Glories procedure (Nadal 2010). Total polyphenol index (TPI) was calculated by the absorbance reading at 280 nm in the supernatant obtained after maceration at pH 3.2 (EEC 1990). All analyses were run in triplicate.

2.7. Antioxidant capacity

Total antioxidant capacity was evaluated on the same must samples used for technological maturity determinations by using the free-radical scavenging activity (α, α-diphenyl-β-picrylhydrazyl (DPPH)) assay (Brand-Williams *et al.* 1995). The free radical scavenging activity, using the free radical DPPH•, was evaluated by measuring the variation in absorbance at 515 nm after 30 min of reaction in Parafilm-sealed glass cuvettes (to avoid methanol evaporation) at 25°C. The reaction was started by adding 20 μL of the corresponding sample to the cuvette containing an 80 μM (methanol solution) (980 μL) of the free radical (DPPH•). The final volume of the assay was 1 mL. The reaction was followed with a spectrophotometer (Jasco V-630; Analytical Instruments, Easton, MD, USA). The calibration curve was made using gallic acid as standard. Results were expressed as mg gallic acid g^{-1} DM.

2.8. Statistical analyses

Statistical analyses were carried out with the statistical software SPSS version 21.0 for Windows (IBM, Armonk, NY, USA). Data were subjected to Kolmogorov–Smirnov normality test due to the small sample size. Data appeared to follow a normal distribution and they were therefore subjected to analysis of variance (ANOVA) within each clone. The test was performed to assess the main effect of the factors temperature (24°C–14°C and 28°C–18°C) and mycorrhization (–M and +M) and the interaction between them (T × M). In the case of mycorrhizal efficiency index (MEI), the main factors were temperature and clone. Means ± standard errors (s.e.) were calculated, and when the F-ratio was significant ($P \leq 0.05$), Duncan's test was applied. Two-way ANOVA was performed to determine significant differences in all parameters. Berry quality parameters and antioxidant activity data were analysed by principal component analysis (PCA) with the same software.

3. Results

3.1. Phenological cycle, plant and berry traits and MEI

In plants grown at 24°C–14°C, the period from fruit set to veraison of the fruit-bearing cuttings of Tempranillo clones was similar to that of the vineyard-grown plants (Tables 1 and 2). However, the time between veraison and harvest ripe was longer in fruit-bearing cuttings than the vineyard-grown plants (Table 2). In our study, temperature was the main factor influencing phenology, and no significant effects could be attributed to AMF inoculation (Table 2). Thus, increasing temperature to 28°C–18°C shortened the time to reach veraison in all clones, but the time from veraison was distinctly affected by raising temperature according to clone. Thus, in CL-260 and CL-1048, fruit maturity was reached earlier at 28°C–18°C, but in CL-1089, no significant variations from veraison to ripening period were found. On a thermal-time scale, most significant changes in phenology disappeared, indicating that the differences are fully accounted for by temperature (Table 2).

Although the intensity of mycorrhizal colonisation was low (10–15%) (Table 2), the MEI allows assessment of the effect obtained after AMF inoculation (Fig. 1). Results showed that AMF inoculation exerted a negative effect on bunch growth (except for CL-1089 grown at 24°C–14°C) but the MEI values were distinctly modified by temperature as a function of clone assayed. Thus, in CL-260 grown at 28°C–18°C, effectiveness of mycorrhizal symbiosis was improved (i.e. MEI was less negative). However, in CL-1048, MEI values were not affected by increased temperature, and in CL-1089, MEI was strongly diminished. This differential pattern was emphasised by two-way ANOVA showing a significant interaction between temperature treatment and clone (Fig. 1). Plant growth (estimated by leaf area) decreased with increased temperature mainly through reduction in leaf number, whereas AMF inoculation did not affect either parameter (Table 3). In the same way, temperature was the main factor influencing yield and berry size, and no significant effects could be attributed to AMF inoculation (Table 3). By contrast, relative skin mass was not significantly modified by temperature or AMF treatments.

Foliar levels of N were not affected by mycorrhizal inoculation and/or increased temperature for CL-260 and CL-1089 (Table 4). In CL-1048, increased temperature and the interaction between high temperature and AMF inoculation ($T \times M$, $P < 0.05$) enhanced the accumulation of N in leaves. Foliar concentrations of P were not significantly affected by mycorrhizal inoculation (Table 4) but clearly increased in plants subjected to increased

temperature. In CL-1048, the application of AMF diminished the increase in P in leaves observed under high temperature (T × M, P ≤ 0.01).

3.2. Phenolic and technological maturity

In CL-260, technological maturity parameters were significantly modified by increased temperature, as evidenced by increased concentration of total soluble sugars and decreased tartaric acid (Table 5). No significant interactions between temperature and mycorrhization were observed in technological maturity (Fig. 2a). Analyses of phenolic maturity showed that increasing temperature significantly reduced TPI, cellular extractability of anthocyanins and seed maturity (Table 5). AMF inoculation also resulted in low cellular extractability of anthocyanins (Table 5), and significant reductions in total and extractable anthocyanins were observed in +M plants grown at 28°C–18°C (Fig. 2b). This differential pattern was emphasised by two-way ANOVA showing significant T × M interaction for total (P ≤ 0.01) and extractable (P ≤ 0.001) anthocyanins.

In CL-1048, analyses of technological maturity showed that temperature was the main factor increasing total soluble solids (Table 5). Moreover, AMF inoculation resulted in significant reduction of must pH and increased tartaric acid. In this clone, phenolic maturity parameters reduced by temperature were TPI and both total and extractable anthocyanins (Table 5). In addition, total anthocyanins were significantly increased by AMF inoculation (Table 5). Two-way ANOVA showed significant T × M interaction for titratable acidity (P ≤ 0.05; Fig. 3a) and for extractability of anthocyanins (P ≤ 0.05; Fig. 3b).

In CL-1089, neither temperature nor mycorrhization affected levels of total soluble solids but titratable acidity as well as malic and tartaric acids were significantly modified by temperature increase (Table 5). Regarding phenolic maturity parameters, in this clone total and extractable anthocyanins were reduced by elevated temperature, but AMF inoculation significantly increased total anthocyanins and extractability of anthocyanins (Table 5). No significant T × M interactions were observed in any case (Fig. 4a, b).

3.3. Antioxidant capacity

In CL-260 and CL-1089, temperature rise was the main factor accounting for diminished total antioxidant capacity of berry extracts (Table 6). On the other hand, AMF inoculation resulted in a significant enhancement of antioxidant capacity in berry extracts of CL-1048.

3.4. Principal component analysis

Principal component analysis was conducted to determine general trends in the different samples. Figure 5a shows the score plot obtained by PCA where samples of each variety are grouped in the plot of the first and second principal components: PC1 *v*. PC2. Here, PC1 accounted for ~28.6% of the total variance and PC2 covered ~15.5%. Different Tempranillo clones could not be clearly distinguished (Fig. 5a). The –M plants grown under both temperature conditions appeared together; however, +M plants were separated along PC1 where a clear distinction was observed between plants grown at 24°C–14°C and plants grown under warming conditions. The loading plot (Fig. 5b) shows the importance of TPI, anthocyanin, pH and acidity levels in explaining variance along PC1. Moreover, the graph highlights that total antioxidant capacity measured was related to anthocyanin and polyphenol levels.

4. Discussion

Clones of *Vitis vinifera* cv. Tempranillo tested in this study have significant diversity for some agronomic traits such as yield, bunch mass and berry mass (Table 1) (Cervera *et al.* 2002). However, clonal selection in viticulture should integrate other criteria related to plant physiological responses to biotic and abiotic factors in order to select interesting clones able to maintain berry properties under future climate scenarios. Under our experimental conditions, a rise of temperature from 24°C–14°C to 28°C–18°C during berry ripening resulted in accelerated phenology, which was more pronounced from fruit set to veraison than from veraison to maturity in CL-260 and CL-1089 (Table 2). Such observations show a consistent trend towards earlier veraison and harvest, commonly observed in previous studies (Duchêne and Schneider 2005; Petrie and Sadras 2008; Duchêne *et al.* 2010). Our data show that temperature effects significantly differed among clones and that the intensity of effects depends on phenological phase, which could be explained by nonlinear effects of temperature on vine phenology (Sadras and Morán 2013).

In the present study, the intensity of mycorrhizal colonisation in grapevine roots never exceeded 15% whether plants were cultivated under either 24°C–14°C or 28°C–18°C (Table 2). These values are clearly lower than measured by Eftekhari *et al.* (2012) in grapevines inoculated with different species of AMF, including *R. intraradices* (70% of root colonisation). Several factors could have been involved. First, differences in root colonisation may be influenced by small soil volume (Poorter *et al.* 2012); 8-L pots were used in Eftekhari *et al.*

(2012) and 6.5-L pots in the present study. In fact, development of extraradical mycelium may be limited by the small size of pots with consequent reduction of root AMF colonisation (Nogales *et al.* 2010). Second, AMF colonisation could be reduced by higher root temperature (Passioura 2006); however, this was avoided by shading pots (see Material and methods). Third, differences in root colonisation may be at least partially due to the type of inoculum applied to grapevine in both studies. Whereas Eftekhari *et al.* (2012) used an inoculum that contained spores, mycelium and root fragments of clover colonised by AMF, in our study the main component of the mycorrhizal inoculum was spores of *R. intraradices*. Similarly, we have found low colonisation percentages when using monoxenically produced spores of *R. intraradices* as mycorrhizal inoculum in onion plants (Bettoni *et al.* 2014). Fourth, percentages of mycorrhizal colonisation are highly dependent on the grape variety (Eftekhari *et al.* 2012). Fifth, because in the grape fruiting-cuttings model several leaves are removed to allow fruit development, the leaf area may have been insufficient to provide enough photoassimilates to satisfy the demand of the main sinks in plants inoculated with *R. intraradices* (fungal structures in roots and bunch), and this would be detrimental to the spread of mycorrhizal colonisation. Such limitation would have resulted in non-improvement of bunch growth brought about by inoculation of plants (low or negative MEI; Fig. 1), even in the only case in which AMF enhanced P status of plants compared with the non-inoculated controls (CL-1048 grown at 24°C–14°C; Table 4). However, the values of root AMF colonisation in our study were similar to those obtained by Nogales *et al.* (2010) working with in-vitro, micro-propagated plantlets of grapevine inoculated with monoxenically produced *R. intraradices*. Although plants in our study were fertilised at a P rate lower than that applied by Petit and Glubler (2006), the foliar concentrations of P were always higher than measured by those authors in shoots of grape rootings. Foliar P concentrations in cv. Tempranillo were also higher than extractable P observed in leaves of cv. Carignane fertilised with P for the full growing season (Skinner and Matthews 1989). Therefore, we can dismiss the possibility that, in our study, P supply was insufficient for correct plant growth.

Recent studies have shown great phenotypic plasticity among grapevine varieties for berry sensory traits in response to elevated temperatures (Sadras *et al.* 2013a, 2013b; Barnuud *et al.* 2014). In the same way, results of this study showed clonal diversity within Tempranillo in response to temperature and AMF inoculation. Thereby, tartaric acid content decreased in CL-260 (Fig. 2a) but increased in CL-1048 at 28°C–18°C (Fig. 3a). These results contrast with general observations that tartaric acid is largely unaffected by temperature. However, some studies agree with our data by showing broad diversity in tartaric acid levels within grapevine varieties and climate conditions (Liu *et al.* 2006; Preiner *et al.* 2013).

On the other hand, in CL-260, TPI and total and extractable anthocyanins decreased in AMF-inoculated plants grown at 28°C–18°C (Fig. 2b), in agreement with other studies (Mori *et al.* 2007; Mira de Orduña 2010; Teixeira *et al.* 2013; Barnuud *et al.* 2014). This loss of anthocyanins has been associated with chemical and/or enzymatic degradation (Mori *et al.* 2007) and with delayed onset of anthocyanin accumulation (Sadras and Morán 2012). Phenolic maturity covers not only the overall concentration of phenolic compounds, but also their structure and capacity to be extracted from grapes during vinification (Boulton *et al.* 1996; Cagnasso *et al.* 2008). In our study, CL-260 showed significant decreases in the cellular extractability of anthocyanins in plants grown at 28°C–18°C, suggesting early phenolic maturity under these conditions (Fig. 2b). As mentioned above, AMF-inoculated plants of CL-260 grown at 28°C–18°C have low anthocyanin content (Fig. 2b) together with increased soluble solids (Fig. 2a), which could lead a decoupling between anthocyanins and sugars (Sadras and Morán 2012). Because these changes could have detrimental consequences for the colour–alcohol balance of wine, we suggest that AMF colonisation may not be beneficial under future warm-temperature scenarios for CL-260.

When grown at 28°C–18°C, CL-1048 showed increased soluble solids (Fig. 3a) together with reduction in anthocyanins regardless of mycorrhizal treatment (Fig. 3b). However, in this clone, mycorrhizal inoculation improved anthocyanin content, suggesting that AMF exerted a positive effect on berry quality (Fig. 3b). To date, there has been little information about the influence of AMF on phenolic composition of grape berries (Karagiannidis *et al.* 2007), but a beneficial role of AMF on anthocyanin content was previously detected in leaves of lettuce (Baslam *et al.* 2013), onion (Bettoni *et al.* 2014) and grapevine (Torres *et al.* 2015). According with these results, we suggest that mycorrhizal association of CL-1048 may improve berry quality under warmer temperatures.

In contrast to other clones, CL-1089 grown at 28°C–18°C did not show changes in total soluble solids (Fig. 4a) as reported in other studies (Coombe 1987; Sadras *et al.* 2013a). However, AMF inoculation of CL-1089 resulted in decreased extractable anthocyanins leading to increased cellular extractability of anthocyanins (~70%), which suggests low potential of colour extraction (Nadal 2010). Overall, our data indicate that CL-1089 could be a good candidate for growing under climate change conditions because of its poor response to temperature, but AMF colonisation could reduce some positive traits.

Phenolic compounds have generated remarkable interest with their antioxidant and free-radical-scavenging properties (Castellarin *et al.* 2012). Catechins, proanthocyanidins and anthocyanins are the most concentrated natural antioxidants present in berries and they may generate significant health benefits (Xia *et al.* 2010; Georgiev *et al.* 2014). The observed

decrease in total antioxidant capacity of AMF-inoculated CL-260 grown at 28°C–18°C was related to a decrease in TPI and anthocyanins (Fig. 2b). By contrast, in CL-1048, the combination of AMF inoculation and elevated temperatures exerted an additive effect in improving antioxidant power (Table 6) that could be explained, at least in part, by improved anthocyanin content in berries (Fig. 3b). In fact, anthocyanins are considered very good antioxidant agents, and a significant relationship has been reported between antioxidant capacity and anthocyanin content in different grapevine varieties (Kallithraka *et al.* 2009; De Nisco *et al.* 2013), as was also observed in our study (Fig. 5b). An additive effect between AMF inoculation and elevated temperature for antioxidant properties of CL-1048 was recently shown in cyclamen (Maya and Matsubara 2013) and grapevine (Torres *et al.* 2015) leaves. Moreover, the enhanced N status of plants from CL-1048 inoculated with AMF and subjected to elevated temperature (Table 4) may have contributed to the synthesis of N-containing compounds (i.e. amino acids) with antioxidant properties.

In conclusion, this study provides evidence for clonal diversity of Tempranillo resulting in different abilities to respond to increasing temperature and mycorrhizal inoculation. Under our experimental conditions, the protective role of AMF inoculation in avoiding warming effects on berry quality was particularly evident in CL-1048. Although under elevated temperatures this clone ripened earlier, it could be an alternative to typical clones of Tempranillo used in the winemaking process. Moreover, the association of CL-1048 with AMF may play a relevant role in a future climate-change scenario to maintain or even improve fruit quality by enhancing antioxidant properties, which demonstrates the importance of adopting measures to protect the indigenous cohorts of AMF in vineyards. However, limitations of controlled environments to assess the effect of temperature on grapevine means that extrapolations to field-grown grapevines should be made with due caution.

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Table 1. Summary of the agronomic characteristics of Tempranillo clones used in the study. Data provided by Institute of Sciences of Vine and Wine (Logroño, Spain) were collected and averaged over the 2009–12 period in an experimental vineyard.

	CL-260	C-1048	CL-1089
City of origin (region)	San Vicente de la Sonsierra (La Rioja)	Laguardia (Álava)	Bargota (Navarra)
	Agronomic classification		
Reproductive cycle	Short	Short	Short
Yield	Low	Medium	High
	Reproductive cycle		
Fruit set–veraison (days)	56	55	52
Veraison–harvest (days)	30	37	33
	Yield components		
Yield (kg vine^{-1})	10.37	12.90	21.91
Bunch mass (g bunch^{-1})	84	133	154
Berry mass (g)	1.24	1.40	2.05

Table 2. Phenology expressed on chronological and thermal-time scales and intensity of mycorrhizal colonisation in roots of fruit-bearing cuttings of Tempranillo clones inoculated with arbuscular mycorrhizal fungi or uninoculated and grown at 24°C–14°C or 28°C–18°C (day–night) temperatures.

	Fruit set–veraison		Veraison–maturity		Mycorrhizal
	(days)	(degree-days)	(days)	(degree-days)	colonisation (%)
			CL-260		
Treatments					
–M24	57	513	57a	513	n.d.
+M24	58	522	55a	495	14.8
–M28	45	585	39b	507	n.d.
+M28	43	559	44b	572	12.6
Main effects					
Temperature:					
24	58a	518	56	504	
28	44b	572	42	540	
Mycorrhization:					
–M	51	549	43	510	
+M	50	541	50	534	
ANOVA					
Temperature	**	n.s.	*	n.s.	n.s.
Mycorrhization	n.s.	n.s.	n.s.	n.s.	n.s.
T × M	n.s.	n.s.	**	n.s.	n.s.
			CL-1048		
Treatments					
–M24	55	495	67	603	n.d.
+M24	54	486	66	594	13.7
–M28	42	546	50	650	n.d.
+M28	43	559	54	702	10.9
Main effects					
Temperature:					
24	58a	518	56a	504	
28	44b	572	42b	539	
Mycorrhization:					
–M	49	521	58	626	
+M	48	523	60	648	
ANOVA					
Temperature	**	n.s.	**	n.s.	n.s.
Mycorrhization	n.s.	n.s.	n.s.	n.s.	n.s.
T × M	n.s.	n.s.	n.s.	n.s.	n.s.
			CL-1089		
Treatments					
–M24	58	522	57	513	n.d.
+M24	56	504	58	522	11.5
–M28	44	572	50	650	n.d.
+M28	43	559	49	637	10.3
Main effects					
Temperature:					
24	57a	513	58	603a	
28	44b	565	50	520b	
Mycorrhization:					
–M	51	547	53	582	
+M	50	532	53	580	
ANOVA					
Temperature	**	n.s.	n.s.	*	n.s.
Mycorrhization	n.s.	n.s.	n.s.	n.s.	n.s.
T × M	n.s.	n.s.	n.s.	n.s.	n.s.

Values represent means (n = 5) separated by Duncan's test (at P = 0.05). Within columns and clones, means followed by different letters are significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, –M) and their interaction (T × M). *P < 0.05; **P < 0.01; n.s., not significant (P > 0.05). n.d., Not detected.

Table 3. Plant and berry characteristics from fruit-bearing cuttings of Tempranillo clones inoculated with arbuscular mycorrhizal fungi or uninoculated and grown at 24°C–14°C or 28°C–18°C (day–night) temperatures.

	Leaf area (m^2 $plant^{-1}$)	Leaf no. (no. $plant^{-1}$)	Yield (g $plant^{-1}$)	Berry mass (g $berry^{-1}$)	Relative skin mass (% berry FM)
			CL-260		
Treatments					
–M24	0.77	59	269.4	1.19	29.5
+M24	0.95	57	246.3	1.41	24.0
–M28	0.39	38	201.0	1.00	30.4
+M28	0.47	35	209.8	1.04	26.1
Main effects					
Temperature:					
24	0.86a	58a	257.9a	1.30a	26.7
28	0.43b	37b	205.4b	1.02b	28.2
Mycorrhization:					
–M	0.58	49	235.2	1.09	29.9
+M	0.71	46	228.1	1.22	25.0
ANOVA					
Temperature	***	**	**	*	n.s.
Mycorrhization	n.s.	n.s.	n.s.	n.s.	n.s.
T × M	n.s.	n.s.	n.s.	n.s.	n.s.
			CL-1048		
Treatments					
–M24	0.97	75	284.1	1.21	33.3
+M24	0.95	91	249.8	1.28	32.2
–M28	0.54	45	227.3	1.00	37.9
+M28	0.58	49	203.0	1.10	28.9
Main effects					
Temperature:					
24	0.96a	83a	266.9a	1.24a	32.7
28	0.56b	47b	215.1b	1.04b	33.9
Mycorrhization:					
–M	0.75	61	255.7	1.10	35.6
+M	0.79	70	226.4	1.20	30.7
ANOVA					
Temperature	***	***	*	*	n.s.
Mycorrhization	n.s.	n.s.	n.s.	n.s.	n.s.
T × M	n.s.	n.s.	n.s.	n.s.	n.s.
			CL-1089		
Treatments					
–M24	0.96	62	324.1	1.27	34.4
+M24	0.92	75	320.6	1.27	31.1
–M28	0.56	54	264.6	1.01	33.5
+M28	0.60	44	241.3	1.01	29.9
Main effects					
Temperature:					
24	0.93a	69a	322.1a	1.27a	32.6
28	0.58b	49b	254.2b	1.01b	31.9
Mycorrhization:					
–M	0.74	58	291.0	1.13	33.9
+M	0.78	60	285.3	1.16	30.6
ANOVA					
Temperature	**	*	*	*	n.s.
Mycorrhization	n.s.	n.s.	n.s.	n.s.	n.s.
T × M	n.s.	n.s.	n.s.	n.s.	n.s.

FM, Fresh matter. Values represent means (n = 5) except for berry mass and relative skin mass (n = 50) separated by Duncan's test ($P \leq 0.05$). Within columns and clones, means followed by different letters are significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, –M) and their interaction (T × M). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s., not significant ($P > 0.05$).

Table 4. Concentrations of nitrogen (N) and phosphorus (P) in leaves from fruiting cuttings of Tempranillo clones inoculated with arbuscular mycorrhizal fungi or uninoculated and grown at 24°C–14°C or 28°C–18°C temperatures.

	Leaf N	Leaf P
	(mg g^{-1} DM)	
	CL-260	
Treatments		
–M24	37.66	2.95
+M24	40.58	2.78
–M28	40.10	4.60
+M28	42.48	4.66
Main effects		
Temperature:		
24	39.34	2.85b
28	41.43	4.63a
Mycorrhization:		
–M	39.41	3.78
+M	41.62	3.71
ANOVA	n.s.	***
Temperature	n.s.	n.s.
Mycorrhization	n.s.	n.s.
T × M		
	CL-1048	
Treatments		
–M24	39.16b	2.38c
+M24	37.62b	3.20b
–M28	40.26b	4.61a
+M28	42.94a	3.99a
Main effects		
Temperature:		
24	38.54	2.83
28	41.66	4.32
Mycorrhization:		
–M	39.84	3.53
+M	40.28	3.62
ANOVA		
Temperature	**	***
Mycorrhization	n.s.	n.s.
T × M	*	**
	CL-1089	
Treatments		
–M24	39.50	2.32
+M24	38.10	2.81
–M28	41.58	3.86
+M28	41.55	4.24
Main effects		
Temperature:		
24	38.46	2.43b
28	41.50	4.11a
Mycorrhization:		
–M	40.77	3.22
+M	40.36	3.61
ANOVA		
Temperature	n.s.	***
Mycorrhization	n.s.	n.s.
T× M	n.s.	n.s.

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

Table 5. Main effects and their interaction on technological and phenolic maturity of berries from fruiting cuttings of Tempranillo clones inoculated with arbuscular mycorrhizal fungi or uninoculated and grown at 24°C–14°C or 28°C–18°C temperatures.

	TSS (°Brix)	Must pH	TA	Malic acid	Tartaric acid ($g\ L^{-1}$)	TPI (AU)	Anthocyanins ($mg\ L^{-1}$) Total	Anthocyanins ($mg\ L^{-1}$) Extractable	EA (%)	SM (%)
					CL-260					
Main effects										
Temperature:										
24	22.0b	4.1	5.6a	5.8	9.1a	46.2a	690.7	376.4	43.6a	66.5a
28	24.4a	4.2	4.7b	4.8	6.8b	30.9b	449.6	321.8	25.3b	55.7b
Mycorrhization:										
–M	23.2	4.2	5.0	5.0	8.3	41.7	548.3	364.7	41.5 a	63.6
+M	23.1	4.1	5.3	5.7	7.9	35.4	592.0	333.5	29.0 b	58.6
ANOVA										
Temperature	***	n.s.	*	n.s.	**	***	**	*	***	*
Mycorrhization	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	n.s.
T × M	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**	***	n.s.	n.s.
					CL-1048					
Main effects										
Temperature:										
24	20.8b	4.2	4.8	5.6	7.5b	39.7a	504.5a	344.0a	32.3	64.7
28	23.3a	4.3	4.6	5.4	10.2a	23.9b	345.8b	234.8b	32.4	60.5
Mycorrhization:										
–M	21.8	4.4a	4.4	5.7	8.2b	28.7	362.4b	274.9	26.5	61.5
+M	22.4	4.1b	5.0	5.3	9.8a	34.9	487.9a	303.8	38.2	63.8
ANOVA										
Temperature	***	n.s.	n.s.	n.s.	***	***	***	**	n.s.	n.s.
Mycorrhization	n.s.	*	n.s.	n.s.	*	n.s.	**	n.s.	**	n.s.
T × M	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.
					CL-1089					
Main effects										
Temperature:										
24	21.2	4.1	6.1a	4.9a	7.5b	40.2	642.9a	328.1a	47.6	66.7
28	21.6	4.2	4.8b	3.7b	9.5a	33.3	505.4b	242.4b	49.5	70.0
Mycorrhization:										
–M	21.7	4.2	5.0	3.9	9.0	35.9	512.5b	305.6	37.3 b	65.3
+M	21.1	4.1	5.9	4.8	8.2	37.3	635.8a	262.6	58.9a	71.3
ANOVA										
Temperature	n.s.	n.s.	*	*	*	n.s.	*	*	n.s.	n.s.
Mycorrhization	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	***	n.s.
T × M	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

TSS, Total soluble solids; TA, titratable acidity; TPI, total polyphenol index; EA, Cellular extractability of anthocyanins; SM, seed maturity index AU, absorbance units. Values represent means (n = 5) separated by Duncan's test ($P \leq 0.05$). Within columns and clones, means followed by different letters are significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, –M) and their interaction (T × M). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s., not significant ($P > 0.05$).

Table 6. Total antioxidant capacity of berry extracts from fruiting cuttings of Tempranillo clones inoculated with arbuscular mycorrhizal fungi or uninoculated and grown at 24°C–14°C or 28°C–18°C temperatures.

		Total antioxidant capacity (mg g DM^{-1})
CL-260	Treatments	
	–M24	0.172
	+M24	0.193
	–M28	0.152
	+M28	0.149
	Main effects	
	Temperature:	
	24	0.183a
	28	0.151b
	Mycorrhization:	
	–M	0.162
	+M	0.171
	ANOVA	
	Temperature	**
	Mycorrhization	n.s.
	T × M	n.s.
CL-1048	Treatments	
	–M24	0.138
	+M24	0.160
	–M28	0.150
	+M28	0.189
	Main effects	
	Temperature:	
	24	0.150
	28	0.169
	Mycorrhization:	
	–M	0.144b
	+M	0.174a
	ANOVA	
	Temperature	n.s.
	Mycorrhization	*
	T × M	n.s.
CL-1089	Treatments	
	–M24	0.178
	+M24	0.129
	–M28	0.117
	+M28	0.122
	Main effects	
	Temperature:	
	24	0.151a
	28	0.119b
	Mycorrhization:	
	–M	0.126
	+M	0.144
	ANOVA	
	Temperature	*
	Mycorrhization	n.s.
	T × M	n.s.

DM, Dry matter. Values represent means (n = 5) separated by Duncan's test ($P \leq 0.05$). Within each clone, means followed by different letters are not significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, –M) and their interaction (T × M). *P < 0.05; **P < 0.01; n.s., not significant (P > 0.05).

Figure legends

Figure 1. Mycorrhizal efficiency index (MEI) from fruit-bearing cuttings of Tempranillo clones grown at normal (24°C–14°C) or elevated (28°C–18°C) (day–night) temperatures during berry growth and ripening. Values are means ± s.e. (n = 5). Means with the same letter are not significantly different (P > 0.05) between treatments according to Duncan's test. Two-way ANOVA was performed to evaluate the effects of temperature (T), clone (C) and their interaction (T × C). ***P < 0.001; n.s., not significant (P > 0.05).

Figure 2. Response of (a) technological and (b) phenolic maturity of berries from fruit-bearing cuttings of Tempranillo CL-260 inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (–M) and grown at 24°C–14°C or 28°C–18°C (day–night) temperatures during berry ripening. Values are means ± s.e. (n = 5). Within each parameter, when interaction between the main factors temperature and mycorrhization was significant, means with the same letter are not significantly different (P > 0.05). TPI, Total polyphenol index; EA, cellular extractability of anthocyanins; SM, seed maturity index; AU, absorbance units.

Figure 3. Response of (a) technological and (b) phenolic maturity of berries from fruit-bearing cuttings of Tempranillo CL-1048 inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (–M) and grown at 24°C–14°C or 28°C–18°C (day–night) temperatures during berry ripening. Values are means ± s.e. (n = 5). Within each parameter, when interaction between the main factors temperature and mycorrhization was significant, means with the same letter are not significantly different (P > 0.05). TPI, Total polyphenol index; EA, cellular extractability of anthocyanins; SM, seed maturity index; AU, absorbance units.

Figure 4. Response of (a) technological and (b) phenolic maturity of berries from fruit-bearing cuttings of Tempranillo CL-1089 inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (–M) and grown at 24°C–14°C or 28°C–18°C (day–night) temperatures during berry ripening. Values are means ± s.e. (n = 5). TPI, total polyphenol index; EA, cellular extractability of anthocyanins; SM, seed maturity index; AU, absorbance units.

Figure 5. Principal component analysis (a) score and (b) loading plot obtained from statistical analysis of technological and phenolic maturity parameters and total antioxidant capacity data of 60 studied samples from fruiting cuttings of Tempranillo clones inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (–M) and grown at 24°C–14°C or 28°C–18°C (day–night) temperatures during berry ripening.

Figure 1.

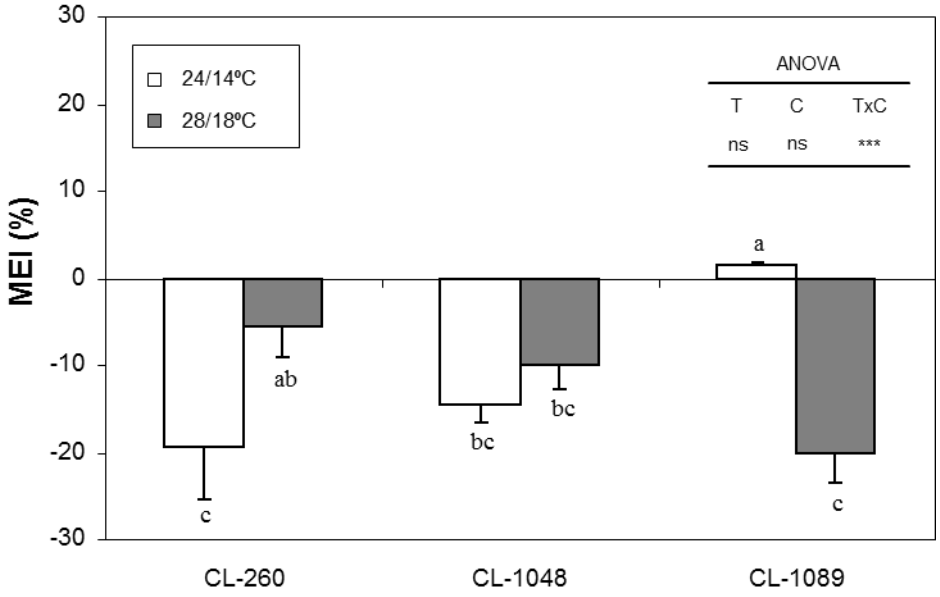
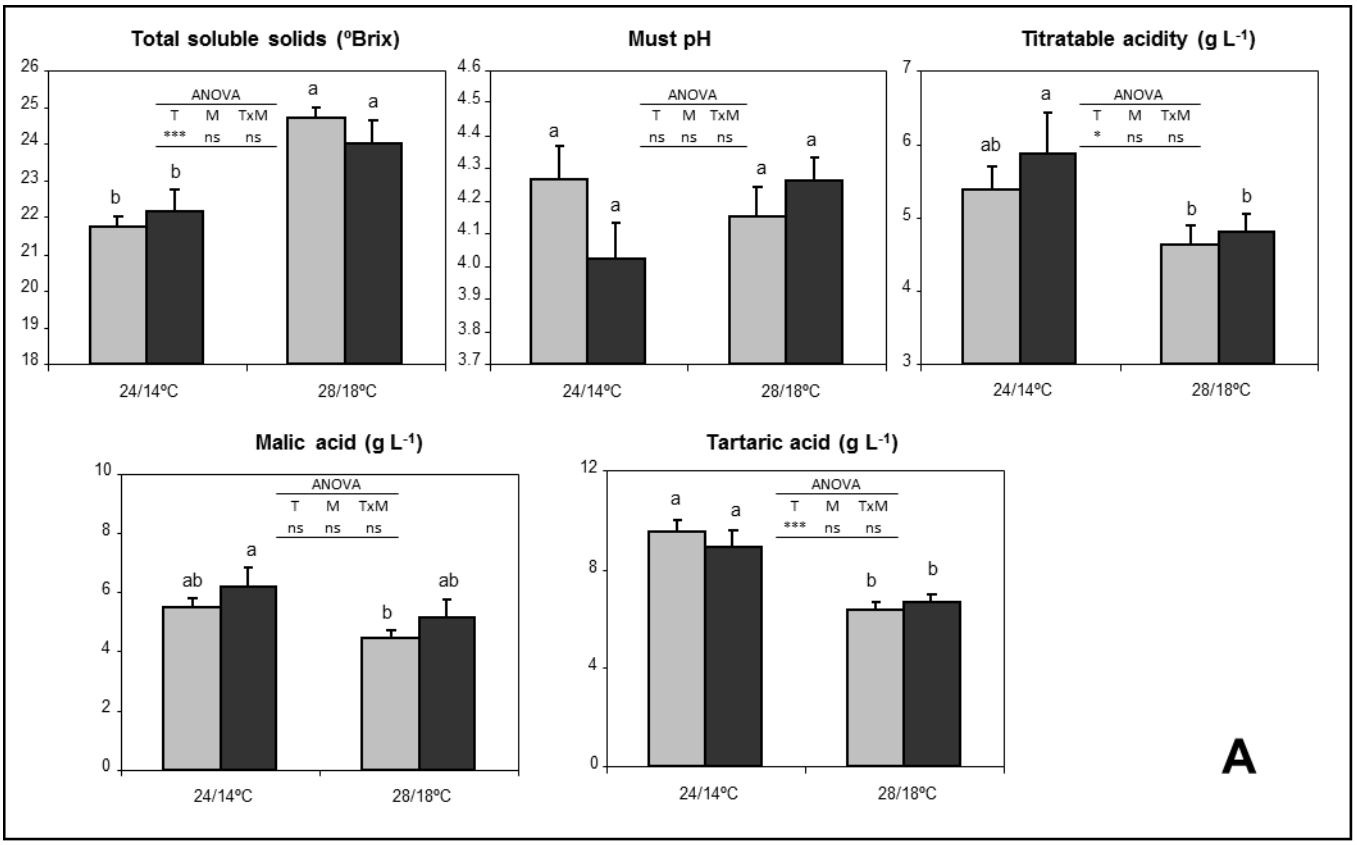

30
20
10
0
-10
-20
-30
MEI (%)
24/14ºC
28/18ºC
ANOVA
T C TxC
ns ns ***
a
ab
bc
bc
c
c
CL-260
CL-1048
CL-1089

Figure 2.


Total soluble solids (ºBrix)
Must pH
Titratable acidity (g L-1)
Malic acid (g L-1)
Tartaric acid (g L-1)
ANOVA
T M TxM
24/14ºC
28/18ºC
A

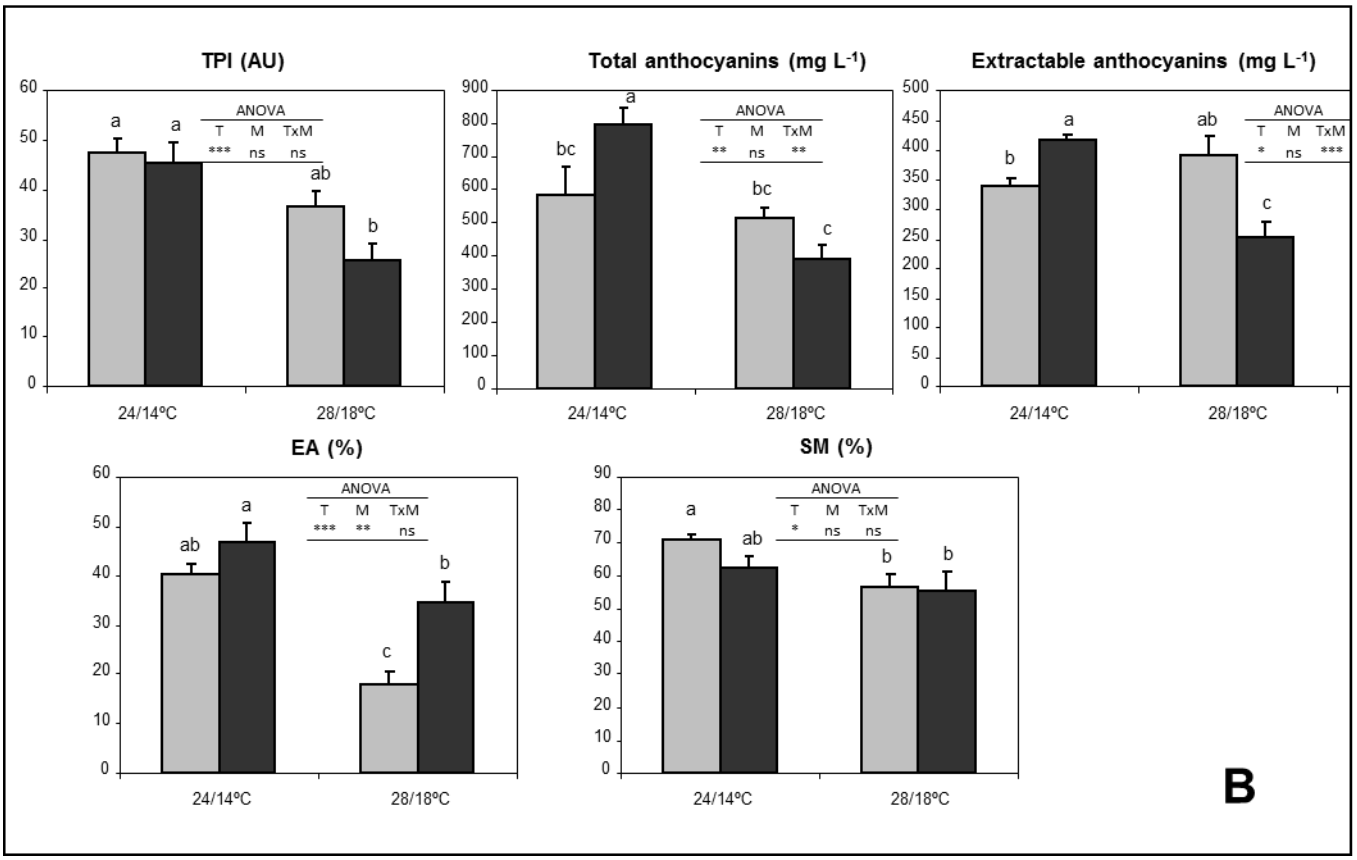
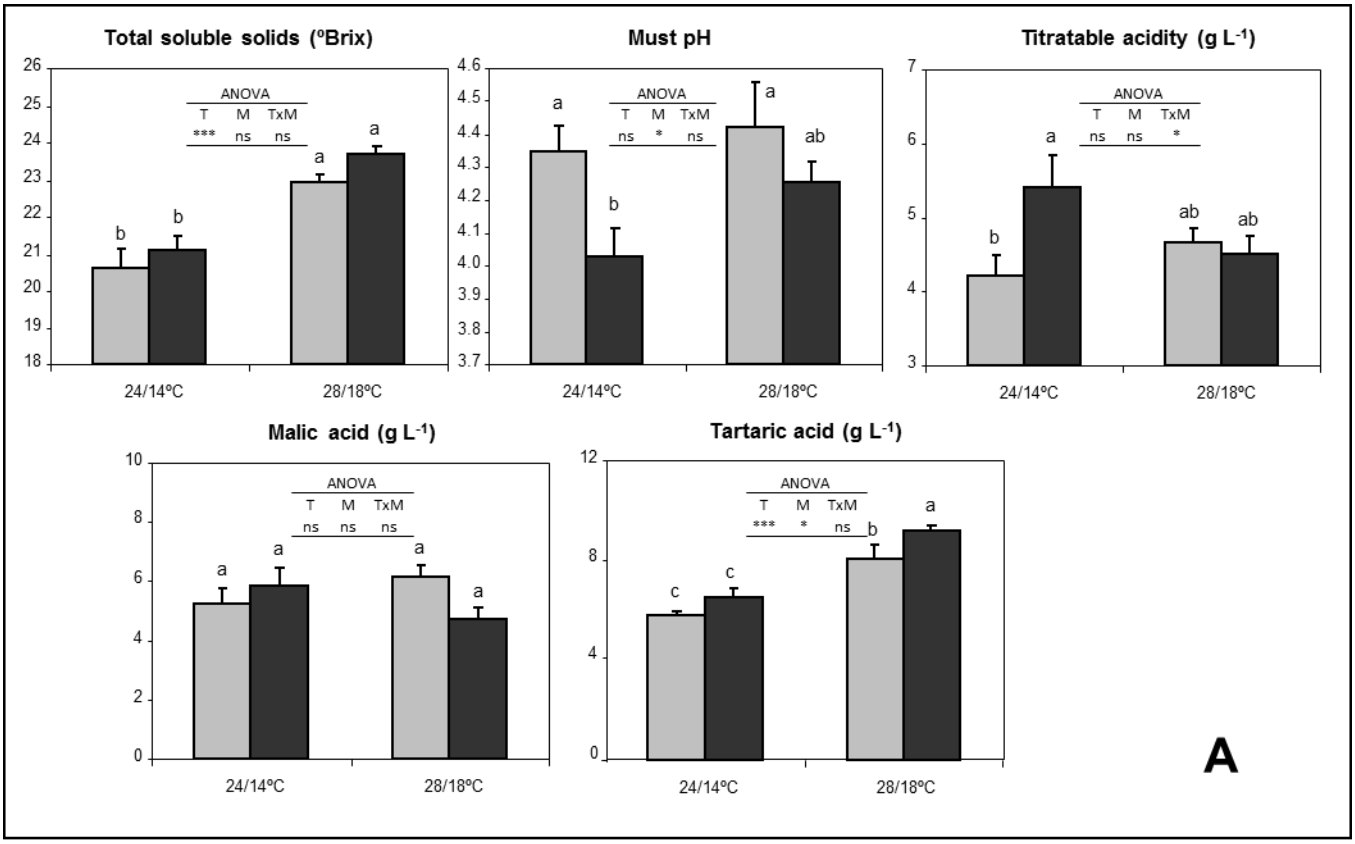

TPI (AU)
Total anthocyanins (mg L-1)
Extractable anthocyanins (mg L-1)
EA (%)
SM (%)
ANOVA
T M TxM
24/14ºC
28/18ºC
B

Figure 3.


Total soluble solids (ºBrix)
Must pH
Titratable acidity (g L-1)
Malic acid (g L-1)
Tartaric acid (g L-1)
ANOVA
T M TxM
24/14ºC
28/18ºC
A

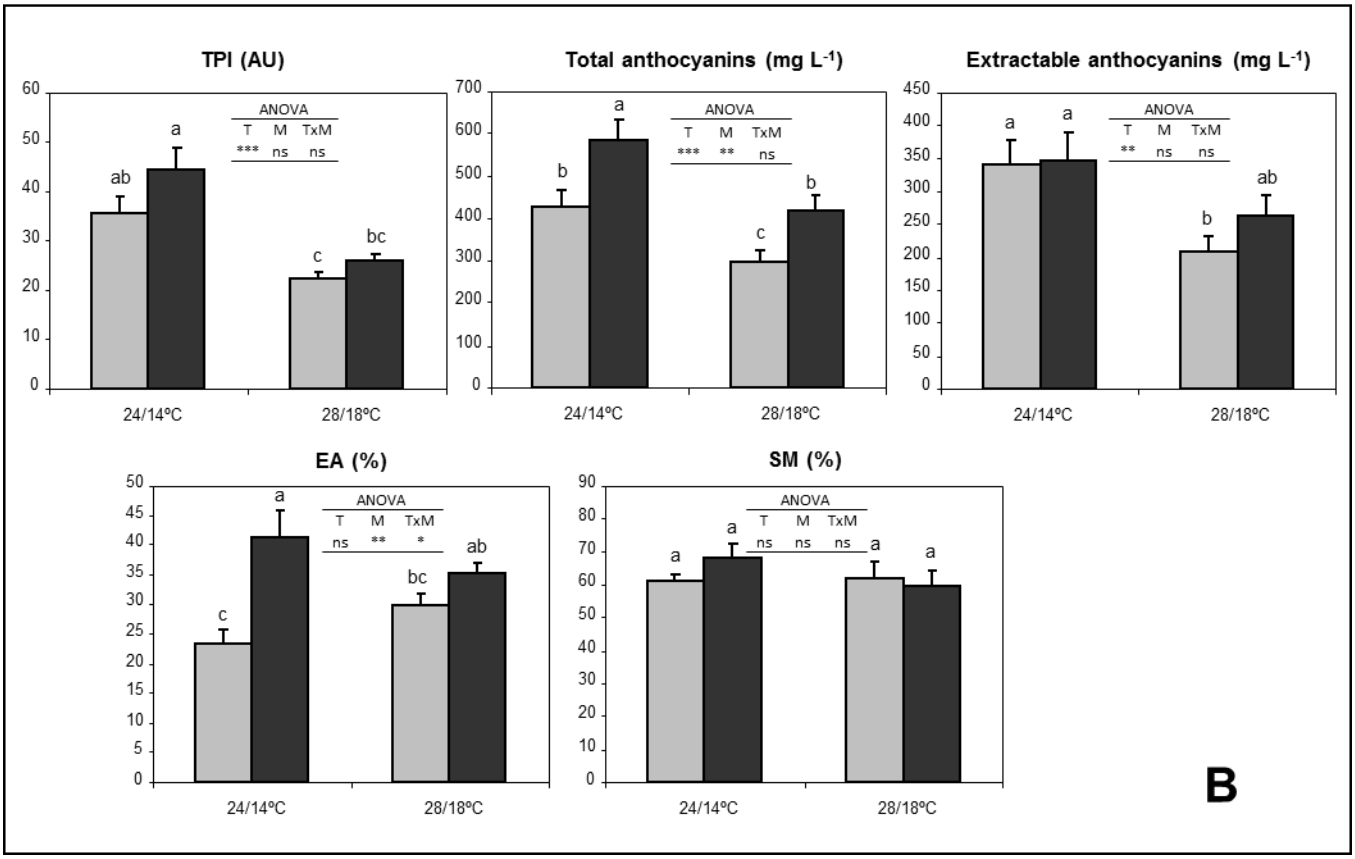
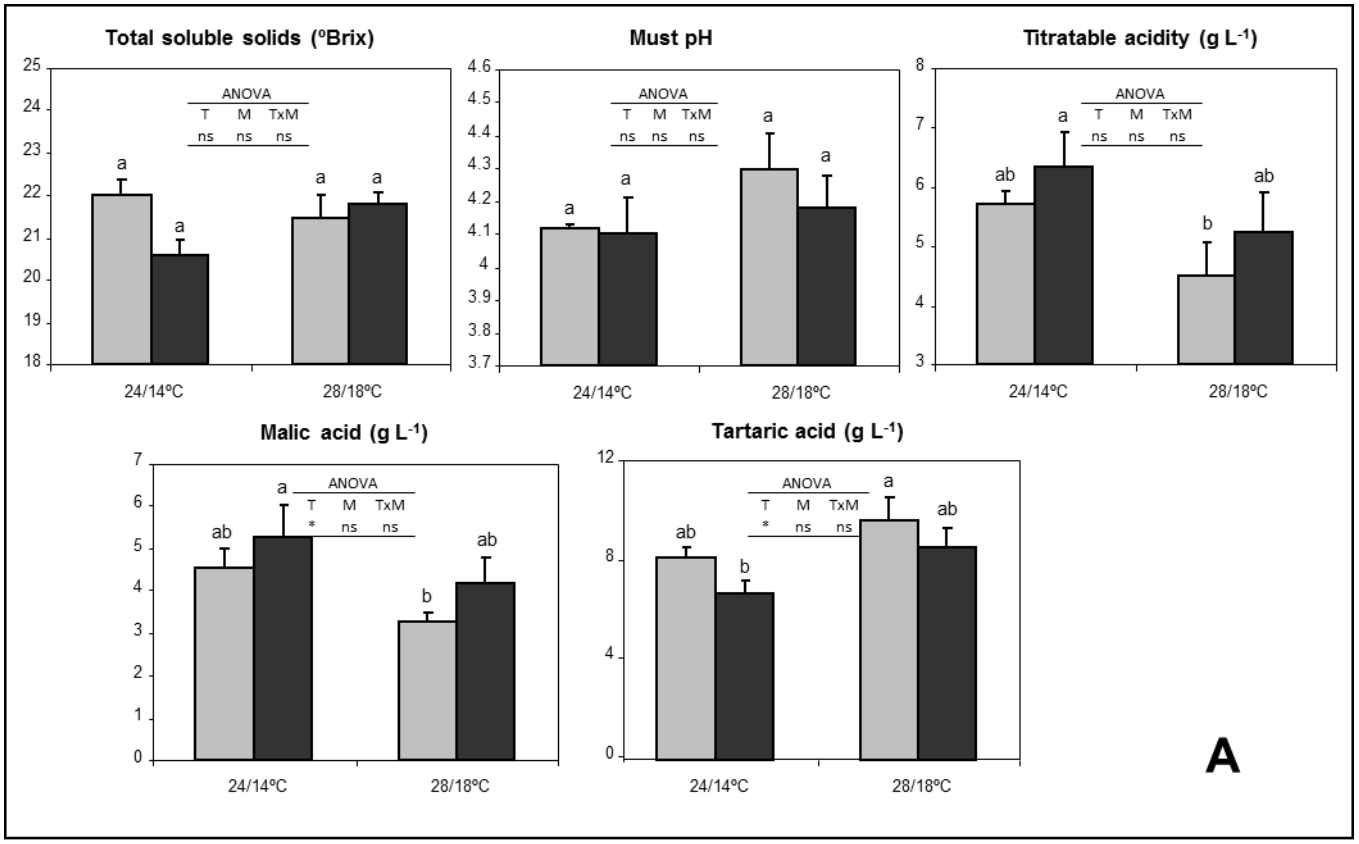

TPI (AU)
Total anthocyanins (mg L-1)
Extractable anthocyanins (mg L-1)
EA (%)
SM (%)
ANOVA
T M TxM
24/14ºC
28/18ºC
B

Figure 4.


Total soluble solids (ºBrix)
Must pH
Titratable acidity (g L-1)
Malic acid (g L-1)
Tartaric acid (g L-1)
ANOVA
T M TxM
24/14ºC
28/18ºC
A

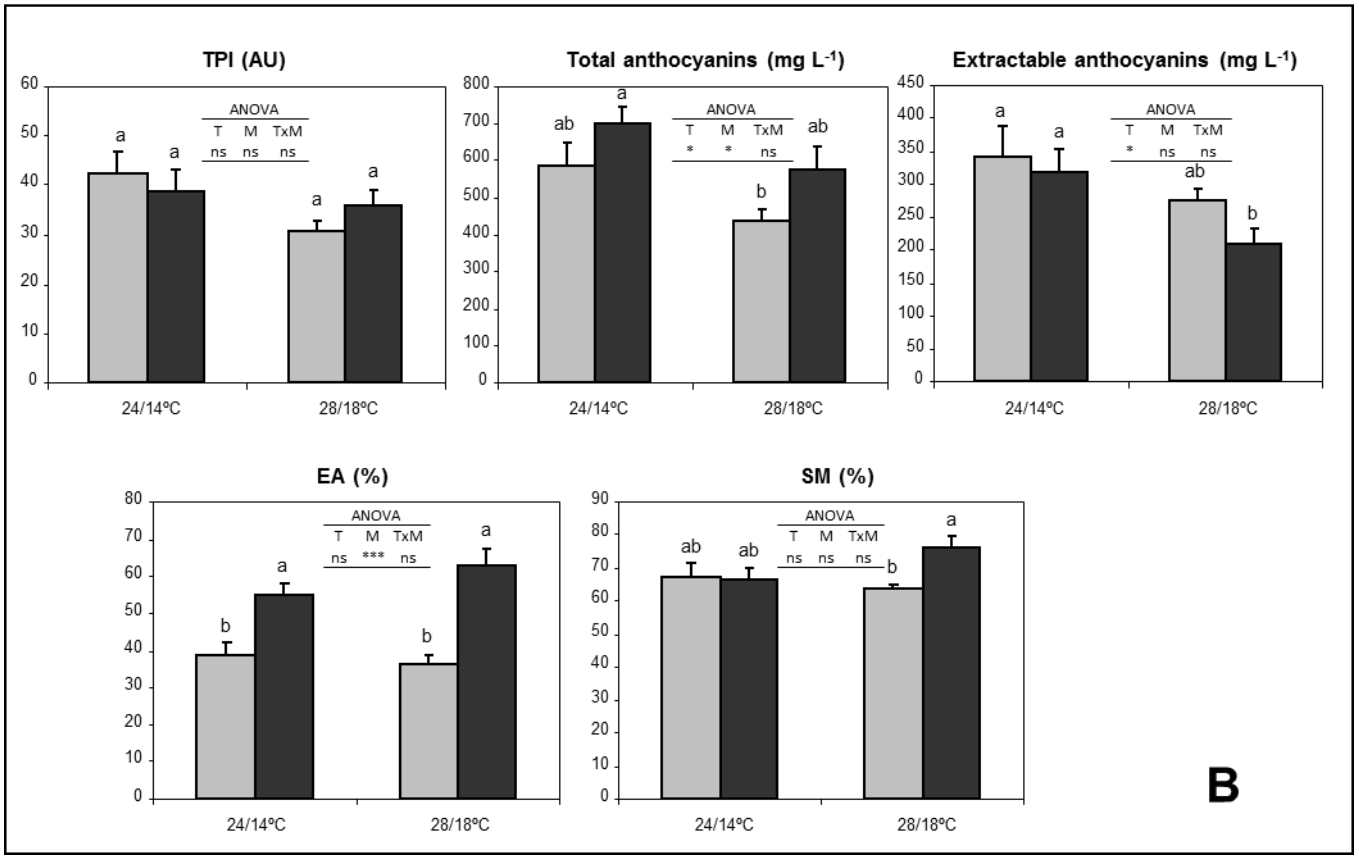
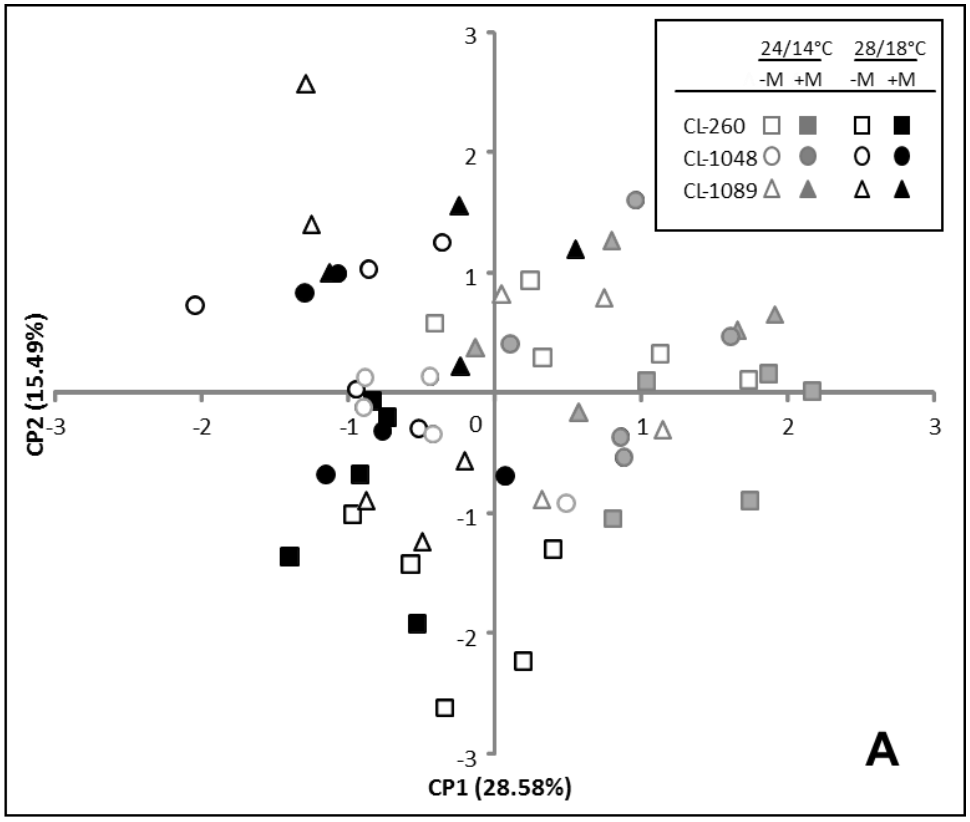
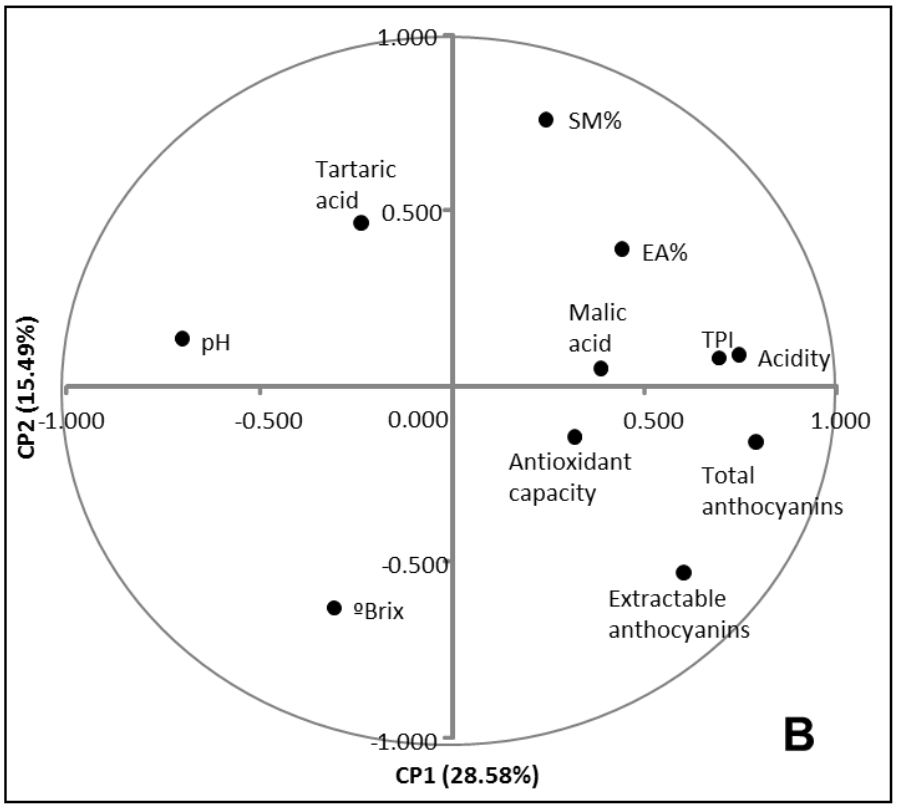

TPI (AU)
Total anthocyanins (mg L-1)
Extractable anthocyanins (mg L-1)
EA (%)
SM (%)
ANOVA
T M TxM
24/14ºC
28/18ºC
B

Figure 5.


24/14°C
28/18°C
-M +M -M +M
CL-260
CL-1048
CL-1089
CP2 (15.49%)
CP1 (28.58%)
A


SM%
Tartaric acid
EA%
Malic acid
pH
TPI
Acidity
Antioxidant capacity
Total anthocyanins
Extractable anthocyanins
ºBrix
CP2 (15.49%)
CP1 (28.58%)
B

CAPÍTULO 3

Influence of irrigation strategy and mycorrhizal inoculation on fruit quality in different clones of Tempranillo grown under elevated temperatures.

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Influence of irrigation strategy and mycorrhizal inoculation on fruit quality in different clones of Tempranillo grown under elevated temperatures

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Abstract

The projected climate scenario for South Mediterranean Europe predicts lower precipitation and higher temperatures that will negatively affect viticulture in the region. The application of moderate deficit irrigation at crucial moments of berry ripening has been found to improve berry quality. Furthermore, grapevine association with arbuscular mycorrhizal fungi (AMF) may improve grapevine's ability to cope with abiotic stresses. Therefore, the aims of this research were: (1) to characterize the response of three clones of *Vitis vinifera* L. cv. Tempranillo to the combination of different water deficit programs and AMF inoculation under elevated temperatures, and (2) to determine whether AMF inoculation can improve berry antioxidant properties under these conditions. The study was carried out on three fruit-bearing cuttings clones of cv. Tempranillo (CL-260, CL-1089 and CL-843) inoculated (+M) or not (-M) with AMF and subjected to two temperature regimes (24/14°C and 28/18°C (day/night)) combined with three irrigation regimes during berry ripening. Irrigation treatments were: (i) water deficit from fruit set to veraison (early deficit, ED); (ii) water deficit from veraison to maturity (late deficit, LD); and (iii) full irrigation (FI). Although each Tempranillo clone seemed to have different abilities to respond to elevated temperatures and water supply, in general, at 24/14°C the LD treatment performed better than ED. Differences among clones were attenuated at 28/18°C. In addition, potential benefits of the LD treatment were improved by AMF inoculation. Thus, in all clones the loss of anthocyanins at 28/18°C detected in –M plants after applying LD did not occur in the +M plants. Moreover, AMF inoculation increased must antioxidant capacity in CL-843 under these environmental conditions. Our results suggest that the implementation of measures to promote the association of grapevines with appropriate AMF for each variety could contribute to optimize effects of irrigation strategy on berry properties under future warming conditions.

***Keywords**: Anthocyanins; arbuscular mycorrhizal fungi (AMF); climate change; clonal variability; DPPH assay; deficit irrigation*

1. Introduction

The grapevine is one of the most widely cultivated crops, with a total global surface area of 7.5 million ha under vines. Most harvested grapes are processed into wine, leading to a global production of 274 million hectoliters in 2015, with Spain being the third largest producer in the world (14% of the total world wine production) (OIV, 2016a). However, the future climate scenario for South Mediterranean Europe is not favorable for agriculture in general and for viticulture in particular (Chaves et al., 2010; Lionello et al., 2014) due to the predicted decreased precipitation, increased air and soil temperatures and extreme climate events (IPCC, 2014). Grapevine development has already suffered from significant impact from global climate change (Teixeira et al., 2013). Thus, a growing body of evidence indicates that as the climate warms, grapevine phenology progresses at a faster rate, grapes ripen earlier (Webb et al., 2012), berry sugar content (and subsequent alcohol in the wine) tends to increase (Petrie and Sadras, 2008) and phenolic ripeness is not always achieved (Mori et al., 2007; Sadras and Moran, 2012). In addition, the tendency toward a decreased acidity of must (Sweetman et al., 2014) has potential effects on wine aging capacity.

In the Mediterranean region, the climate may be quite dry during the grapevine growing season and vines may require additional irrigation to counteract water deficit stress (Chaves et al., 2007; 2010).Currently, irrigation of vineyards is below 10% of the total area in Europe, but the tendency towards irrigation is increasing in order to mitigate the negative impact of climate change (Costa et al., 2016). Severe water deficit exacerbates the accelerated accumulation of sugars in grapes caused by warm temperatures (Bonada et al., 2015), which results in an imbalance between the levels of sugars and the phenolic ripening in berries (Sadras and Moran, 2012). In contrast, several studies (Santesteban et al., 2011; Zarrouk et al., 2012; Niculcea et al., 2014) have demonstrated that, under moderate water restriction, berries from red wine varieties (such as Tempranillo) had increased levels of sugars and anthocyanins. For several years, moderate deficit irrigation has been applied in order to improve cluster microclimate, increase water use efficiency, control the vegetative development of grapevines, reduce berry size and induce the accumulation of sugars and polyphenols in fruits (Wample and Smithyman, 2002). Different deficit irrigation programs maintain plants at some degree of water deficit for a prescribed part of the season (Basile et al., 2011; Intrigliolo et al., 2012). Nevertheless, high temperatures can constitute a relevant constraint to the implementation and success of the deficit irrigation (Shellie, 2011) and the timing of water deficit might need to be revised to account for the deleterious effects of elevated temperatures in water-stressed plants (Edwards et al., 2011). Thus, it has been reported that alterations in berry primary

metabolism (such as sugars, organic acids and amino acids) due to warm temperatures was higher than in secondary metabolism (i.e., anthocyanins and flavonoids), which was mainly affected by timing of water deficit throughout ripening (Torres et al., 2017).

Soil microorganisms 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). For this reason, considerable progress has been made in the last decade towards the use of these symbiotic fungi to improve grapevine growth and yield. Mycorrhizal symbiosis has been associated with improved growth, increased tolerance against drought and/or enhanced mineral uptake from soils (Trouvelot et al., 2015). Moreover, mycorrhizal plants can accumulate higher levels of phenolic compounds in their tissues than non-mycorrhizal plants and this phenomenon is more evident when plants undergo water deficit rather than optimal irrigation (Baslam and Goicoechea, 2012). In grapevines, mycorrhizal colonization enhances water use efficiency under drought (Valentine et al., 2006) and induces the accumulation of phenolics in leaves (Eftekhari et al., 2012; Torres et al., 2015) and berries (Torres et al., 2016) under optimal irrigation, with these latter results being highly dependent on intravarietal differences of grapevines and air temperatures throughout grapevine cultivation. The phenolic compounds detected in grapes have generated remarkable interest because they have antioxidant properties that are beneficial for human health (Georgiev et al., 2014).

Currently, deficit irrigation in viticulture can be managed in order to increase the concentrations of phenolics in berries with attain the final objective of enhancing must quality and its nutraceutical properties. However, to our knowledge, no studies have assessed the contribution of AMF for improving or maintaining the benefits that different deficit irrigation programs can exert on berry quality in a future scenario of climate change. In our previous studies, we showed that the effects of warm temperatures on berry composition of Tempranillo depended on the deficit irrigation system applied and on the clone chosen (Torres et al., 2017). Moreover, under warm temperatures, the benefits of AMF inoculation on berry properties were also modulated by type of clone (Torres et al., 2016). Taking into account all these precedents, the aims of the current research were (1) to characterize the response of three clones of *Vitis vinifera* L. cv. Tempranillo to the combination of different water deficit programs (pre- and post-veraison deficit irrigation) and AMF inoculation under elevated day/night temperatures; and (2) to determine whether AMF inoculation can improve berry antioxidant properties under different climatic scenarios. Previous research (Antolín et al.,

2010; Morales et al., 2016) has demonstrated that fruit-bearing cuttings are a meaningful and useful model system to study the response of berry ripening to environmental factors. Thus, potted vines were used to ensure that all clones experienced the same conditions and to control mycorrhizal inoculation and to have comparable non-inoculated plants.

2. Material and Methods

2.1. Biological material and growth conditions

Dormant 400-500 mm long *Vitis vinifera* (L.) cuttings from different clones of Tempranillo were collected during the winter of 2014 from an experimental vineyard of the Institute of Sciences of Vine and Wine (Logroño, Spain) (Denomination of Origin Rioja, North of Spain). Three clones (CL-260, CL-1089 and CL-843) were selected in the field on the basis on their different agronomic traits (Table S1) and on the basis of our previous finding, which showed that phenolic content and antioxidant activity were stimulated by the combination of elevated temperature and AMF inoculation (Torres et al., 2015; 2016). Cuttings of each clone were selected for fruit-bearing according to the steps originally outlined by Mullins (1966) with some modifications as described in Ollat et al. (1998) and Antolín et al. (2010). Briefly, rooting was made in a heat-bed (27°C) kept in a cool room (4°C). One month later, the cuttings were planted in 6.5-L plastic pots containing a mixture of vermiculite–sand–light peat (2.5:2.5:1, v:v:v). Properties of the peat (Floragard, Vilassar de Mar, Barcelona, Spain) were pH 5.2–6.0, nitrogen 70-150 mg L^{-1}, P_2O_5 80-180 mg L^{-1}, and K_2O 140-220 mg L^{-1}. The peat was previously sterilised at 100°C for 1 h on 3 consecutive days. At transplanting, half of the plants were inoculated with the mycorrhizal inoculum GLOMYGEL Vid, Olivo, Frutales (Mycovitro S.L., Pinos Puente, Spain) (+M plants). The concentrated commercial inoculum was derived from an *in-vitro* culture of AMF *Rhizophagus intraradices* (Schenck & Smith) Walker & Schüßler comb. nov. (Krüger et al., 2012) that contained 2,000 mycorrhizal propagules (inert pieces of roots colonised by AMF, spores and vegetative mycelium) per mL inoculum. The selection of *in vitro*-produced inoculum of *R. intraradices* was based on two expected benefits: easy application of the product and low colonization of grape roots by contaminant fungi (Vimard et al., 1999). In order to facilitate its application, the concentrated commercial inoculum was diluted with distilled water to a mycorrhizal inoculum of 250 propagules mL^{-1}. Each +M plant received 8 mL diluted mycorrhizal inoculum close to the roots, thus making 2,000 propagules in total. In order to compensate for a possible partial disinfection of mycorrhizal spores during the production of high quantities of the commercial inoculum, a filtrate was added to plants that

did not receive any inoculum (–M plants) with the objective of restoring the helper microorganisms accompanying spores and hyphae of AMF and which 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). The filtrate was obtained by passing diluted 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), and each –M plant received 8 mL filtrate close to the roots.

After transplanting, the fruit-bearing cuttings were transferred to greenhouses, which were adapted to simulate climate change conditions (see details in Morales et al., 2014) until berry maturity was reached. 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} at the level of the inflorescence. Humidity and temperature were controlled using M22W2HT4X transmitters (Rotronic Instrument Corp., Hauppauge, USA). PPFD was monitored with a LI-190SZ quantum sensor (LI-COR, Lincoln, USA). Under these conditions, bud-break took place after one week. Careful control of vegetative growth before flowering improves the partitioning of stored carbon towards the roots and the reproductive structures. Thus, only a single flowering stem was allowed to develop on each plant during growth. Until fruit set, plants were watered twice per day with a nutrient solution (140 mL day^{-1}) with phosphorus level 0.30 mM (Ollat et al., 1998) alternated with water to maintain the soil water content at 80% of pot capacity. 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 Instruments SA, Alella, Spain).

2.2. Experimental design

At fruit set (Eichhorn and Lorenz (E-L) growth stage 27) (Coombe, 1995), which took place one month after bud-break, we established a three-factorial design where two temperature regimes were combined with three water regimes that were applied to plants inoculated (+M) or non-inoculated (-M) with AMF. At fruit set (Eichhorn and Lorenz (E-L) growth stage 27) (Coombe, 1995) fruit-bearing cuttings from +M or -M treatments (36 plants per clone) were exposed to two different temperature regimes: 24/14°C (day/night) and 28/18°C (day/night). At this stage, plants have 4-5 fully expanded leaves. The 24/14°C temperature regime was selected according to average temperatures recorded in La Rioja (1981-2010) (AEMET, Spain) during the growing season. The 28/18°C temperature regime was selected according to

predictions of a rise of 4.0°C at the end of the present century (IPCC, 2014). Both temperature regimes were maintained until berry ripened (21-23°Brix) (E-L 38 stage). To avoid excessive soil overwarming, which can negatively affect roots, and maintain a stable temperature, pots were shaded by wrapping their lateral surface with a reflecting material (Passioura, 2006). Soil temperature was measured at 5 cm soil depth using temperature probes PT100 (Coreterm, Valencia, Spain) and reached 23±0.5°C and 28±0.5°C for 24/14°C and 28/18°C air temperature regimes, respectively (Figure S1).

Within each temperature regime, +M and -M plants from each clone were divided into three groups that were subjected to different irrigation programs. Two deficit irrigation strategies were compared with full irrigation (FI). In the FI treatment, pots were maintained at 80% of pot capacity (volumetric soil water content between 40 and 50%, (m^3 H_2O m^{-3} soil) × 100) from fruit set to harvest. In the water deficit treatments, plants received 50% of the water given to FI plants from fruit set (E-L 27 stage) to onset of veraison (E-L 35 stage) (early deficit, ED) or from onset of veraison (E-L 35 stage, 20-24 fully expanded leaves) to maturity (E-L 38 stage) (late deficit, LD). After ED and before LD, plants were subjected to full irrigation. Volumetric soil water content was monitored with an EC 5 water sensor (Decagon Devices, Inc., Pullman, WA, USA) placed within each pot. Pot capacity was previously assessed by determining water retained after free-draining water had been allowed to pass through the holes in the bottom of the pot. The surface of the plant containers was covered with quartz stones during the experiments to avoid water loss because of evaporation. Water volume supplied to the FI treatment was adjusted to increase plant development according to the daily measurements of the EC 5 water sensor (Figure S2). Watering was performed with nutrient solution or deionised water in order to supply the different treatments with the same amount of nutrients during water deficit. There were three replicates for each combination of mycorrhizal, temperature, irrigation treatment and clone.

2.3. Mycorrhizal colonization and relative mycorrhizal dependency (RMD)

Root samples were cleared and stained (Koske and Gemma, 1989), and AMF colonization was determined by examining 1-cm root segments (45 per pot) under the microscope. Then, parameters of AMF colonisation were calculated for each pot as described previously (Torres et al., 2015). The relative mycorrhizal dependency (RMD) index was estimated as reported by Bagyaraj (1994): RMD = (bunch fresh matter of +M plant) × 100/ (bunch fresh matter of -M plant). Determination of RMD allows assessment of the dependency of a crop on the mycorrhizal condition to achieve its maximum growth or yield at a given level of soil fertility.

When yield in +M plants is very similar to that achieved in –M plants, the value of RMD is equal to 100%. Values beyond 100% indicate that mycorrhizal symbiosis has improved plant yield. RMD values lower than 100% indicate that mycorrhizal association has reduced yield.

2.4. Plant determinations

Predawn leaf water potential (Ψ_{pd}) was measured with a SKYE SKPM 1400 pressure chamber (Skye Instruments Ltd, Llandrindod, Wales) on three fully expanded leaves per treatment at four stages of berry development: 1) when berries began to soften (Eichhorn and Lorenz(E-L) growth stage 34, green berries) (Coombe, 1995); 2) when berries began to colour and enlarge (E-L 35 stage, veraison); 3) seven days after veraison (E-L 36 stage, veraison+7); and 4) fourteen days after veraison (E-L 37 stage, veraison+14). When fruit maturity was reached (E-L 38 stage) plants were harvested separately based on sugar level (21-23°Brix) from berry subsamples (2-3 berries) taken weekly.

The length of phenological phases was recorded as the number of days from fruit set (E-L 27 stage) to veraison (E-L 35 stage), and from veraison (E-L 35 stage) to maturity (E-L38 stage). To obtain yield, bunches were weighed and then 10 berries from each plant were collected and weighed individually. Mean fresh berry mass was determined and berries were separated into skin and flesh. The remaining berries were counted, weighed and frozen at -80°C for further analysis. The relative skin mass was calculated as the quotient between skin FM and total berry FM expressed as percentage.

2.5. Grape berry determinations

A subsample of 25 berries was crushed and then extracts were centrifuged at 4,300 × *g* at 4°C for 10 min. The supernatant was used for the following determinations: total soluble solids (mainly sugars) measured with a temperature-compensating refractometer (Zuzi model 315; Auxilab, Beriáin, Spain) and expressed as °Brix; must pH measured with a pH meter (Crison Instruments, Barcelona, Spain) standardised to pH 7.0 and 4.0; titratable acidity measured by titration with NaOH according to International Organisation of Vine and Wine methods (OIV, 2016b) and expressed as g tartaric acid L^{-1}; and L-malic acid measured by an enzymatic method (Enzytec L-Malic Acid; R-Biopharm, Darmstadt, Germany).

Another 25-berry subsample per plant was weighed and taken for the analysis of anthocyanins and total phenols. Anthocyanins were calculated according to the procedure described by Saint-Cricq et al. (1998). The samples of the non-filtered, crushed grape

homogenate were macerated for 4 h at pH 3.2 (tartaric acid). Once maceration was over, the macerated samples were centrifuged at 4,300 × *g* at 4°C for 10 min. Anthocyanin content was determined in the supernatant by measuring absorbance at 520 nm (Ribéreau-Gayon and Stonestreet, 1965). Total polyphenol index (TPI) was calculated by the absorbance reading at 280 nm in the supernatant obtained after maceration at pH 3.2 (EEC, 1990). All analyses were run in triplicate.

2.6. Antioxidant capacity

Total antioxidant capacity was evaluated on the same must samples used for berry quality determinations by using the free-radical scavenging activity (α, α-diphenyl-β-picrylhydrazyl, DPPH) assay (Brand-Williams et al., 1995). The free radical scavenging activity, using the free radical DPPH•, was evaluated by measuring the variation in absorbance at 515 nm after 30 min of reaction in Parafilm sealed glass cuvettes (to avoid methanol evaporation) at 25°C. The reaction was started by adding 20 µL of the corresponding sample to the cuvette containing 80 mM (methanol solution) (980 µL) of the free radical (DPPH*). The final volume of the assay was 1 mL. The reaction was followed with a spectrophotometer (Jasco V-630; Analytical Instruments, Easton, MD, USA). The calibration curve was made using gallic acid as a standard.

2.7. 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. Data were subjected to Kolmogorov-Smirnov normality test due to the small sample size (n= 3). Data appeared to follow a normal distribution and were thus subjected to analysis of variance (ANOVA) within each clone. The test was performed to assess the main effect of the factors temperature (T) (24/14°C, 24 and 28/18°C, 28), AMF inoculation (+M and –M) and irrigation program (FI, ED and LD) and the interaction between them. Means ± standard errors (SE) were calculated and when the F ratio was significant (P≤0.05), a Duncan test was applied. Three-way ANOVA was performed to determine significant differences in all measured parameters. Berry quality parameters and antioxidant activity data were analyzed by principal component analysis (PCA) with the same software to determine general trends in the different samples.

3. Results and discussion

3.1. Mycorrhizal colonization and relative mycorrhizal dependency (RMD)

Under moderate temperatures and optimal irrigation, 100% of plants from CL-260 and CL-1089 had mycorrhizal structures (mainly hyphae and vesicles) (Figure 1), but percentages of root mycorrhizal colonization did not exceed 10%. In FI plants from CL-843 grown at moderate temperatures, the percentage of root colonization reached 15%, but only 35% of plants showed mycorrhizal structures. Imposition of drought (ED or LD) and/or elevated temperatures (28/18°C) negatively affected mycorrhizal symbiosis in the three clones of Tempranillo. These low percentages of mycorrhizal colonization achieved at the final stage of fruit ripening could be due to several causes: (1) disinfection of the AMF spores during the production of *in vitro*-mycorrhizal inoculum may have eliminated or at least reduced the bacteria associated with the fungus, thus harming both the survival and the well-timed germination and growth of spores and hyphae included in the applied inoculum (Roesti et al., 2005; Agnolucci et al., 2015); (2) low photosynthetic rates measured at fruit ripening (data not shown) could not allow the production of sufficient carbohydrates to support the mycorrhizal symbiosis; and/or (3) several sinks, such as fruits and mycorrhizal fungus in roots, would be competing for the scarce synthetized photoassimilates during this period of the grapevine life cycle.

In our study, no clear effect of the irrigation regimes and/or air temperatures on the percentage of mycorrhizal colonization was observed. However, several studies have revealed that climate change can affect indirectly (through changes in host plant growth and physiology) and directly the fungal development and the rates of root colonization by AMF (Compant et al., 2010). Although fungal responses to environmental factors can be strain-dependent, in most cases, increased soil temperature favors the mycorrhizal colonization of roots and the spread of hyphal networks while drought reduces AMF colonization. The future scenarios of climate change, however, predict that increased temperature will increase soil drying, which subsequently will reduce the length of the growing season for mycorrhizal fungal hyphae (Allen et al., 2014).

On the other hand, it is important to note that percentages of mycorrhizal colonization provides information about the root length colonized by AMF but does not indicate the functionality of the symbiosis and thus its benefits for the host plant. A low correlation between the root length colonized by the fungus and the mycorrhizal responsiveness of the host plant means that genetic factors other than those allowing colonization are determining

the responsiveness of the host plant to the percent of its root colonized by the mycorrhizal fungus (Parke and Kaeppler, 2000). Calculation of RMD allowed the assessment of the degree to which the studied Tempranillo clones were dependent on the mycorrhizal conditions to produce their maximum yield. Results were highly dependent on the Tempranillo clone and environmental conditions (Figure 2). In CL-260, mycorrhizal inoculation always decreased fruit yield (RMD values lower than 100%), which may be as a consequence of a sink effect caused by the presence of the fungus in roots that would have increased the translocation of sugars from the aerial part to the roots in detriment to fruit development. The lower yield of +M24-FI plants from CL-260 in comparison with that of -M24-FI was accentuated by the ED treatment, but attenuated when ED was imposed under elevated temperatures(28ED). Pischl and Barber (2017) also found that non-mycorrhizal bell peppers grown at optimal irrigation and ambient temperatures produced more fruits than mycorrhizal plants. According to these authors, under non-stressed conditions the AMF treatments incurred a cost that resulted in reduced growth and fitness relative to uncolonized plants. In agreement with our results, Pischl and Barber (2017) also observed that warm temperatures and drought reduced differences between treatments.

In contrast with findings in CL-260, mycorrhizal association improved yield in plants from CL-1089 grown at 24/14°C, except for those undergoing LD (Figure 2). The highest benefit of AMF inoculation on yield was observed when ED was applied.. In this case, RMD values almost reached 200%. In CL-843, at 24/14°C mycorrhizal inoculation enhanced yield in drought stressed plants (24ED and 24LD) . However, at 28/18°C RMD only was significantly improved under full irrigation (28FI). Arbuscular mycorrhizal dependency can strongly vary not only among different plant species (Tawaraya, 2003) but also among cultivars belonging to the same plant species (Tawaraya, 2003; Fahey et al., 2016), which has also been assessed between different grape varieties (Eftekhari et al., 2012; Torres et al., 2015, 2016). Root morphology (root length, length and frequency of the root hairs) seems to be the main factor for determining the mycorrhizal dependency of a given plant species or cultivar because it strongly affects the efficiency of the host plant to acquire resources from the soil (Tawaraya, 2003). Any environmental factor that may alter the root morphology of host plants could also modify their mycorrhizal dependency.

In our study, despite the interclonal differences between CL-1089 and CL-843, in general terms, mycorrhizal inoculation improved yield in plants undergoing adverse conditions (drought, elevated temperatures or both), which reinforces the idea that mycorrhizal symbiosis may be more beneficial for host plants under stressful conditions (Goicoechea et al.,

2004) and supports the results indicating that AMF can improve the drought/heat tolerance of vines (Schreiner and Linderman, 2005; Valentine et al., 2006; Schreiner et al., 2007).

3.2. Phenology and physiological characteristics during berry ripening

Clones of grapevine cv. Tempranillo tested in this study have significant diversity for some agronomic traits such as length of cycle, yield, bunch mass and berry mass (Table S1). With regard to phenology, vineyard-grown plants of CL-260 and CL-1089 were characterized by a shorter reproductive cycle than CL-843. In our experimental conditions, temperature was the main factor influencing time to reach veraison that was shortened by increasing temperature to 28/18°C (Table S2). Such observations show a consistent trend towards earlier veraison commonly observed at warm temperatures (Petrie and Sadras, 2008; Web et al., 2012; Torres et al., 2016). However, in CL-843, at 24/14°C the ED treatment also contributed to reduce time to reach veraison, as showed by significant interaction between both factors (T×I, $P \leq 0.05$). In the same way, several studies provided support for acceleration of the ripening process due to ED water restriction that was shown by the accelerated onset of anthocyanin synthesis (Castellarin et al., 2007; Herrera and Castellarin, 2016). The hormone abscisic acid (ABA) plays a critical role in regulating berry ripening because berry ABA concentration increases remarkably at veraison, and stimulates anthocyanin synthesis by promoting expression of key biosynthetic genes (Fortes et al., 2015). Under water deficit, it has been reported that ABA pattern accumulation was altered and at veraison, peak of ABA was significantly higher in ED than FI (Niculcea et al., 2014). Possibly, such changes could have contributed to reducing time to reach veraison in the ED treatment. On the other hand, temperature and irrigation were the main factors affecting the time course from veraison to maturity in CL-260 and CL-843. In CL-1089, there was also an additive effect of AMF inoculation, as indicated by the interaction between temperature, AMF inoculation and irrigation ($T \times M \times I$, $P \leq 0.05$).

Grapevine water status throughout fruit ripening was significantly different per treatments as indicated by the decrease in predawn Ψ_{pd} measured in plants subjected ED or LD compared with FI plants (Figure 3). During the ED treatment, Ψ_{pd} values reached in CL-260, CL-1089 and CL-843 varied between −1.2 and -1.5 MPa and were not modified by AMF inoculation and/or elevated temperature. In contrast, in the LD treatment, AMF inoculation and elevated temperature modified plant water status. Moreover, significant interactions among factors were observed at stages EL-37 and EL-38 in CL-260 ($T \times I$ and $M \times I$, $P \leq 0.001$), CL-1089 ($T \times M$ and $T \times I$, $P \leq 0.05$) and CL-843 ($T \times I$, $P \leq 0.01$, $M \times I$, $P \leq 0.05$ and $T \times M \times I$, $P \leq 0.01$). Thus, the lowest values

of Ψ_{pd} were always recorded in the +M28 treatment and were reached at stage E-L37 (ca. -1.4 MPa) in CL-260 and at stage EL-38 (ca. -1.7 MPa) in CL-1089 and CL-843 (Figure 3). Similarly, Edwards et al. (2011) showed an additive effect of temperature and water stress on plant water status, which in our study seemed to be more accentuated in +M plants. This low Ψ_{pd} in +M28 treatment could be result from elevated water losses due to high leaf area and/or high transpiration rates. However, this was not the case because leaf area was considerably smaller in +M28 than in +M24 plants (0.80-0.90 m^2 $plant^{-1}$ in +M24 and 0.25-0.40 m^2 $plant^{-1}$ in +M28). Furthermore, +M28 plants had the lowest leaf conductance rates (data not shown). In disagreement with our results, Nikolaou et al. (2003) measured higher Ψ_{pd} in leaves of mycorrhizal 'Cabernet Sauvignon' than in those of non-mycorrhizal plants under drought conditions and the improved water relations in mycorrhizal grapevines were attributed to their higher cytokinin concentrations. However, the exposure of plants to high temperatures can cause reductions in the levels of cytokinins (Todorova et al., 2005; Yang et al., 2016), which may explain the low Ψ_{pd} measured in the +M28 treatment. In addition, it should be noted that although decreases in the tissue Ψ_{pd} depend mostly upon leaf dehydration, it also depend both on initial osmolarity of the cell sap and on cell wall elasticity. Previous studies of our group showed that Ψ_{pd} of mycorrhizal plants involved an increase in the percentage of apoplastic water without a decrease in osmotic Ψ (Goicoechea et al., 1997). This mechanism may allow mycorrhizal plants to tolerate drought without investing solutes into osmotic adjustment, which may be of special importance when the production of carbohydrates to support several sinks (fruits and mycorrhizal fungus) is limited, especially at the end of berry ripening in +M28 plants.

3.3. Berry characteristics

Berry size was mainly modulated by both AMF inoculation and irrigation in CL-260 and CL-1089 (Table 1). Moreover, a significant interaction between irrigation and temperature was highlighted for these clones (T×I, $P \leq 0.01$ and $P \leq 0.05$, respectively), showing that the LD treatment resulted in small berries when plants grew at 28/18°C. Our findings are in contrast with those presented by Bonada et al. (2015), which showed additive effects (i.e. lack of interaction) between temperature and water for berry mass. In our study, ED was the treatment that most reduced berry mass regardless of temperature and clone, which agrees with previous studies in Tempranillo (Girona et al., 2009; Santesteban et al., 2011; Intrigliolo et al., 2012; Niculcea et al., 2014; Torres et al., 2017). On the other hand, relative skin mass in CL-

260 and CL-843 was affected by the three factors applied (T×M×I, P≤0.001 for CL-260 and P≤0.01 for CL-843) (Table 1). In these clones, AMF inoculation and/or elevated temperature accentuated the ability of the ED treatment to increase relative skin mass (Table S3), which could be due to a reduction in berry flesh mass (Roby and Matthews, 2004; Bonada et al., 2015). In CL-1089, temperature had no effect on relative skin mass, with this parameter being mainly modulated by AMF inoculation (P≤0.05) and irrigation (P≤0.001). The increase of relative skin mass could be an interesting response under climate change conditions because flavour and colour compounds, which determine wine quality, are located principally in the berry skin. On that basis, at 28/18°C, berries from 28ED (CL-260 and CL-843) and +M28 (CL-843) treatments will be subject to less dilution of skin compounds during winemaking (Table 1).

3.4. Berry quality

Some studies have observed great phenotypic plasticity in berry composition between grapevine varieties in response to warm temperatures (Sadras et al., 2013; Barnuud et al., 2014) and to water deficit irrigation (Basile et al., 2011; Niculcea et al., 2014). Our previous research provided evidence for clonal diversity within Tempranillo that resulted in different abilities to respond to AMF inoculation (Torres et al., 2016) or to the irrigation schedule applied (Torres et al., 2017) under elevated temperatures. Data from the current study showed that interaction between three factors (temperature, irrigation and AMF inoculation) modulated fruit composition to a different extent in each clone. Zarrouk et al. (2016) demonstrated that the interaction between irrigation regime and high temperature controls berry ripening in Tempranillo. Similarly, in CL-260, temperature and irrigation were the main factors modulating berry quality parameters (Table 2). At 28/18°C, total soluble solids were increased in the water deficit treatments (T×I, P<0.001), which was explained by the fact that faster accumulation of sugars at warm temperatures may be intensified under water deficit conditions (Bonada et al., 2015). The ED treatment caused the most significant changes in must pH and titratable acidity whereas malic acid was affected by all treatments and growth conditions. This pattern was underlined by a significant interaction among factors (T×M×I, P≤0.01). One of the clearest relationships between temperature and fruit quality occurs with grape berry acidity, whereby high temperatures reduce the concentration and/or increase the breakdown of malic acid. Our data coincide previous studies that showed that warm temperatures produced malate losses in Shiraz (Sweetman et al., 2014), Cabernet Sauvignon and Chardonnay (Barnuud et al., 2014). This has been related to an increased NAD-dependent

malic enzyme activity and decreased phosphoenolpyruvate carboxylase and pyruvate kinase activities (Sweetman et al., 2014). Furthermore, total polyphenol index (TPI) and anthocyanin content of CL-260 were also significantly modulated by all factors (Table 2). Thus, TPI tended to be low in response to elevated temperatures, AMF inoculation or ED irrigation, and total anthocyanins decreased in plants grown at 28/18°C. The decline of anthocyanins under water stress and elevated temperature likely results from the repression of anthocyanin biosynthesis at the onset of ripening and by a high degradation rate at later stages of berry ripening (Bonada et al., 2015; Zarrouk et al., 2016). However, this effect was clearly mitigated in +M28-LD plants, which achieved higher anthocyanins than –M28-LD plants (Table S4). This finding indicates that the beneficial effects produced by AMF inoculation on berry phenolic content under warming conditions (Torres et al., 2016) was higher under LD in comparison to ED, obtaining wines with improved nutritional and nutraceutical value (Gabriele et al., 2016). Similarly, Baslam and Goicoechea (2012) reported that AMF colonization improved the accumulation of anthocyanins in leaves of lettuce especially under water deficit conditions, which may be due to ability of AMF to stimulate the expression of key genes of the phenylpropanoid pathway (Bruisson et al., 2016).

In CL-1089, total soluble solids, must pH and malic acid were modulated by the three factors and/or their interactions, but titratable acidity was only affected by elevated temperatures (Table 3). At 24/14°C, total soluble solids were reduced by the ED treatment, but such differences disappeared at 28/18°C (T×I, P≤0.01). Malic acid decreased in response to elevated temperatures and AMF inoculation under ED and LD, which underlines the interactive effects of all factors on berry quality (T×M×I, P≤0.05) (Bonada et al., 2015). Furthermore, TPI was significantly modified by the combination of different factors (T×M, P≤0.01, T×I, P≤0.05, and M×I, P≤0.01) (Table 3). In general, TPI diminished in response to AMF inoculation or ED irrigation, but interestingly, the deleterious effect of the –M-ED treatment did not appear in +M-ED plants. Furthermore, total anthocyanin content was significantly modulated by the interaction between all factors (T×M×I, P≤0.05). At 24/14°C, AMF inoculation and/or the ED treatment reduced the content of anthocyanins in berries. However, such differences were alleviated at warm temperatures (Table 3) which is in agreement with our previous observations showing that CL-1089 was quite tolerant of elevated temperatures (Torres et al., 2016). Interestingly, although in the study of Torres et al. (2016) AMF colonization deteriorated some aspects of berry quality of CL-1089, the current study shows that the irrigation schedule may modify this pattern. Thus, the loss of anthocyanins detected in –M28-LD plants in comparison with –M24-LD plants did not occur in +M-LD plants when cultivated at

28/18°C instead of 24/14°C. This observation highlights the potential benefit of AMF inoculation on phenolic content maintenance under LD conditions applied to CL-1089 (Table S5).

In CL-843, the berry quality parameters were significantly modified by warm temperatures and ED irrigation, as indicated by increased concentration of total soluble sugars, must pH and decreased titratable acidity (T×I, P≤0.001, P≤0.01, and P≤0.01, respectively) (Table 4). In contrast to other clones, the content of malic acid was modulated by mycorrhization and irrigation but not by temperature, which highlights a clonal diversity within the Tempranillo variety in malate metabolism under warm temperatures. Similarly, Sadras et al. (2013) observed great phenotypic plasticity in the responsiveness for some traits such as titratable acidity and must pH. Furthermore, all factors contributed to changes in TPI and anthocyanins as highlighted by the interaction between them (T×M×I, P≤0.01 for TPI and P≤0.001 for anthocyanins). Regarding TPI, data showed that the +M28-LD treatment performed better than the +M28-ED treatment (Table S6). With regard to anthocyanins accumulated in berries of plants grown at 24/14°C, both water restriction regimes (ED and LD) resulted in decreased anthocyanin contents in comparison with levels found in FI plants (-M24-FI or +M24-FI), this effect being more pronounced in –M than in +M plants (Table S6). In general, anthocyanin content was reduced under elevated temperatures but the impact was also lower in +M than in –M plants., In fact, in CL-843, the loss of anthocyanins detected in –M28-LD plants was not found in +M28-LD plants, in comparison with –M24-LD and +M24LD respectively. This finding supports the notion that benefits of AMF under elevated temperature on berry composition (Torres et al., 2016) could be accentuated in combination with water deficit.

3.5. Antioxidant capacity

Figure 4 shows the results obtained from the DPPH assay performed in must in order to test its total antioxidant capacity. In CL-260 and CL-1089, all factors accounted for modifying total antioxidant capacity of berry extracts (T×M×I, P≤0.05). In CL-260, there were small changes in total antioxidant capacity, and only + +M28-FI plants exhibited a significant reduction in this parameter under warm temperatures. However, in CL-1089, AMF inoculation exerted a positive effect on antioxidant capacity, which was especially accentuated in +M28-FI and +M28-ED treatments. In CL-843, total antioxidant capacity was modulated by the interaction between temperature and mycorrhization (T×M, P≤0.001). Our data also revealed that elevated temperatures reduced total antioxidant capacity in –M28 plants but it was

clearly improved in +M28 plants regardless irrigation regime applied (Figure 4). These findings suggest that in this clone, the combination of AMF inoculation and elevated temperatures exerted an additive effect in improving antioxidant power (Maya and Matsubara, 2013; Torres et al., 2016).

3.6. Principal component analysis

Figure 5A shows the score plot obtained by PCA where samples of each clone are grouped in the plot of the first and second principal components: PC1 versus PC2. Here, PC1 accounted for about 27.66% of the total variance while PC2 covered 20.30%. Different Tempranillo clones and AMF inoculated or uninoculated plants could not be clearly distinguished (Figure 5A). However a clear distinction between plants subjected to different irrigation treatments was shown across PC1. On the other hand, temperature treatments were separated along PC2. These findings reveal that, in our experimental model, abiotic factors (air temperature and water regime) were more determinant than biotic factors (Tempranillo clones and mycorrhization) for berry quality. However, the selection of a given clone of Tempranillo and/or the application of the mycorrhizal inoculum under specific environmental conditions modulated some concrete aspects related to characteristics (Table 1) and quality (Figure 4, Tables 2, 3 and 4) of berries, which was demonstrated by the significant interactions between the main factors reflected in the results of ANOVA.

The loading plot (Figure 5B) highlighted the importance of Ψ_{pd} E-L35, TPI, anthocyanin, pH and acidity levels in explaining variance across PC1. Separation between temperature treatments was related to Ψ_{pd} at the final stages of berry ripening (E-L37 and E-L38), the days between veraison to berry maturity and total soluble solids. This analysis also showed that LD and FI appeared together under both temperature conditions, reinforcing the idea that LD irrigation could continue being valid under a projected warming scenario in order to maintain berry quality and antioxidant properties. With the aim to understand the different performance of biotic factors (clones and mycorrhization) in each temperature two more PCA were conducted (Figure 6). Figure 6A and 6B show the score and the loading plots generated at 24/14°C while Figure 6C and 6D correspond to 28/18°C conditions. At 24/14°C irrigation treatments were separated along PC1 which explained the 33.36% of the variance whereas PC2 covered 15.95%. Separation was due to the content of total anthocyanins, TPI, titratable acidity, Ψ_{pd} E-L34, pH, and the number of days between veraison and maturity. Results highlight the similarities between LD and FI at 24/14°C in the quality parameters and phenology data, but clones and mycorrhizal treatments were not clearly distinguished (Figure

6A). On the other hand, the two first principal components from data of warming conditions covered 27.39% and 14.95% of the total variance, respectively. ED was distinguished from FI and LD due to PC1, according to their Ψ_{pd} E-L34, Ψ_{pd} E-L35, total anthocyanins, titratable acidity and days from veraison to maturity. However, PC2 allowed establishing a difference between FI and LD treatments (Figure 6C) in relation with Ψ_{pd} E-L37, Ψ_{pd} E-L38 and the °Brix (Figure 6D). This result is not surprising due to the treatments were similar till berries reached E-L37 and this period matched with the sugar accumulation period which has been recently shown to be faster under water deficit and warming (Bonada et al., 2015). Additionally, the PC2 conducted at 28/18°C showed a slightly separation between –M and +M within each irrigation treatment (FI and LD) (Figure 6D), suggesting that the effect of AMF is more pronounced at 28/18°C. Under elevated air temperature and LD irrigation the association of Tempranillo grapevine with the mycorrhizal fungus *R. intraradices* favoured the antioxidant capacity of berries (Figure 4) in CL-843, and induced the accumulation of anthocyanins in berries of CL-260 (Table S4) and CL-843 (Table S6). Because Tempranillo clones responded differently to the inoculation with *R. intraradices* and responses varied according to the environmental conditions, we completely agree with Sinclair et al. (2014) when they concluded that it may be profitable to identify the AMF inoculants most suitable for a given cultivar in a given environment.

4. Conclusions

In a scenario of climate change, effective water management should accomplish the dual purpose of reducing water supply at certain phenological stages to improve grape quality traits while ensuring plant water status to counteract the deleterious effects of elevated temperature in water-stressed plants. The findings in this study indicate that AMF inoculation may alleviate the negative effects of water restriction and warming conditions in some clones of Tempranillo grapevines. In general, post-veraison (LD) water deficit performed better than pre-veraison (ED) water deficit but such differences were attenuated at elevated temperatures. Furthermore, at 28/18°C, the potential benefits of LD can be improved by AMF inoculation because the loss of anthocyanins detected in the non-mycorrhizal plants did not occur when plants were inoculated with AMF; this benefit, however, was dependent on Tempranillo intravarietal differences. Our results suggest that the implementation of measures to promote the association of grapevines with AMF could contribute to optimize effects of irrigation strategy on berry properties under future warming conditions. This research also offers the first evidence that the combined effect of water and temperature stress varied

depending on AMF inoculation and the clone studied. Future assays with different mycorrhizal inoculants may help to select the most adequate fungal species for benefiting yield and/or berry quality of each clone of Tempranillo cultivated under predicted warming scenarios.

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Table 1. Main effects and their interactions on berry characteristics from fruit-bearing cuttings of Tempranillo clones (CL-260, CL-1089, CL-843) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

	CL-260		CL-1089		CL-843	
	Berry mass (g FM berry^{-1})	Relative skin mass (% berry FM)	Berry mass (g FM berry^{-1})	Relative skin mass (% berry FM)	Berry mass (g FM berry^{-1})	Relative skin mass (% berry FM)
Main effects						
Temperature (T)						
24	0.99	30.6	1.00	28.1	0.89	29.5
28	0.86	29.5	0.90	28.1	0.99	28.4
Mycorrhization (M)						
-M	0.99	31.2	0.88 b	30.4 a	0.97	30.0
+M	0.87	28.9	1.03 a	25.8 b	0.91	27.9
Irrigation (I)						
FI	1.16	30.2	1.19	22.9 b	1.09 a	25.8
ED	0.54	36.6	0.47	35.4 a	0.52 b	36.7
LD	1.08	23.3	1.19	26.0 b	1.21 a	24.3
Two-factor interactions						
-M24	1.06	31.4	0.91	30.7	0.90	34.0 a
+M24	0.92	29.7	1.08	25.5	0.88	24.9 b
-M28	0.92	30.9	0.84	30.1	1.04	25.9 b
+M28	0.81	28.1	0.97	26.1	0.94	30.9 a
24FI	1.18 a	34.6 b	1.19 ab	23.1	1.06	28.3 b
24ED	0.51 c	33.2 b	0.46 c	33.9	0.55	33.7 b
24LD	1.29 a	23.8 c	1.35 a	27.3	1.06	26.5 bc
28FI	1.14 a	25.7 c	1.20 ab	22.6	1.12	23.3 c
28ED	0.58 c	40.1 a	0.48 c	37.0	0.50	39.7 a
28LD	0.88 b	22.7 c	1.03 b	24.7	1.36	22.2 c
-M-FI	1.20	34.5	1.10	23.7	1.03	27.3
-M-ED	0.62	35.0	0.47	36.7	0.64	39.3
-M-LD	1.15	24.1	1.06	30.7	1.24	23.3
+M-FI	1.12	25.9	1.29	22.0	1.15	24.3
+M-ED	0.46	38.3	0.47	34.2	0.40	34.1
+M-LD	1.01	22.5	1.32	21.3	1.18	25.3
ANOVA						
Temperature (T)	*	ns	ns	ns	ns	ns
Mycorrhization (M)	*	ns	*	*	ns	ns
Irrigation (I)	***	***	***	***	***	***
T×M	ns	ns	ns	ns	ns	***
T×I	**	**	*	ns	ns	*
M×I	ns	ns	ns	ns	ns	ns
T×M×I	ns	***	ns	ns	ns	**

Values represent means separated by Duncan's test (at P = 0.05). Within columns, means followed by different letters are significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, –M), irrigation (FI, ED, LD) and their interactions. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant (P > 0.05). FM indicates fresh matter.

Table 2. Main effects and their interactions on berry quality parameters from fruit-bearing cuttings of Tempranillo CL-260 inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

	Total soluble solids (°Brix)	Must pH	Titratable acidity (g L^{-1})	Malic acid (g L^{-1})	TPI (AU)	Anthocyanins (mg L^{-1})
Main effects						
Temperature (T)						
24	22.0	4.17	5.7 a	7.6	40.0	505.5
28	22.3	4.22	4.6 b	5.6	36.5	456.2
Mycorrhization (M)						
-M	22.0	4.23	5.0	6.6	40.8	484.9
+M	22.4	4.16	5.2	6.5	35.4	473.4
Irrigation (I)						
FI	21.1	3.97 b	5.8 a	6.5	44.4	615.3
ED	22.6	4.46 a	4.1 b	6.5	31.3	341.1
LD	22.8	4.16 b	5.5 a	6.8	41.3	530.9
Two-factor interactions						
-M24	22.0	4.20	5.5	7.1 b	40.5 a	550.9
+M24	22.0	4.10	5.9	8.1 a	39.6 a	467.7
-M28	22.0	4.20	4.6	6.2 b	41.0 a	437.8
+M28	22.7	4.20	4.6	5.0 c	30.9 b	479.7
24FI	21.8 b	4.00	6.3	7.0	48.9 a	713.4 a
24ED	22.4 b	4.50	4.6	8.1	29.0 b	287.9 c
24LD	21.8 b	4.10	6.2	7.8	49.5 a	660.2 a
28FI	20.4 c	3.90	5.4	6.0	41.1 a	541.7 b
28ED	22.8 ab	4.50	3.6	4.9	33.9 b	400.2 b
28LD	23.8 a	4.30	4.8	5.9	35.1 b	433.9 b
-M-FI	20.6	4.10	5.5	7.1 b	51.2 a	630.7
-M-ED	22.7	4.50	4.0	4.7 c	28.4 b	351.6
-M-LD	22.6	4.10	5.6	8.1 a	42.9 ab	472.5
+M-FI	21.6	3.80	6.2	5.9 c	35.5 b	594.9
+M-ED	22.5	4.50	4.2	8.3 a	33.4 b	333.4
+M-LD	23.0	4.20	5.3	5.5 c	39.1 b	608.7
ANOVA						
Temperature (T)	ns	ns	***	***	**	*
Mycorrhization (M)	ns	ns	ns	ns	*	ns
Irrigation (I)	***	***	***	ns	***	***
T×M	ns	ns	ns	**	*	ns
T×I	***	ns	ns	ns	***	***
M×I	ns	ns	ns	***	***	ns
T×M×I	ns	ns	ns	**	ns	*

Values represent means separated by Duncan's test (at P = 0.05). Within columns, means followed by different letters are significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, –M), irrigation (FI, ED, LD) and their interactions. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant (P > 0.05). AU indicates absorbance units.

Table 3. Main effects and their interactions on berry quality parameters from fruit-bearing cuttings of Tempranillo CL-1089 inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

	Total soluble solids (°Brix)	Must pH	Titratable acidity (g L^{-1})	Malic acid (g L^{-1})	TPI (AU)	Anthocyanins (mg L^{-1})
Main effects						
Temperature (T)						
24	21.0	4.15	5.7 a	6.4	36.7	443.8
28	22.6	4.37	3.7 b	4.9	45.1	459.1
Mycorrhization (M)						
-M	20.9	4.23	4.8	6.2	47.4	481.8
+M	22.7	4.30	4.6	5.0	33.9	420.2
Irrigation (I)						
FI	21.8	4.16	4.8	5.6	44.3	508.2
ED	20.4	4.36	4.3	5.2	35.4	285.7
LD	23.1	4.27	5.0	6.1	43.1	586.8
Two-factor interactions						
-M24	19.8	4.10	5.8	6.3 a	39.2 b	490.4
+M24	22.1	4.20	5.7	6.5 a	34.2 b	397.2
-M28	22.0	4.40	3.9	6.2 a	56.6 a	472.4
+M28	23.3	4.40	3.5	3.5 b	33.6 b	445.8
24FI	21.6 a	4.10 c	5.8	5.4 b	34.8 b	584.1 a
24ED	18.6 b	4.30 b	5.2	5.9 b	34.0 b	199.4 c
24LD	22.7 a	4.10 c	6.3	7.9 a	42.1 ab	629.3 a
28FI	22.1 a	4.20 bc	3.9	5.8 b	53.7 a	432.4 b
28ED	22.3 a	4.50 a	3.3	4.4 b	37.3 b	400.8 b
28LD	23.5 a	4.40 a	3.8	4.4 b	44.1 ab	544.3 a
-M-FI	20.6	4.10	5.4	5.6 b	57.9 a	572.0 b
-M-ED	20.1	4.40	4.2	5.6 b	38.1 bc	252.3 c
-M-LD	22.0	4.20	5.0	7.6 a	47.8 ab	659.6 a
+M-FI	23.1	4.20	4.4	5.6 b	30.7 c	444.5 b
+M-ED	20.8	4.40	4.4	4.7 b	32.8 c	319.2 c
+M-LD	24.2	4.30	5.1	4.6 b	38.5 bc	513.9 b
ANOVA						
Temperature (T)	***	***	***	***	***	ns
Mycorrhization (M)	***	*	ns	***	***	*
Irrigation (I)	***	***	ns	ns	**	***
T×M	ns	ns	ns	***	***	ns
T×I	**	*	ns	***	**	***
M×I	ns	ns	ns	**	***	*
T×M×I	ns	ns	ns	*	ns	*

Values represent means separated by Duncan's test (at P = 0.05). Within columns, means followed by different letters are significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, –M), irrigation (FI, ED, LD) and their interactions. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant (P > 0.05). AU indicates absorbance units.

Table 4. Main effects and their interactions on berry quality parameters from fruit-bearing cuttings of Tempranillo CL-843 inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

	Total soluble solids (°Brix)	Must pH	Titratable acidity (g L^{-1})	Malic acid (g L^{-1})	TPI (AU)	Anthocyanins (mg L^{-1})
Main effects						
Temperature (T)						
24	20.8	4.13	5.3	6.2	39.0	569.9
28	21.6	4.35	4.0	5.9	40.9	427.0
Mycorrhization (M)						
-M	21.2	4.25	4.6	6.9 a	40.6	509.2
+M	21.2	4.23	4.7	5.2 b	39.2	483.3
Irrigation (I)						
FI	21.5	4.13	5.0	6.1 b	49.0	619.4
ED	20.7	4.43	3.7	5.1 c	32.6	316.1
LD	21.4	4.16	5.3	7.0 a	40.0	599.3
Two-factor interactions						
-M24	19.94 c	4.10	5.2	6.8	36.8 b	581.1
+M24	21.61 ab	4.10	5.3	5.7	41.4 a	557.4
-M28	22.41 a	4.40	4.0	7.1	44.5 a	437.3
+M28	20.82 bc	4.30	4.1	4.8	37.2 b	416.7
24FI	21.37 ab	4.00 b	5.7 a	6.2	49.6	784.6 a
24ED	20.77 b	4.40 a	3.8 bc	4.8	30.5	304.6 d
24LD	20.20 b	4.00 b	6.3 a	7.7	38.3	664.6 b
28FI	21.67 ab	4.30 a	4.4 b	6.1	48.5	454.3 c
28ED	20.58 b	4.40 a	3.5 c	5.3	34.5	326.2 d
28LD	22.65 a	4.30 a	4.2 bc	6.4	41.7	534.0 b
-M-FI	21.48	4.20	4.9	6.7	51.1	678.6
-M-ED	20.95	4.40	3.7	6.2	30.6	305.0
-M-LD	21.10	4.20	5.2	7.8	43.4	612.1
+M-FI	21.50	4.10	5.1	5.5	47.0	560.3
+M-ED	20.40	4.50	3.6	3.9	34.8	328.9
+M-LD	21.75	4.10	5.3	6.3	36.6	586.5
ANOVA						
Temperature (T)	***	***	***	ns	ns	***
Mycorrhization (M)	ns	ns	ns	***	ns	ns
Irrigation (I)	*	***	***	***	***	***
T×M	***	ns	ns	ns	*	ns
T×I	***	**	**	ns	ns	***
M×I	ns	ns	ns	ns	ns	ns
T×M×I	ns	ns	ns	ns	**	***

Values represent means separated by Duncan's test (at P = 0.05). Within columns, means followed by different letters are significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, –M), irrigation (FI, ED, LD) and their interactions. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant (P > 0.05). AU indicates absorbance units.

Figure legends

Figure 1. Microscopic image (× 100) of fungal structures from *Rhizophagus intraradices* colonizing roots of inoculated grape plants. h = hyphae; v = vesicle.

Figure 2. Relative mycorrhizal dependency (RMD) for yield of fruit-bearing cuttings of Tempranillo clones grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation). Values represent means ± SE (n = 3). Data exceeding 100% indicate that mycorrhizal symbiosis improved yield. A three-way ANOVA analysis was performed to evaluate the effects of temperature (T), irrigation (I), clone (C) and their interaction. ns and *** indicate non-significance or significance at 0.1% probability levels, respectively. Histograms with different letter indicate that values differed significantly (P≤0.05).

Figure 3. Pre-dawn leaf water potential (Ψ_{pd}) recorded at different stages of berry ripening in fruit-bearing cuttings of Tempranillo clones inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/night) temperatures and subjected to different irrigation regimes (FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation). Values represent means ± SE (n = 3). A three-way ANOVA analysis was performed to evaluate the effects of temperature (T), mycorrhizal inoculation (M), irrigation (I) and their interactions. ns, *, **, and *** indicate non-significance or significance at 5%, 1%, and 0.1% probability levels, respectively.

Figure 4. Total antioxidant capacity of berries from fruit-bearing cuttings of Tempranillo clones inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/night) temperatures and subjected to different irrigation regimes (FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation). Values represent means ± SE (n = 3). A three-way ANOVA analysis was performed to evaluate the effects of temperature (T), mycorrhizal inoculation (M), irrigation (I) and their interactions. ns, *, **, and *** indicate non-significance or

significance at 5%, 1%, and 0.1% probability levels, respectively. When interaction between the main factors 'temperature, T', 'mycorrhizal inoculation, M' and 'irrigation, I' was significant, histograms with different letter indicate that values differed significantly (P≤0.05).

Figure 5. Principal component analysis score (A) and loading plot (B) obtained from the statistical analysis of plant and berry characteristics and total antioxidant capacity data of 72 studied samples from fruit-bearing cuttings of Tempranillo clones inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation).

Figure 6. Principal component analysis score (A, C) and loading plot (B, D) obtained from the statistical analysis of plant and berry characteristics and total antioxidant capacity data of 36 studied samples from fruit-bearing cuttings of Tempranillo clones grown at 24/14°C (day/ night) (A, B) or at 28/18°C (day/ night) (C, D), inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M) and subjected to different irrigation regimes (FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation).

Figure 1.

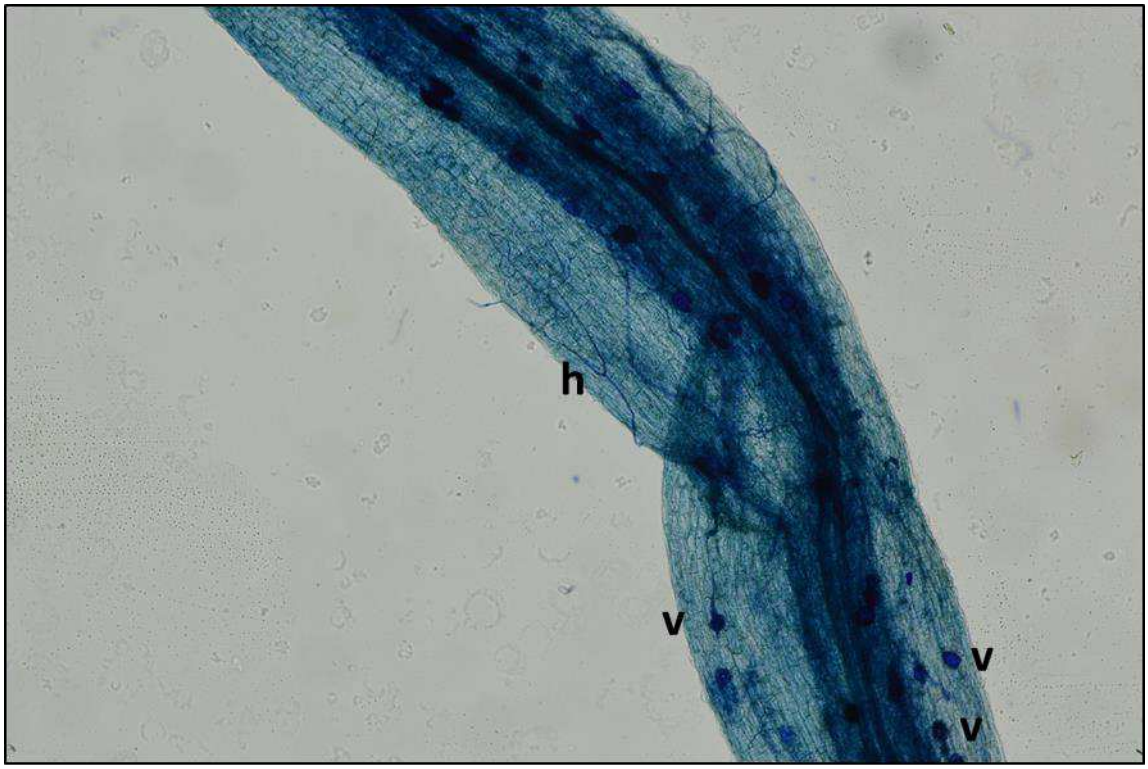


Figure 2

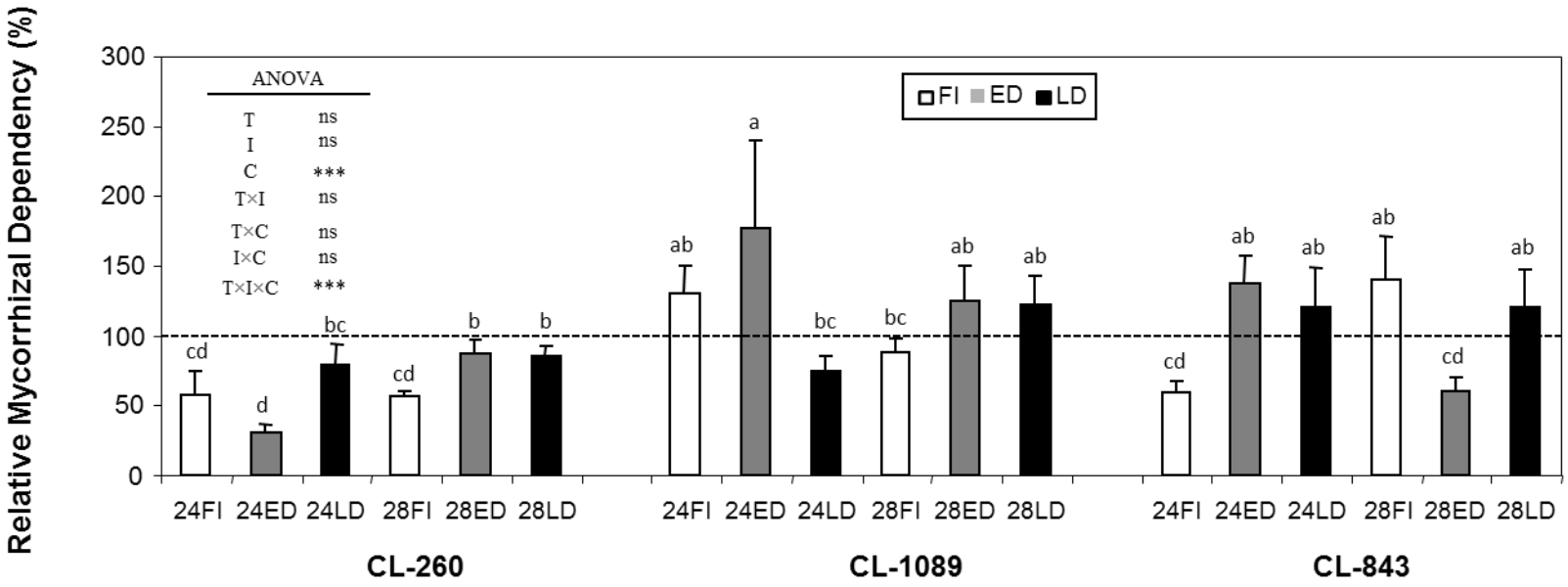

Relative Mycorrhizal Dependency (%)
300
250
200
150
100
50
0
ANOVA
T ns
I ns
C ***
T×I ns
T×C ns
I×C ns
T×I×C ***
FI ED LD
cd d bc cd b b
ab a bc bc ab ab
cd ab ab ab cd ab
24FI 24ED 24LD 28FI 28ED 28LD
CL-260
CL-1089
CL-843

Figure 3.

T	ns	ns	***	***	**	ns	*	***	***	**	ns	***	***	***	***
M	ns	ns	ns	**	**	ns	ns	ns	*	ns	ns	ns	ns	ns	ns
I	***	ns	ns	***	***	***	ns	ns	***	***	***	***	**	***	***
TxM	ns	ns	***	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	*	ns
TxI	ns	ns	*	ns	***	ns	ns	ns	ns	*	*	*	ns	ns	**
MxI	ns	ns	***	***	***	ns	*	ns	ns	ns	ns	ns	ns	ns	*
TxMxI	*	**	***	***	ns	ns	ns	ns	ns	ns	ns	***	ns	**	ns

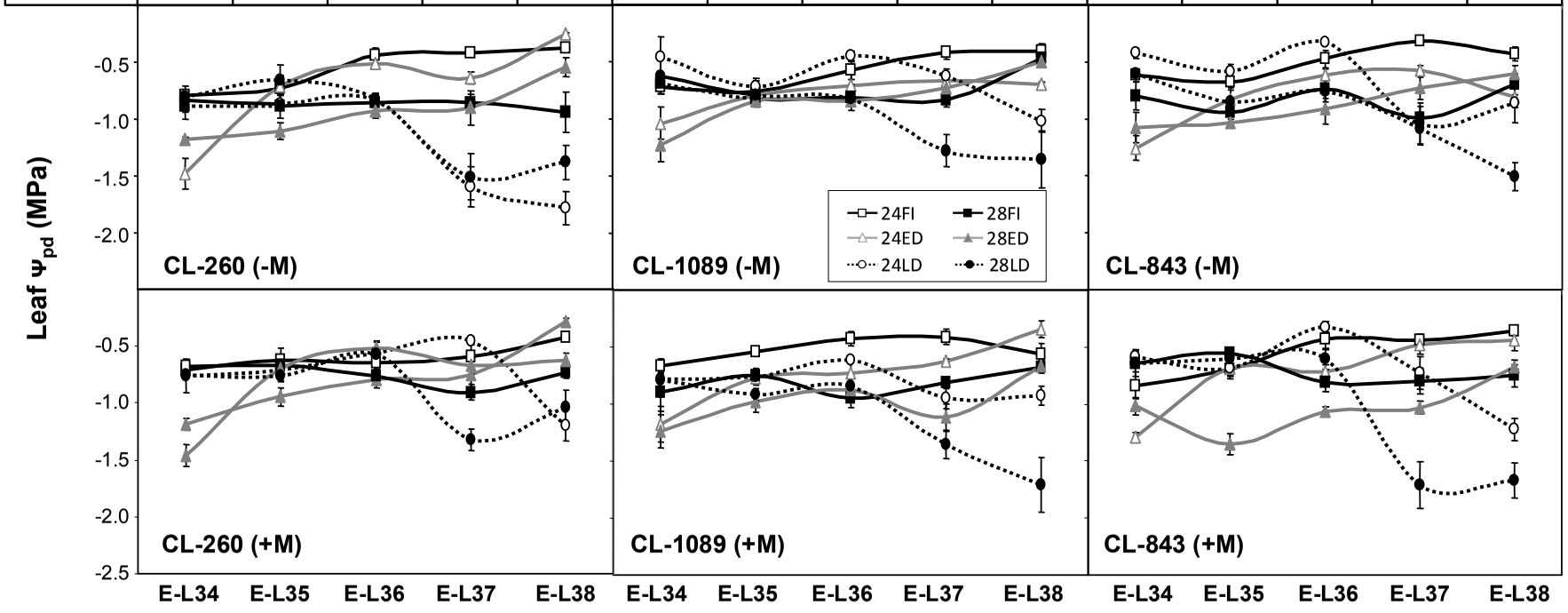
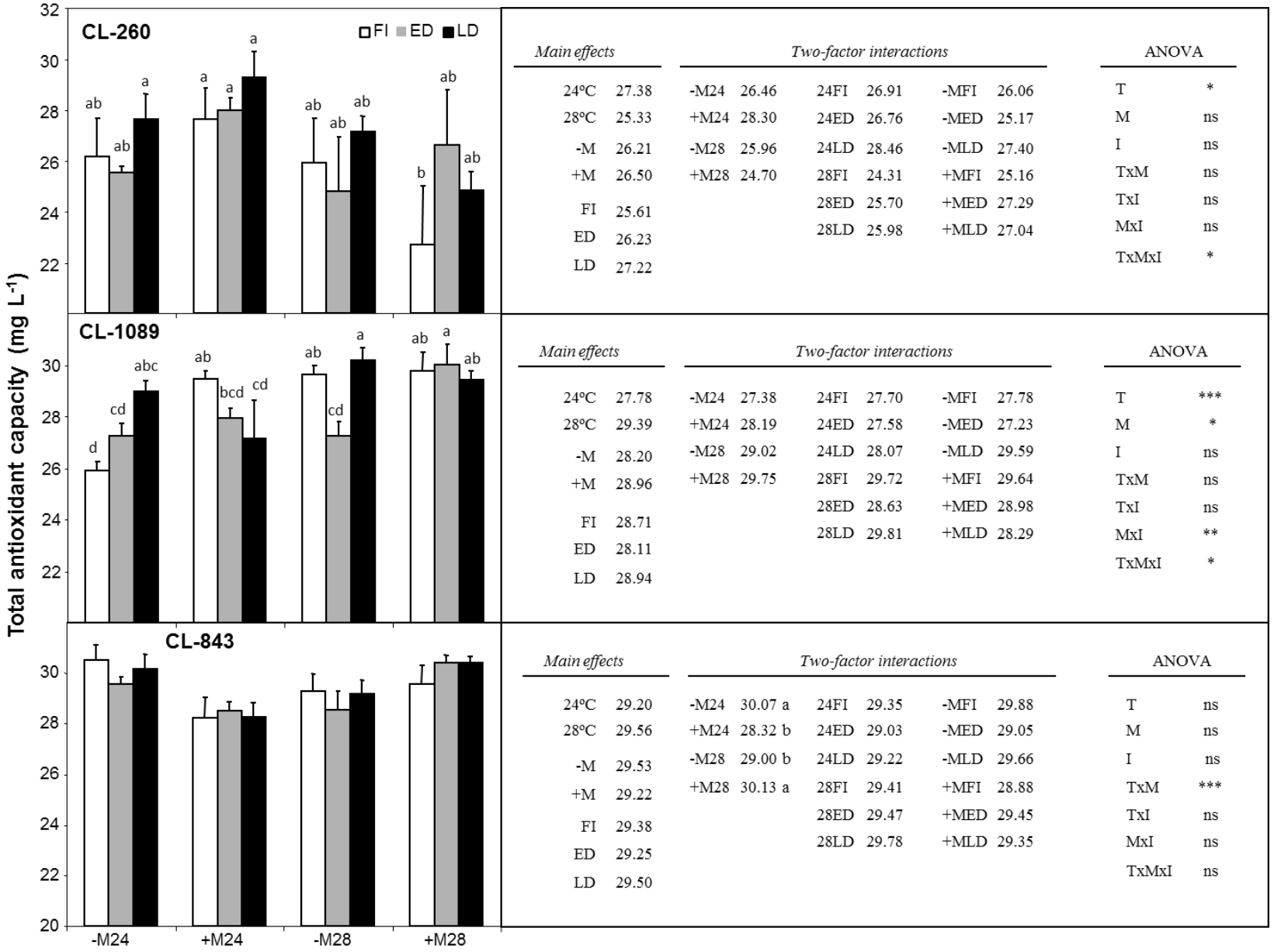


Figure 4.



CL-260

Main effects		*Two-factor interactions*				ANOVA	
24ºC	27.38	-M24	26.46	24FI	26.91	-MFI	26.06	T	*
28ºC	25.33	+M24	28.30	24ED	26.76	-MED	25.17	M	ns
-M	26.21	-M28	25.96	24LD	28.46	-MLD	27.40	I	ns
+M	26.50	+M28	24.70	28FI	24.31	+MFI	25.16	TxM	ns
FI	25.61			28ED	25.70	+MED	27.29	TxI	ns
ED	26.23			28LD	25.98	+MLD	27.04	MxI	ns
LD	27.22							TxMxI	*

CL-1089

Main effects		*Two-factor interactions*				ANOVA	
24ºC	27.78	-M24	27.38	24FI	27.70	-MFI	27.78	T	***
28ºC	29.39	+M24	28.19	24ED	27.58	-MED	27.23	M	*
-M	28.20	-M28	29.02	24LD	28.07	-MLD	29.59	I	ns
+M	28.96	+M28	29.75	28FI	29.72	+MFI	29.64	TxM	ns
FI	28.71			28ED	28.63	+MED	28.98	TxI	ns
ED	28.11			28LD	29.81	+MLD	28.29	MxI	**
LD	28.94							TxMxI	*

CL-843

Main effects		*Two-factor interactions*				ANOVA	
24ºC	29.20	-M24	30.07 a	24FI	29.35	-MFI	29.88	T	ns
28ºC	29.56	+M24	28.32 b	24ED	29.03	-MED	29.05	M	ns
-M	29.53	-M28	29.00 b	24LD	29.22	-MLD	29.66	I	ns
+M	29.22	+M28	30.13 a	28FI	29.41	+MFI	28.88	TxM	***
FI	29.38			28ED	29.47	+MED	29.45	TxI	ns
ED	29.25			28LD	29.78	+MLD	29.35	MxI	ns
LD	29.50							TxMxI	ns

Figure 5.

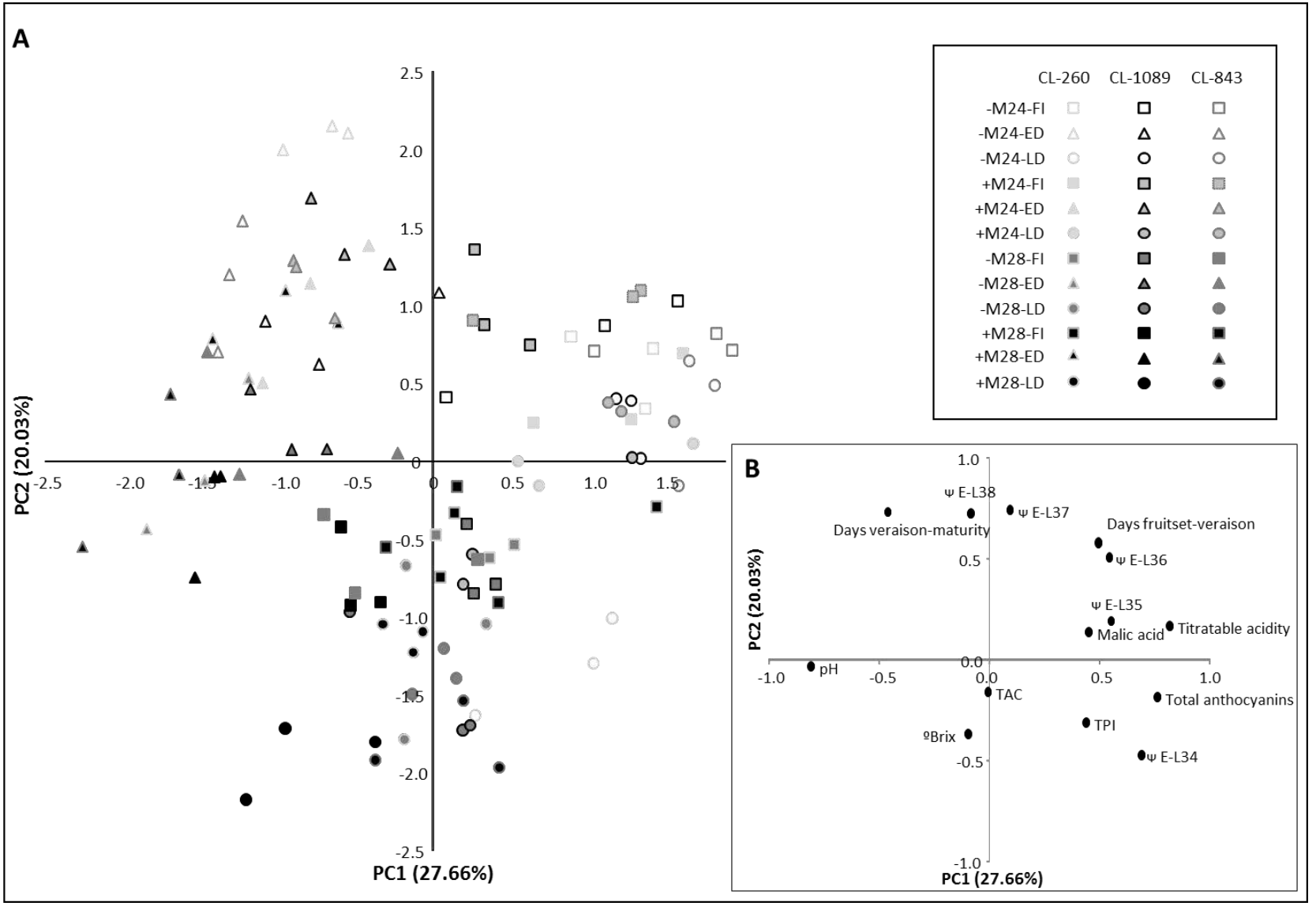
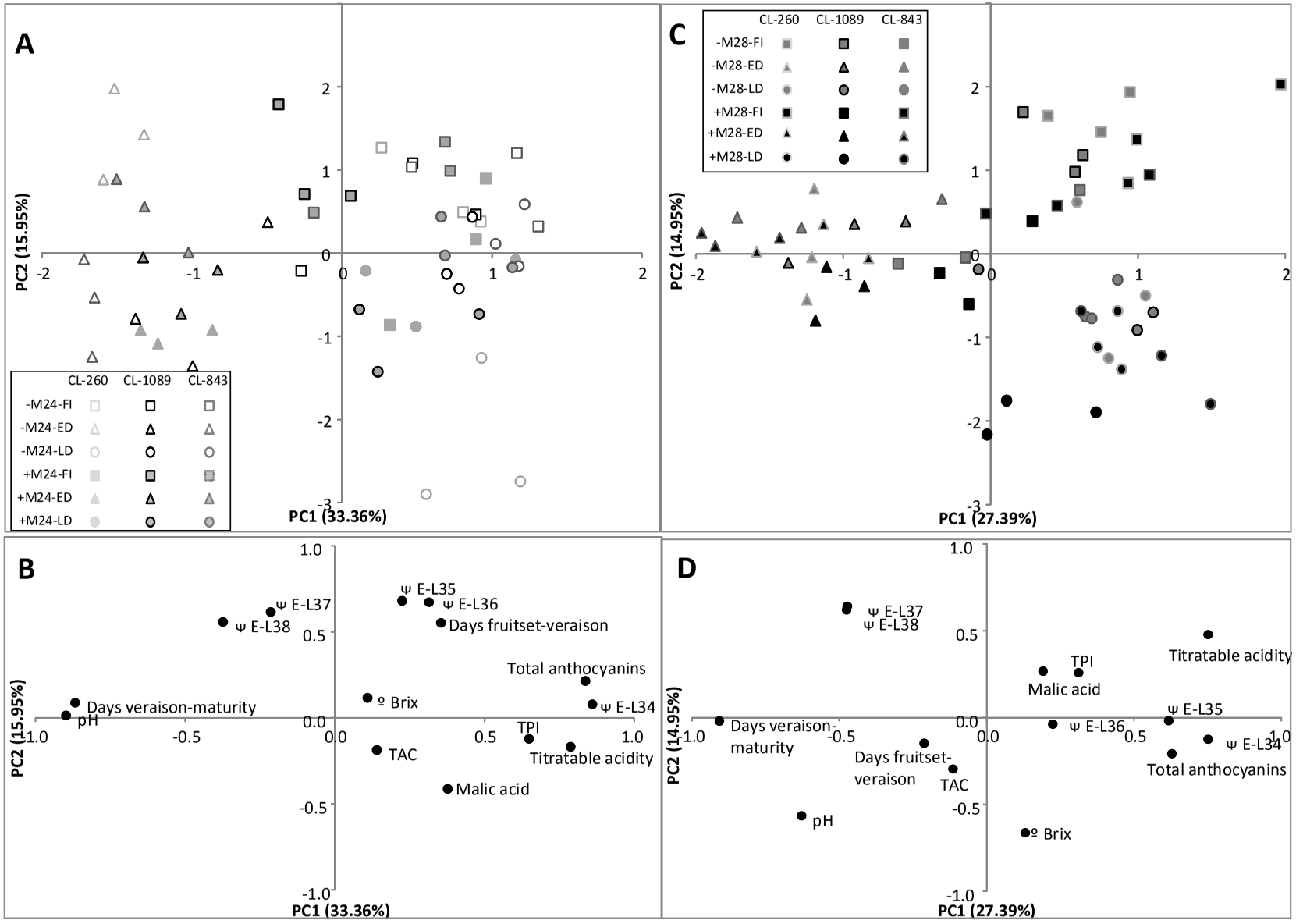

A
PC2 (20.03%)
PC1 (27.66%)
CL-260
CL-1089
CL-843
-M24-FI
-M24-ED
-M24-LD
+M24-FI
+M24-ED
+M24-LD
-M28-FI
-M28-ED
-M28-LD
+M28-FI
+M28-ED
+M28-LD
B
Ψ E-L38
Ψ E-L37
Days veraison-maturity
Days fruitset-veraison
Ψ E-L36
Ψ E-L35
Malic acid
Titratable acidity
pH
TAC
Total anthocyanins
TPI
ºBrix
Ψ E-L34
PC2 (20.03%)
PC1 (27.66%)

Figure 6.


A
PC2 (15.95%)
PC1 (33.36%)
CL-260
CL-1089
CL-843
-M24-FI
-M24-ED
-M24-LD
+M24-FI
+M24-ED
+M24-LD
C
PC2 (14.95%)
PC1 (27.39%)
CL-260
CL-1089
CL-843
-M28-FI
-M28-ED
-M28-LD
+M28-FI
+M28-ED
+M28-LD
B
PC2 (15.95%)
PC1 (33.36%)
Ψ E-L35
Ψ E-L37
Ψ E-L36
Ψ E-L38
Days fruitset-veraison
Total anthocyanins
º Brix
Ψ E-L34
Days veraison-maturity
pH
TPI
TAC
Titratable acidity
Malic acid
D
PC2 (14.95%)
PC1 (27.39%)
Ψ E-L37
Ψ E-L38
Titratable acidity
TPI
Malic acid
Ψ E-L35
Ψ E-L36
Ψ E-L34
Days veraison-maturity
Days fruitset-veraison
TAC
Total anthocyanins
pH
º Brix

Original research article

Influence of irrigation strategy and mycorrhizal inoculation on fruit quality in different clones of Tempranillo grown under elevated temperatures

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Table S1. Summary of the agronomic characteristics of the Tempranillo clones used in this study. Data provided by Institute of Sciences of Vine and Wine (Logroño, Spain) were collected and averaged over the 2009-2012 period from plants grown in the field.

Table S2. Phenology from fruit-bearing cuttings of Tempranillo clones (CL-260, CL-1089, CL-843) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

Table S3. Berry characteristics from fruit-bearing cuttings of Tempranillo clones (CL-260, CL-1089, CL-843) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

Table S4. Berry quality parameters from fruit-bearing cuttings of Tempranillo CL-260 inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different

irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

Table S5. Berry quality parameters from fruit-bearing cuttings of Tempranillo CL-1089 inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

Table S6. Berry quality parameters from fruit-bearing cuttings of Tempranillo CL-843 inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

Figure S1. Soil temperature recorded during a week each hour in eight pots subjected to different temperature treatments. Values represent means ± SE (n = 4).

Figure S2. Volumetric soil water content recorded daily from fruit set to maturity in fruit-bearing cuttings of Tempranillo clones grown at 24/14°C or 28/18°C (day/night) temperatures and subjected to different irrigation regimes (FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation). Values represent means ± SE (n = 18).

Table S1. Summary of the agronomic characteristics of the Tempranillo clones used in this study. Data provided by Institute of Sciences of Vine and Wine (Logroño, Spain) were collected and averaged over the 2009-2012 period from plants grown in the field.

	CL-260	CL-1089	CL-843
City of origin (region)	San Vicente de la Sonsierra (La Rioja)	Bargota (Navarra)	Oyón (Álava)
Agronomic classification			
Reproductive cycle	Short	Short	Long
Yield	Low	High	High
Reproductive cycle			
Fruit set-veraison (days)	56	52	61
Veraison-maturity (days)	30	33	56
Yield components			
Yield (kg vine^{-1})	10.37	21.91	12.65
Bunch mass (g bunch^{-1})	84	154	199
Berry mass (g)	1.24	2.05	1.50

Table S2. Phenology from fruit-bearing cuttings of Tempranillo clones (CL-260, CL-1089, CL-843) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

	CL-260		CL-1089		CL-843	
	Fruit set-veraison (days)	Veraison-maturity (days)	Fruit set-veraison (days)	Veraison-maturity (days)	Fruit set-veraison (days)	Veraison-maturity (days)
Treatments						
-M24-FI	46	49	43	60 cd	50	56
-M24-ED	47	92	38	70 bc	38	100
-M24-LD	42	46	45	60 cd	52	57
+M24-FI	42	51	46	76 b	52	66
+M24-ED	40	82	40	98 a	43	91
+M24-LD	36	65	38	54 de	44	55
-M28-FI	34	32	34	42 ef	35	50
-M28-ED	34	74	37	66 bcd	38	76
-M28-LD	34	43	32	46 ef	34	41
+M28-FI	28	37	34	38 f	33	50
+M28-ED	39	71	34	63 bcd	33	81
+M28-LD	36	40	32	45 ef	34	37
Main effects						
Temperature (T)						
24	42 a	64 a	42 a	70	47	71 a
28	34 b	49 b	34 b	50	34	56 b
Mycorrhization (M)						
-M	39	56	38	57	41	63
+M	37	58	37	62	40	63
Irrigation (I)						
FI	37	42 b	39	54	43	55 b
ED	40	80 a	37	74	38	87 a
LD	37	48 b	37	51	41	48 b
Two-factor interactions						
-M24	45	62	42	63	47	71
+M24	43	66	41	76	47	71
-M28	34	49	34	51	35	56
+M28	34	49	33	49	33	56
24FI	44	50	45	68	51 a	61
24ED	44	87	39	84	41 b	96
24LD	39	55	42	57	48 a	56
28FI	31	34	34	40	34 b	50
28ED	36	72	35	64	35 b	79
28LD	35	41	32	45	34 b	39
-M-FI	40	40	39	51	43	53
-M-ED	41	83	37	68	38	88
-M-LD	38	44	39	53	43	49
+M-FI	35	44	40	57	43	58
+M-ED	39	77	37	80	38	86

+M-LD	36	52	35	50	39	46
ANOVA						
Temperature (T)	***	***	***	***	***	***
Mycorrhization (M)	ns	ns	ns	*	ns	ns
Irrigation (I)	ns	***	ns	***	ns	***
T×M	ns	ns	ns	*	ns	ns
T×I	ns	ns	ns	*	*	ns
M×I	ns	ns	ns	ns	ns	ns
T×M×I	ns	ns	ns	*	ns	ns

Values represent means (n = 3) separated by Duncan's test (at P = 0.05). Within columns, means followed by different letters are significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, –M), irrigation (FI, ED, LD) and their interactions. *P < 0.05; ***P < 0.001; ns, not significant (P > 0.05).

Table S3. Berry characteristics from fruit-bearing cuttings of Tempranillo clones (CL-260, CL-1089, CL-843) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

	CL-260		CL-1089		CL-843	
	Berry mass (g FM berry^{-1})	Relative skin mass (% berry FM)	Berry mass (g FM berry^{-1})	Relative skin mass (% berry FM)	Berry mass (g FM berry^{-1})	Relative skin mass (% berry FM)
Treatments						
-M24-FI	1.16	45.9 a	1.08	20.8	1.06	33.2 b
-M24-ED	0.59	25.9 bc	0.47	36.6	0.63	43.9 a
-M24-LD	1.44	22.5 c	1.19	34.8	1.01	25.1 c
+M24-FI	1.20	23.3 c	1.31	25.4	1.06	23.4 c
+M24-ED	0.42	40.6 a	0.44	31.2	0.47	23.5 c
+M24-LD	1.13	25.2 bc	1.51	19.8	1.11	27.8 c
-M28-FI	1.24	23.1 c	1.13	26.6	1.00	21.5 c
-M28-ED	0.65	44.1 a	0.46	36.9	0.65	34.6 b
-M28-LD	0.86	25.7 c	0.93	26.7	1.46	21.6 c
+M28-FI	1.03	28.4 bc	1.27	18.6	1.24	25.1 c
+M28-ED	0.50	36.1 ab	0.50	37.2	0.34	44.7 a
+M28-LD	0.89	19.7 c	1.13	22.7	1.25	22.8 c

Values represent means (n = 3). When interaction between the main factors 'temperature, T', 'mycorrhizal inoculation, M' and 'irrigation, I' was significant, histograms with different letter indicate that values differed significantly (P≤0.05). FM indicates fresh matter.

Table S4. Berry quality parameters from fruit-bearing cuttings of Tempranillo CL-260 inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

	Total soluble solids (°Brix)	Must pH	Titratable acidity (g L^{-1})	Malic acid (g L^{-1})	TPI (AU)	Anthocyanins (mg L^{-1})
Treatments						
-M24-FI	21.7	4.11	5.9	6.6 b	53.7	823.9 a
-M24-ED	22.9	4.47	4.3	6.6 b	21.3	268.0 e
-M24-LD	21.4	4.06	6.2	8.2 ab	53.0	655.1 ab
+M24-FI	22.0	3.90	6.6	7.5 b	44.2	602.9 b
+M24-ED	21.8	4.43	4.8	9.6 a	34.1	301.2 e
+M24-LD	22.3	4.04	6.2	7.3 b	46.1	665.3 ab
-M28-FI	19.6	4.09	5.1	7.7 ab	49.7	514.7 bc
-M28-ED	22.5	4.44	6.7	2.7 c	35.5	435.3 cd
-M28-LD	23.8	4.19	5.0	8.1 ab	36.8	362.9 d
+M28-FI	21.2	3.77	5.7	4.3 c	26.7	586.8 bc
+M28-ED	23.1	4.49	3.5	7.0 b	32.6	372.1 d
+M28-LD	23.7	4.35	4.5	3.7 c	32.1	552.1 bc

Values represent means (n = 3). When interaction between the main factors 'temperature, T', 'mycorrhizal inoculation, M' and 'irrigation, I' was significant, histograms with different letter indicate that values differed significantly ($P \leq 0.05$). FM indicates fresh matter. AU indicates absorbance units.

Table S5. Berry quality parameters from fruit-bearing cuttings of Tempranillo CL-1089 inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

	Total soluble solids (°Brix)	Must pH	Titratable acidity (g L^{-1})	Malic acid (g L^{-1})	TPI (AU)	Anthocyanins (mg L^{-1})
Treatments						
-M24-FI	20.6	3.98	6.3	5.4 bc	43.1	676.7 ab
-M24-ED	17.7	4.24	5.1	5.1 c	32.6	134.3 d
-M24-LD	21.3	4.10	5.9	8.4 a	45.0	778.8 a
+M24-FI	22.5	4.24	5.3	5.4 bc	27.5	491.4 b
+M24-ED	19.5	4.26	5.3	6.7 b	35.4	264.5 d
+M24-LD	24.1	4.12	6.6	7.3 ab	39.3	479.8 bc
-M28-FI	20.7	4.16	4.4	5.7 bc	73.6	467.3 bc
-M28-ED	22.5	4.51	3.2	6.1 b	45.5	409.3 c
-M28-LD	22.7	4.40	4.0	6.8 b	50.6	540.4 b
+M28-FI	23.6	4.25	3.4	5.8 bc	33.8	397.4 c
+M28-ED	22.1	4.44	3.4	2.8 d	29.2	392.0 c
+M28-LD	24.3	4.49	3.6	1.9 d	37.7	548.0 b

Values represent means (n = 3). When interaction between the main factors 'temperature, T', 'mycorrhizal inoculation, M' and 'irrigation, I' was significant, histograms with different letter indicate that values differed significantly ($P \leq 0.05$). FM indicates fresh matter. AU indicates absorbance units. AU indicates absorbance units.

Table S6. Berry quality parameters from fruit-bearing cuttings of Tempranillo CL-843 inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C or 28/18°C (day/ night) temperatures and subjected to different irrigation regimes (FI: full irrigation; ED, early season water deficit; LD, late season water deficit).

	Total soluble solids (°Brix)	Must pH	Titratable acidity (g L^{-1})	Malic acid (g L^{-1})	TPI (AU)	Anthocyanins (mg L^{-1})
Treatments						
-M24-FI	20.6	3.94	5.9	6.4	48.9 ab	936.0 a
-M24-ED	20.5	4.42	3.8	6.1	22.1 c	196.7 e
-M24-LD	18.7	4.03	6.0	7.8	44.2 ab	738.8 b
+M24-FI	22.1	4.01	5.6	5.9	50.2 a	633.2 b
+M24-ED	21.0	4.41	3.8	3.6	41.7 b	448.6 cd
+M24-LD	21.7	3.97	6.6	7.6	32.4 bc	590.3 bc
-M28-FI	22.3	4.40	4.0	7.0	53.3 a	421.2 d
-M28-ED	21.4	4.37	3.6	6.4	39.2 bc	413.3 d
-M28-LD	23.5	4.35	4.4	7.7	42.7 b	485.3 cd
+M28-FI	20.9	1.20	4.7	5.0	43.7 b	487.4 cd
+M28-ED	19.8	4.51	3.4	4.3	29.7 c	239.1 e
+M28-LD	21.8	4.29	4.0	5.0	40.8 b	582.8 bc

Values represent means (n = 3). When interaction between the main factors 'temperature, T', 'mycorrhizal inoculation, M' and 'irrigation, I' was significant, histograms with different letter indicate that values differed significantly (P≤0.05). FM indicates fresh matter. AU indicates absorbance units.

Figure S1. Soil temperature recorded during a week each hour in eight pots subjected to different temperature treatments. Values represent means ± SE (n = 4).

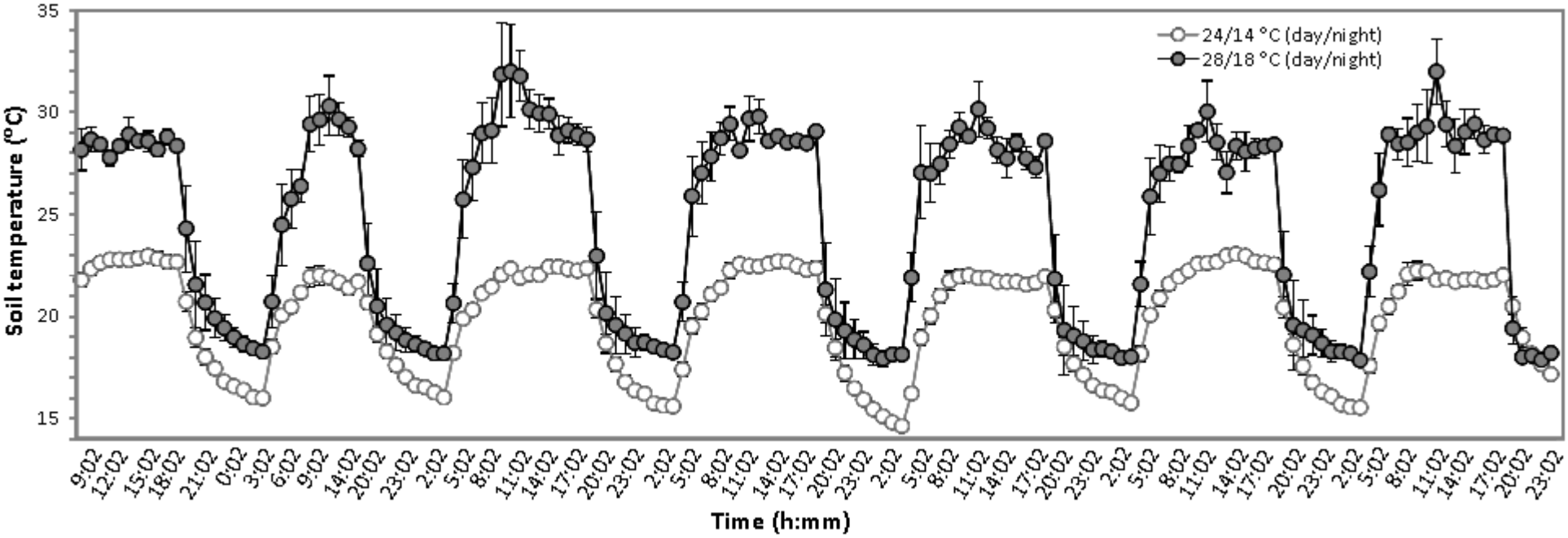
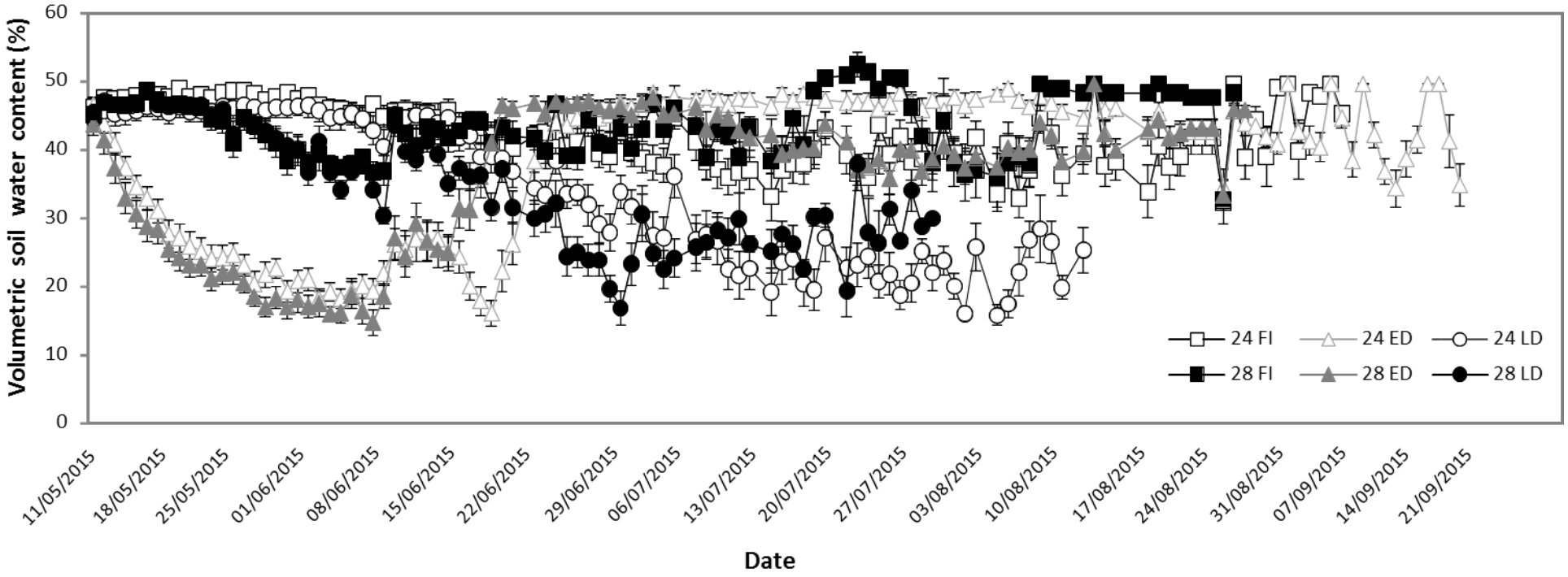


Figure S2. Volumetric soil water content recorded daily from fruit set to maturity in fruit-bearing cuttings of Tempranillo clones grown at 24/14°C or 28/18°C (day/night) temperatures and subjected to different irrigation regimes (FI, full irrigation; ED, early season deficit irrigation; LD, late season deficit irrigation). Values represent means ± SE (n = 18).



CAPÍTULO 4

Mycorrhizal symbiosis affects ABA metabolism during berry ripening in *Vitis vinifera* L. cv. Tempranillo grown under climate change scenarios. Published in *Plant Science* (doi: 10.1016/j.plantsci.2018.06.009)

Mycorrhizal symbiosis affects ABA metabolism during berry ripening in *Vitis vinifera* L. cv. Tempranillo grown under climate change scenarios

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Abstract

Arbuscular mycorrhizal symbiosis is a promising tool for improving the quality of grapes under changing environments. Therefore, the aim of this research was to determine if the ability of arbuscular mycorrhizal fungi (AMF) to enhance phenolic content (specifically, anthocyanins) in a climate change framework could be mediated by alterations in berry ABA metabolism during ripening. The study was carried out on fruit-bearing cuttings of cv. Tempranillo (CL-1048 and CL-1089) inoculated (+M) or not (−M) with AMF. Two experimental designs were implemented. In the first experiment +M and -M plants were subjected to two temperatures (24/14°C or 28/18°C (day/night)) from fruit set to berry maturity. In the second experiment, +M and -M plants were subjected to two temperatures (24/14°C or 28/18°C (day/night)) combined with two irrigation regimes (late water deficit (LD) and full irrigation (FI)). At 28/18°C AMF contributed to an increase in berry anthocyanins and modulated ABA metabolism, leading to higher ABA-GE and 7'OH-ABA and lower phaseic acid (PA) in berries compared to –M plants. Under the most stressful scenario (LD and 28/18°C), at harvest +M plants exhibited higher berry anthocyanins and 7´OH-ABA and lower PA and dihydrophaseic acid (DPA) levels than –M plants. These findings highlight the involvement of ABA metabolism into the ability of AMF to improve some traits involved in the quality of grapes under global warming scenarios.

***Keywords**: Abscisic acid; anthocyanins; arbuscular mycorrhizal fungi; restricted irrigation; global warming; grapevines*

1. Introduction

Global warming is expected to reduce food production in the future. Viticulture is one of those sectors most sensitive to both short- and long-term climate changes due to the narrow cultivation niches of vines [1]. Climate scenarios for South Mediterranean Europe predict an increase in temperature, alterations in rainfall patterns and an increasing frequency of extreme climate events, all of which will negatively affect viticulture in the region [2, 3]. In spite of accounting for 14% of the surface area of vineyards in the world, the Spanish surface area has fallen from 1196 kha in 1995 to 975 kha in 2016 and in this context future projections are unlikely to be positive without adaptation. Almost 90% of the total Spanish grape production is used to produce wine, with a production of 33.5 MhL in 2017, which represents a reduction of 5.8% with respect to 2016 [4]. Among red wine varieties, Tempranillo is one of the dominant varieties in Spain, where it accounts for 21% of the total Spanish vineyard surface [4]. The Tempranillo variety is characterized by early ripening with a short vegetative cycle. Although these traits are not relevant for coping with climate change, it is difficult to change the established grapevine cultivars in a specific region because of the narrow dependency on consumer preferences which are often linked to a certain particular wine taste [5]. In this context, producers and markets are clearly aware of the risks and opportunities of climate change and demand information on future choices [6].

Climate change affects winemaking because it reduces grapevine yields and modifies berry composition due to its great impact on berry growth and ripening [7]. Among the most important warming-related effects on grapevines are the significant advance in phenology (i.e., budburst, flowering and veraison dates) [8, 9], increases in berry sugar concentrations that lead to high wine alcohol levels, lower acidity levels, delays in the synthesis of phenolic compounds [10] and changes in berry skin metabolite profiles [11-13]. In addition to temperature, the predicted reductions in rainfall imply that vines may require supplemental irrigation to limit water deficit stress during the grapevine growing season [14]. As a result, different irrigation programs have been implemented in South Mediterranean areas, which allow the control of vegetative development and reduction in berry size, improvement of the cluster microclimate, increases in water use efficiency, and the enhancement of the sugar and phenolic content of berries [15-18].

Within the climate change scenario, new strategies are crucial to maintaining grape quality under the future environmental constraints. Maintaining soil quality in order to improve the

beneficial relationships between plants and arbuscular mycorrhizal fungi (AMF) could be a suitable option. Considerable progress has been made in the last decade in the use of AMF to improve plant growth and yield for crop plants in general, but also for grapevines, particularly as AMF symbiosis plays a major role in the survival of grapevines in their natural habitats [19]. Thus, the adaptation of viticulture to climate change may benefit from AMF since root colonization increases grapevine growth and nutrition, tolerance to abiotic stresses, protects against biotic stresses, and increases soil stability [20]. Furthermore, AMF colonization induces changes in plant secondary metabolism leading to enhanced biosynthesis of polyphenols, carotenoids or flavonoids [21-25].

Phytohormone abscisic acid (ABA) has been considered as the main mediator of grapevine response to abiotic stress, such as water deficit [26] or elevated temperature [27, 28]. Furthermore, ABA plays a crucial role in grape berry development and ripening [29, 30]. Thus, the accumulation of ABA around veraison is accompanied by sugar accumulation, colour development, and berry softening, suggesting that ABA may play a major role in controlling several ripening-associated processes [30-33]. Nevertheless, a lack of correlation between free-ABA in berries and 9-cis-epoxycarotenoid dioxygenase (NCED), the enzyme involved in the first step of ABA synthesis suggests that compounds derived from ABA catabolism/conjugation could also be involved in berry ripening [32, 34]. Active ABA can be metabolized in a variety of ways [35]. It can be conjugated to glucose forming the inactive ABA-glucose ester (ABA-GE), which is stored or transported [36] or alternatively, ABA can be catabolized by hydroxylation at the positions 7', 8' and 9'. Hydroxylation at the 7' position produces 7-hydroxy-ABA (7'OH-ABA) and at the 8' position phaseic acid (PA) and subsequently dihydrophaseic acid (DPA). Recent research has shown that ABA concentrations and catabolites were also regulated by the intensity and/or timing of water deficit [31, 37, 38] and temperature [39].

Plant hormones also interact to regulate the establishment and functioning of symbiotic associations with AMF [40]. Specifically, it has been demonstrated that ABA is essential for root colonization and for the functionality of the fungal structures [41]. In view of the role of ABA in the regulation of some berry ripening processes, and given that ABA concentration is enhanced by AMF in leaves, especially under abiotic stress [42, 43], it is of interest to investigate the possible role of AMF inoculation on the levels of free ABA and its catabolites throughout berry ripening. Therefore, the aim of this research was to determine if the ability of arbuscular mycorrhizal fungi (AMF) for inducing the accumulation of anthocyanins in grapes

under a climate change framework could be mediated by alterations in the metabolism of ABA during berry ripening. Previous research has demonstrated that fruit-bearing cuttings are a useful model system to study the response of berry ripening to environmental factors [24, 25, 44-46]. Hence, potted vines were used to control mycorrhizal inoculation and to have comparable non-inoculated plants.

2. Material and Methods

2.1. Plant material and growth conditions

Vitis vinifera (L.) cuttings from different clones of Tempranillo were obtained from an experimental vineyard of the Institute of Sciences of Vine and Wine (Logroño, Spain) (Denomination of Origin Rioja, North of Spain) during the winter. The study was performed in two clones (CL) of different origins (CL-1048, from Laguardia (Álava), and CL-1089, from Bargota (Navarra)) that were selected in the field on the basis on their different agronomic traits and plant material availability. Both clones have a short reproductive cycle but differed in yield, which was medium for CL-1048 and high for CL-1089). Dormant 400-500 mm long cuttings of each clone were selected for fruit-bearing according to the steps originally outlined by Mullins [47] with slight modifications as described in Ollat et al. [48] and Antolín et al. [49]. Briefly, rooting was induced with indole butyric acid (400 mg L^{-1}) in a heat-bed (27°C) kept in a cold room (4°C). Once cuttings had developed roots, they were transplanted to 6.5-L plastic pots containing a mixture of vermiculite–sand–light peat (2.5:2.5:1, v:v:v). The properties of the peat (Floragard, Vilassar de Mar, Barcelona, Spain) were pH 5.2–6.0, nitrogen 70-150 mg L^{-1}, P_2O_5 80-180 mg L^{-1}, and K_2O 140-220 mg L^{-1}. The peat was previously sterilised at 100°C for 1 h on 3 consecutive days.

At transplantation, the fruit-bearing cuttings were transferred to two growth chamber-greenhouses (GCG) adapted to provide different climate change scenarios [50] until berry maturity was reached. In both GCG, initial growth conditions were a 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} at the level of the inflorescence. Humidity and temperature were controlled using M22W2HT4X transmitters (Rotronic Instrument Corp.,

Hauppauge, USA). PPFD was monitored with a LI-190SZ quantum sensor (LI-COR, Lincoln, USA). Under these conditions, plants reached bud-break one week later. In order to improve the partitioning of stored carbon towards the roots and the reproductive structures, vegetative growth before flowering was controlled carefully. Thus, only a single flowering stem was allowed to develop on each plant during growth. Until fruit set, plants were watered twice a day with a nutrient solution (140 mL day^{-1}) with a phosphorus level of 0.30 mM [48] alternated with water. 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 Instruments SA, Alella, Spain). Under these conditions, fruit set (Eichhorn and Lorenz (E-L) growth stage 27) [51] took place 30 days after bud break and plants had 4-5- fully expanded leaves.

2.2. Mycorrhizal inoculation

At transplantation, half of the plants were inoculated with the commercial mycorrhizal inoculum Bioradis Gel (Bioera SLU, Tarragona, Spain) (+M plants). The inoculum consisted of a mixture of five AMF fungi (*Septglomus deserticola*, *Funneliformis mosseae, Rhizoglomus intraradices, Rhizoglomus clarum* and *Glomus aggregatum*), containing 100 spores per g of inoculum and a mixture of rhizobacteria belonging to the *Bacillus* and *Paenibacillus* genera (2 x 10^6 cfu g^{-1}). The microbial preparation was diluted in distilled water (1:20) so that each plant received 1 g of product. The roots of +M fruit bearing cuttings were submerged in the gel for 15 min, and then plants were placed in the pots. In order to discriminate the effects on plant metabolism due to the action of the mycorrhizal symbiosis, half of the plants were kept as non-inoculated controls. Uninoculated plants (-M plants) were submerged directly for 15 minutes in the filtrate of mycorrhizal inocula with the objective of restoring rhizobacteria and other soil free-living microorganisms accompanying AMF and which play an important role in the uptake of soil resources as well as in the infectivity and efficiency of AMF isolates [52]. The filtrate was obtained by passing diluted 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). All plants (-M and +M) were fertilized as described previously. Studies carried out by our group [24] had demonstrated that a phosphorus level of 0.30 mM was sufficient to ensure an adequate development of -M plants, even under water deficit [25], and not too high to impede the correct establishment of the mycorrhizal symbiosis.

2.3. Experimental design

Two separate experimental designs were performed to assess the grapevine responses under different climate change scenarios. In a first experiment (experiment 1), we chose Tempranillo CL-1048 because previous data had shown that AMF inoculation improved berry properties in this clone when subjected to elevated temperatures [24]. Fruit-bearing cuttings from +M or -M treatments were divided into two GCG to be exposed to each temperature regime: 24/14°C (day/night) and 28/18°C (day/night) from fruit set (E-L 27 stage) to berry maturity (E-L 38 stage). The 24/14°C temperature regime was selected according to average temperatures recorded in La Rioja (1981-2010) [53] during the growing season. The 28/18°C temperature regime was selected according to predictions of a rise of 4.0°C at the end of the present century [54]. To avoid excessive soil overwarming, which can negatively affect roots, and maintain a stable temperature, pots were shaded by wrapping their lateral surface with a reflecting material. Soil temperature was monitored at a depth of 5 cm soil using temperature probes PT100 (Coreterm, Valencia, Spain) and reached 24±0.5°C and 28±0.5°C for 24/14°C and 28/18°C air temperature regimes, respectively. Berry samples were collected at five stages of berry development: 1) when berries began to soften (Eichhorn and Lorenz (E-L) growth stage 34, green berries); 2) when berries began to colour and enlarge (E-L 35 stage, mid-veraison); 3) one week after mid-veraison (E-L 36 stage); 4) two weeks after mid-veraison (E-L 37 stage); and 5) at commercial maturity (22°Brix) (E-L 38 stage).

In a second experiment (experiment 2), we chose the clone CL-1089 because previous data had shown that AMF inoculation improved anthocyanin accumulation under deficit irrigation and elevated temperatures [25]. Thus, we established a three-factorial design where the two temperature regimes (24/14°C and 28/18°C) were combined with two water regimes. Within each temperature regime, fruit-bearing cuttings of CL-1089 from +M or -M treatments were divided into two groups: 1) plants under full irrigation (FI) from fruit set (E-L 27 stage) to maturity (E-L 38 stage) and 2) plants that received 50% of the water given to FI plants from veraison (E-L 35 stage) to maturity (E-L 38 stage) (late deficit, LD). Until the beginning of treatment, LD plants were maintained to full irrigation. Soil moisture sensors (EC-5 Soil Moisture Sensors, Decagon Devices Inc., Pullman, WA, USA) were placed in the pots. FI plants were maintained at ca. 80% of pot capacity (sensor value between 40 and 50%, (m^3 H_2O m^{-3} soil) × 100) until fruit harvest. Pot capacity was previously assessed by determining water retained after free-draining water had been allowed to pass through the holes in the bottom of the pot. The surface of the plant containers was covered with quartz stones during the

experiments to avoid water loss because of evaporation. The water volume supplied to the FI treatment was adjusted to increase plant development according to the daily measurements of the EC 5 water sensor. Watering was performed with nutrient solution or deionised water in order to supply the different treatments with the same amount of nutrients during water deficit. Predawn leaf water potential (Ψ_{pd}) was measured with a SKYE SKPM 1400 pressure chamber (Skye Instruments Ltd, Llandrindod, Wales, UK) on three fully expanded leaves per treatment at each sampling date just prior to irrigation (Figure S1). In this experiment, berry samples were collected at three stages of berry development: 1) one week after mid-veraison (E-L 36 stage); 2) two weeks after mid-veraison (E-L 37 stage); and 3) at commercial maturity (22°Brix) (E-L 38 stage).

2.4. Mycorrhizal colonization

Root samples were cleared and stained following the procedure described in Koske and Gemma [55]. A potassium hydroxide solution (10% w:v) was added to the roots which were placed in an oven at 70°C for 2 h. After being rinsed with water, roots were clarified by the addition of H_2O_2 (3% v:v) and subsequent washing with water. Then, they were acidified by soaking in HCl (1% v:v) for 5-15 minutes and stained in a solution of methyl blue: lactic acid (1% w:v) at 70°C for 1 h. Stained roots were stored in a mixture of glycerol, water and HCl 1% (500:450:50, v:v:v) until quantification. The percentage of mycorrhizal colonization was determined under a stereoscopic microscope by the plate intersection method [56].

2.5. Berry determinations

For each stage, berries were collected and frozen in liquid nitrogen and kept at -80°C until determinations. When fruit maturity was reached, plants were harvested separately based on sugar level from berry subsamples (2-3 berries) taken weekly. The length of phenological phases was recorded as the number of days from fruit set (E-L 27 stage) to each of the abovementioned berry stages.

A subsample of 5 berries was crushed and then extracts were centrifuged at 4300 *g* at 4°C for 10 min. The supernatant was used for determination of total soluble solids (mainly sugars) measured with a temperature-compensating refractometer (Zuzi model 315; Auxilab, Beriáin, Spain) and expressed as °Brix. Another subsample of 5 berries was taken for the analysis of anthocyanins, total phenols and abscisic acid (ABA) metabolites. Berries were ground separately to a powder in a mortar with liquid nitrogen and weighed. Anthocyanins were calculated according to the procedure described by Saint-Cricq et al. [57]. Two samples of the non-filtered, crushed grape homogenate were macerated for 4 h at pH 1 (hydrogen chloride) and pH 3.2 (tartaric acid), respectively. Once maceration was over, the macerated samples were centrifuged at 4300 *g* at 4°C for 10 min. Total anthocyanins were determined in supernatant (macerated at pH 1) according to Ribéreau-Gayon and Stonestreet [58] by reading absorbance at 520 nm. Calibration was performed by using malvidin-3-glucoside as a standard and anthocyanins were expressed as mg g^{-1} DM. Phenolic substances were estimated by reading absorbance at 280 nm in the supernatant obtained after maceration at pH 3.2 and results were expressed as gallic acid equivalent (mg g^{-1} DM) [59]. All analyses were run in triplicate.

2.6. ABA and catabolite analyses

The extraction, purification, and quantification of abscisic acid (ABA) and its catabolites (abscisic acid glucosylester (ABA-GE), 7-hydroxyl-ABA (7'OH-ABA), dihydrophaseic acid (DPA) and phaseic acid (PA)) were carried out in 0.1 g of the frozen powdered material as recently described by Chini et al. [60] with some modifications. Briefly, 1 mL of precooled (-20°C) methanol:water:formic acid (90:9:1, v/v/v with 2.5 mM Na-diethyldithiocarbamate) and 10 µL of deuterium labelled internal standards [([$^2H_4$]-ABA), ([$^2H_5$]-ABA-GE), ([$^2H_3$]-DPA), ([$^2H_3$]-PA) and ([$^2H_4$]-7-OH-ABA) provided by The National Research Council of Canada, Saskatoon, Saskatchewan, Canada) in methanol, were added to each sample. After shaking in a Multi Reax shaker (Heidolph Instruments) at 2,000 r.p.m. for 60 min at room temperature, solids were separated by centrifugation at 20,000 *g* for 10 min at room temperature in a Sigma 4-16K Centrifuge (Sigma Laborzentrifugen), and re-extracted with an additional 0.5 mL extraction mixture, followed by shaking (20 min) and centrifugation. 1 mL of the pooled supernatants was separated and evaporated at 40°C using a RapidVap Evaporator (Labconco Co). The residue was redissolved in 0.5 mL of methanol: 0.133% acetic acid (40:60, v/v). The solution was centrifuged at 20,000 *g* for 10 min at room temperature before injection into the high resolution accurate mass spectrometry (HPLC-ESI-HRMS) system.

The quantification was carried out using a Dionex Ultimate 3000 UHPLC device coupled to a Q Exactive Focus Mass Spectrometer (Thermo Fisher Scientific) equipped with an HESI(II) source, a quadrupole mass filter, a C-trap, a HCD collision cell and an Orbitrap mass analyser, using a reverse-phase column (Synergi 4 mm Hydro-RP 80A, 150 × 2 mm; Phenomenex). A linear gradient of methanol (A), water (B) and 2% acetic acid in water (C) was used: 38% A for 3 min, 38% to 96% A in 12 min, 96% A for 2 min, and 96% to 38% A in 1 min, followed by stabilization for 4 min. The percentage of C remained constant at 4%. The flow rate was 0.30 mL min^{-1}, injection volume was 40 µL, and column and sample temperatures were 35 and 15°C, respectively. Ionization source working parameters were optimized (Table S1). The detection and quantification were performed by a full MS experiment with MS/MS confirmation in the negative-ion mode, employing multilevel calibration curves with deuterated hormones as internal standards. MS1 extracted from the full MS spectrum was used for quantitative analysis and MS2 for confirmation of target identity. For full MS, a m/z scan range from 62 to 550 was selected, resolution was set at 70,000 full width at half maximum (FWHM), automatic gain control (AGC) target at $1e^6$ and maximum injection time (IT) at 250 ms. A mass tolerance of 5 ppm was accepted. The MS/MS confirmation parameters were resolution of 17,500 FWHM, an isolation window of 3.0 m/z, AGC target of $2e^5$, maximum IT of 60 ms, loop count of 1 and minimum AGC target of $3e^3$. Instrument control and data processing were carried out with TraceFinder 3.3 EFS software. Accurate masses of ABA, its metabolites and internal standard, as well as their principal fragments are reported in Table S2 and a chromatographic profile of ABA and its metabolites is shown in Figure S2.

2.7. 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. Data were subjected to Kolmogorov-Smirnov normality test due to the small sample size (n= 4). Data appeared to follow a normal distribution and were thus subjected to analysis of variance (ANOVA). In the first experiment, tests were performed to assess the main effect of the factors temperature (T) (24/14°C, 24 and 28/18°C, 28), AMF inoculation (+M and –M) and the interaction between these factors. In the second experiment, tests evaluated the main effect of the factors temperature (T) (24/14°C, 24 and 28/18°C, 28), AMF inoculation (+M and –M) and irrigation program (FI and LD) and the interaction between them. Means ± standard errors (SE) were calculated and when the F ratio was significant (P≤0.05), a Duncan test was applied.

Two-way or three-way ANOVAs were performed to determine significant differences in all measured parameters in CL-1048 or CL-1089, respectively.

3. Results and discussion

Previous research has shown the ability of Tempranillo grapevine to adapt to different environmental constraints associated with climate change, such as elevated air temperature and water deficit, both of which ultimately benefited berry properties [13]. In this context of heat and drought, AMF inoculation has been shown to be an appropriate resource to maintain or improve Tempranillo berry quality [24, 25]. For these reasons, a detailed study into berry ABA metabolism was performed to explore the mechanism underlying this effect.

3.1. Climate change scenario: effects of warming temperature

The results showed that mycorrhizal colonization of CL-1048 Tempranillo reached high values (to ca. 40 %) and that this percentage increased significantly at 28/18°C (Table 1). Several authors have reported that elevated temperature increased the abundance of mycorrhizas [61], mycorrhizal colonization and hyphal length [62] by enhancing carbon allocation of AMF and increasing phosphorus acquisition [63]. In contrast, Wilson et al. [64] reported that increased temperature diminished mycorrhizal colonization, and this effect was consistent across the Mediterranean climate gradient. These inconsistencies could be due to the role that AMF ultimately play in the alteration of the carbon storage capacity of soils and could be dependent on changes in the structure of the AMF network and the flux of labile photosynthetates from plants to the fungus [65].

In the current study, no significant effect of AMF on phenolic content was found (Table 1), which contrasts with results of Torres et al. [24]. These discrepancies could be due to differences in the rates of AMF colonization in the former study, which were much lower than those presented here. Thus, the high carbon cost of symbiosis maintenance could have resulted in limited carbon available for phenolic biosynthesis under elevated temperatures [61, 65]. Moreover, the type of mycorrhizal inocula could have also exerted an influence because Torres et al. [24] used a commercial inoculum derived from an in vitro culture of *Rhizophagus intraradices*. In the present study, grapevines received a mixture of five AMF (see Material and Methods section), which reinforces the idea that it may be useful to identify the AMF inoculants most suitable for a given variety or cultivar in a given environment [66]. On the

other hand, the temperature was the main factor reducing the content of phenolic substances in berries (Table 1). Similarly, other studies have reported significant reductions in phenolic content in berries at high temperatures [10], which have recently been linked to increased peroxidase activity under these conditions [12, 67].

At 28/18°C berries of CL-1048 reached berry maturity (estimated as total soluble sugars) 20 days earlier than plants exposed to 24/14°C (Figure 1), which agrees with the known effect of temperature to accelerate grapevine phenology [68]. AMF inoculation dampened the effect of elevated temperature, with berry sugars being similar under both temperature conditions (T×M, P≤0.05) (Figure 1). Nevertheless, in –M28 plants, the acceleration of phenology led to higher levels of sugars than those obtained at 24/14°C. Furthermore, the pattern of anthocyanin accumulation throughout berry ripening was significantly modified by AMF inoculation and/or temperature (Figure 2). The study of the main factors (temperature and mycorrhization) revealed that, while AMF induced the accumulation of anthocyanins in grapes at veraison (E-L 35 stage), elevated temperature favoured the accumulation of these phenolic compounds at the E-L 37 stage. At harvest (E-L 38), however, the highest levels of anthocyanins were found in berries from +M plants cultivated at 24/14°C. Mycorrhizal symbiosis also increased the content of anthocyanins in strawberry fruits [69, 70], which has been attributed to the up-regulation of some genes responsible for phenylpropanoid biosynthesis, such as phenylalanine ammonia lyase (PAL), a key enzyme involved in the synthesis of many phenolic compounds [71].

The influence that AMF symbiosis and temperature exerted on ABA metabolism was examined profiling ABA and its catabolites throughout berry ripening (Figure 3). The endogenous free ABA content is determined by the dynamic balance between biosynthesis and catabolism [35]. In our study, the concentrations of ABA and its catabolites were assessed at the same time as sugars and anthocyanins. As expected, free ABA content peaked at veraison (E-L 35 stage) and decreased thereafter in all treatments (Figure 3). The concentrations of ABA-GE increased during berry ripening, reaching a maximum content at maturity, whereas the concentrations of 7'OH-ABA were reduced at the E-L 38 stage and PA and DPA continuously diminished from the E-L 34 to E-L 38 stages. Current research is focusing on ABA catabolites, which have been recently highlighted as key molecules in grapevine development [34] and in its physiological responses to environmental stresses [37-39]. The ABA-GE acts as a reservoir of ABA and controls its concentration via the release of ABA by β–glucosidase. Moreover, ABA can be catabolized by hydroxylation at the positions 7' and 8', which produces 7'OH-ABA or PA and

DPA, respectively. Our results showed that mycorrhizal inoculation was the main factor modulating levels of ABA derivatives by increasing ABA-GE, 7´OH-ABA and DPA and by decreasing PA content in most of the stages studied (Table 2). Temperature also modulated the free ABA content of berries, since warm temperatures resulted in increased concentrations of ABA in the most stages analysed. This could be related to the up-regulation of NCED genes under these conditions suggesting the participation of ABA in berry acclimation responses to high temperature [28]. Similarly, temperature also contributed to increasing levels of ABA-GE and 7´OH-ABA, and to a lesser extent, PA and DPA (Table 2). At the late stages of berry maturation (E-L 37 and E-L 38) both temperature and AMF influenced ABA metabolism, which led to higher ABA-GE (T×M, P≤0.05) and 7'OH-ABA (T×M, P≤0.001) and lowers PA (T×M, P≤0.01) in the +M28 treatment (Table 2).

It has been reported that in grapes ABA hydroxylation at the 8´position predominates over the 7´position [34]. In contrast, our results suggest that, at the end of berry ripening (E-L37 and E-L 38 stages), AMF inoculation under warming temperatures promoted ABA catabolism by means of 7´OH-ABA (Figure 3). Other authors have indicated that 7´OH-ABA may be active in some hormonal processes, showing ABA-like activity and up-regulating secondary metabolism-related genes [72]. Although little is known about the role of this catabolite in berry ripening, Owen et al. [73] suggested that the increase in some ABA metabolites could make ABA unnecessary. This idea could help to explain some effects of AMF inoculation such as the enhancement of anthocyanin content related to lower levels of the inactive conjugated (ABA-GE) in +M24 treatment at maturity (E-L 38 stage) or the advancement of anthocyanin biosynthesis after veraison (E-L 37 stage) related to higher 7'OH-ABA in +M28 plants (Figures 2 and 3).

3.2. Climate change scenario: combined effects of deficit irrigation and warming temperature

Mycorrhizal colonization of Tempranillo CL-1089 reached high values (ca. 60 %) at 24/14°C and this percentage increased significantly at 28/18°C, attaining values up to 75%, (Table 3) in accordance with references discussed above. On the other hand, total phenolic content in berries was not significantly modified by any of the three factors applied (AMF, temperature or irrigation regime), corroborating that CL-1089 could be a good candidate to cope with global warming due to its ability to maintain certain fruit quality traits under these conditions [24].

As indicated in experiment 1, temperature was the main factor accelerating berry maturity (estimated as total soluble sugars) regardless of the AMF inoculation or irrigation level applied

(Figure 4). In contrast, the anthocyanin content was mostly affected by the three factors applied (T×M×I, P ≤ 0.001 and P ≤ 0.01 for E-L 37 and E-L 38, respectively) (Figure 5). Thus, under the most stressful climate change conditions (LD and 28/18°C) anthocyanin accumulation was significantly improved at the E-L 38 stage, especially in AMF inoculated plants, in agreement with preceding results obtained in this clone [25]. These observations could be explained by the ability of AMF symbiosis to stimulate the production of secondary metabolites in plants [22], together with the suitability of a post-veraison water deficit (LD) schedule to improve anthocyanin content [13, 15, 46].

The influence of AMF symbiosis, temperature and deficit irrigation on ABA metabolism was assessed profiling ABA and its catabolites from the end of veraison until berry maturity (Figure 6). Our results showed that under elevated temperature AMF inoculation reduced ABA-GE concentrations in all phenological stages studied and DPA concentration in the E-L 38 stage, (T×M, P≤0.05) (Table 4). Moreover, at berry maturity, AMF inoculation contributed to increasing 7´OH-ABA concentrations under LD conditions (M×I, P≤0.05). Recent studies indicated that the ABA catabolism/conjugation processes play an important role under environmental constraints. Thus, Balint and Reynolds [37, 38] reported that ABA was mainly catabolized by conjugation to form ABA-GE in plants subjected to water deficit, which agrees with the high rates of ABA-GE detected in the LD treatment, especially under elevated temperature (Figure 6).

It has been reported that in grape berries, the patterns of mRNA expression associated with ABA metabolism were altered under water deficit that, in turn, this could modify the endogenous ABA content of berry [18, 31, 44, 74]. Similarly, our results showed that the imposition of LD altered the pattern of ABA accumulation, consisting of a significant prolongation of ABA production over time (Figure 6). In a previous study we have reported that in the LD treatment ABA accumulation lasted until to the end of veraison [46]. The data presented here show that under LD, ABA accumulation was prolonged to berry maturity (E-L 38 stage), especially under warming conditions. However, at the E-L 37 stage ABA was modulated by the three factors applied that led to lower ABA concentrations in the berries of +M plants subjected to 28LD (T×M×I, P ≤ 0.001) (Table 4).

Overall, under the most stressful climate change conditions (LD and 28/18°C) ABA catabolism was seriously altered by AMF inoculation. Indeed, at the E-L 38 stage, +M plants showed a preferential pathway of ABA degradation to 7´OH-ABA that was modulated by all the factors applied (T×I, P≤0.001 and M×I, P≤0.05) (Table 4). However, in –M plants ABA catabolism

seemed to occur mainly by means of degradation to PA and DPA (8'hydroxilation pathway), as shown by the higher PA and DPA concentrations in the 28LD treatment at maturity (T×M×I, $P \leq 0.001$ and $P \leq 0.05$, respectively). Similar findings were obtained in non-mycorrhizal Tempranillo plants subjected to a combination of water deficit and high temperature and were related to lower anthocyanin content at maturity [39]. Furthermore, the observed differences in ABA catabolism pathways between –M and +M plants in our study could explain why berries in the –M28LD treatment reached lower anthocyanins than those of +M28LD (Figure 5). Since phytohormonal homeostasis in the plant host is also modulated by AMF symbiosis [40, 42, 43], our study suggests that the observed changes in ABA metabolism under climate change conditions could contribute to explain the positive effects of AMF inoculation of Tempranillo on berry anthocyanins. Although this study does not provide information on the contents of minerals in grapes, AMF may exert a positive effect by increasing the uptake of some macro (mainly phosphorus) and micronutrients (such as iron, copper, manganese or zinc) which are accumulated in berries during growth and ripening [75].

4. Conclusions

The main findings of this study showed that ABA catabolism/conjugation throughout berry development was modified by AMF inoculation and by the climate change conditions and that 7´OH-ABA plays an important role in the anthocyanin content of Tempranillo berries. Thus, under elevated temperature, AMF inoculation contributed to increase berry anthocyanins and modulated ABA metabolism, which led to higher ABA-GE and 7'OH-ABA and lower PA concentrations in comparison with those in fruits of –M plants. Under the most stressful climate change conditions (elevated temperature and deficit irrigation) AMF-inoculated plants reached higher berry anthocyanins and evidenced some modifications in berry ABA catabolism, leading to increased ABA hydroxylation at the position 7' in detriment of position 8'. Our findings provide an explanation of the ability of AMF to maintain and/or improve berry characteristics under future climatic conditions. To our knowledge, data presented in this study offer the first evidence on the implication of mycorrhizal symbiosis on grape ABA metabolism and how changes induced by AMF could affect some traits which determine the quality of grapes. Moreover, these results provide information on the role that AMF may play under future conditions of climate change. Several authors have studied the mycorrhizal communities associated with grapevines in the field. Results obtained in vineyards from USA, Italy, France or Central Europe subjected to agricultural practices such as high fertilizer inputs,

tillage, weed control or pest management [76-79], have shown reduced diversity of AMF in comparison with that found in the rhizosphere of European wild grapevine [80]. Evidence on the role that AMF may play on berry hormonal status and thus on berry ripening in different environmental scenarios reinforces the need to protect the natural mycorrhizal fungal communities present in vineyards.

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Table 1. Experiment 1: percentage of mycorrhizal colonization and total phenolics recorded at the harvest of fruit-bearing cuttings of Tempranillo (CL-1048) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M) and grown at 24/14°C (24) or 28/18°C (28) (day/night) temperatures (T).

Treatments	Mycorrhizal colonization (%)	Soluble phenolic substances (mg g^{-1} DM)
Treatments		
-M24	-	40.4
+M24	45.3 b	42.9
-M28	-	32.6
+M28	62.3 a	29.4
Main effects		
Temperature (T)		
24	-	41.7 a
28	-	31.0 b
Mycorrhization (M)		
-M	-	36.5
+M	-	36.2
ANOVA		
T×M	ns	ns

Values represent means (n = 4) separated by Duncan's test (at P≤0.05). Within columns, means followed by different letters are significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, –M) and their interaction. ns, not significant (P>0.05). DM: dry matter.

Table 2. Experiment 1: main effects and their interactions on berry ABA and its catabolites quantified during ripening in fruiting cuttings from Tempranillo (CL-1048) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M) and grown at 24/14°C (24) or 28/18°C (28) (day/ night) temperatures (T).

	Main effects				ANOVA		
	Temperature (T)		Mycorrhization (M)		T	M	T×M
	24	28	-M	+M			
ABA (nmol g^{-1} DM)							
E-L 34	2.7	3.0	3.3 a	2.4 b	ns	*	ns
E-L 35	5.2 b	9.5 a	7.3	7.4	***	ns	ns
E-L 36	3.7 b	5.6 a	4.7	4.5	*	ns	ns
E-L 37	1.8 b	3.6 a	2.9	2.5	***	ns	ns
E-L 38	0.4 b	0.6 a	0.5	0.5	**	ns	ns
ABA-GE (nmol g^{-1} DM)							
E-L 34	3.8	3.7	3.2	4.3	ns	ns	*
E-L 35	3.2 b	6.1 a	4.2 b	5.1 a	***	*	ns
E-L 36	3.9 b	8.4 a	4.9 b	7.4 a	**	*	ns
E-L 37	5.2 b	13.9 a	8.8 b	10.3 a	***	ns	ns
E-L 38	6.2	12.3	8.1	10.4	***	**	*
7'OH-ABA (nmol g^{-1} DM)							
E-L 34	0.39	0.43	0.30 b	0.52 a	ns	*	ns
E-L 35	0.50 b	0.71 a	0.52	0.59	**	ns	ns
E-L 36	0.36 b	0.54 a	0.39	0.51	**	ns	ns
E-L 37	0.34	0.60	0.37	0.56	***	***	***
E-L 38	0.17 b	0.30 a	0.19 b	0.28 a	**	*	ns
PA (pmol g^{-1} DM)							
E-L 34	22.4	24.3	31.8 a	23.0 b	ns	*	ns
E-L 35	15.6	28.2	21.7	22.0	*	ns	**
E-L 36	12.8 b	20.1 a	19.3 a	13.7 b	*	*	ns
E-L 37	10.0	13.2	14.4 a	8.9 b	ns	*	ns
E-L 38	4.2	5.9	5.2	4.9	*	ns	**
DPA (nmol g^{-1} DM)							
E-L 34	2.97	2.12	1.97 b	3.13 a	ns	*	ns
E-L 35	0.52 b	0.95 a	0.63	0.84	*	ns	ns
E-L 36	0.25	0.50	0.47	0.27	***	***	***
E-L 37	0.14 b	0.27 a	0.24	0.17	*	ns	ns
E-L 38	0.13	0.17	0.08 b	0.22 a	ns	**	ns

Values represent means (n = 4) separated by Duncan's test (at P≤0.05). Within rows, means followed by different letters are significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, –M) and their interaction. *P≤0.05; **P≤0.01; ***P≤0.001; ns, not significant (P>0.05). *ABA*: abscisic acid, *ABA-GE*: abscisic acid glucosylester; *7'OH-ABA*: 7-hydroxy-ABA; *DPA*: dihydrophaseic acid; *PA*: phaseic acid; DM: dry matter.

Table 3. Experiment 2: percentage of mycorrhizal colonization and total phenolics recorded at the harvest of fruit-bearing cuttings from fruit-bearing cuttings of Tempranillo (CL-1089) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C (24) or 28/18°C (28) (day/ night) temperatures (T) and subjected to different irrigation (I) regimes (FI: full irrigation; LD: late season water deficit).

	Mycorrhizal colonization (%)	Soluble phenolic substances (mg g^{-1} DM)
Treatments		
-M24-FI	-	41.9
-M24-LD	-	40.6
+M24-FI	65.7	39.8
+M24-LD	46.3	43.4
-M28-FI	-	37.1
-M28-LD	-	39.5
+M28-FI	67.0	38.2
+M28-LD	83.7	48.5
Main effects		
Temperature (T)		
24	56.0 b	41.4
28	75.3 a	40.8
Mycorrhization (M)		
-M	-	39.8
+M	-	42.5
Irrigation (I)		
FI	66.3	39.3
LD	65.0	43.0
ANOVA		
T×M×I	-	ns

Values represent means (n = 4) separated by Duncan's test (at P≤0.05). Within columns, means followed by different letters are significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, −M), irrigation (FI, LD) and their interactions. ns, not significant (P>0.05). DM indicates dry matter.

Table 4. Experiment 2: main effects and their interactions on berry ABA and its catabolites quantified during ripening in fruiting cuttings from Tempranillo (CL-1089) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M), grown at 24/14°C (24) or 28/18°C (28) (day/ night) temperatures (T) and subjected to different irrigation (I) regimes (FI: full irrigation; LD: late season water deficit).

	Main effects						*Two factor interactions*												*ANOVA*						
	Temperature (T)		Mycorrhization (M)		Irrigation (I)														T	M	I	T×M	T×I	M×I	T×M×I
	24	28	-M	+M	FI	LD	-M24	+M24	-M28	+M28	24FI	24LD	28FI	28LD	-MFI	-MLD	+MFI	+MLD							
ABA (nmol g^{-1} DM)																									
E-L36	3.6 b	5.3 a	4.3	4.5	-	-	3.7	3.6	5.1	5.4	-	-	-	-	-	-	-	-	***	ns	-	ns	-	-	-
E-L37	2.6	2.8	2.9	2.5	2.2	3.2	2.8	2.3	3.0	2.7	2.1	3.0	2.2	3.4	2.5	3.2	1.9	3.1	ns	ns	***	ns	ns	ns	***
E-L38	2.6 b	4.4 a	3.2	3.7	0.7 b	6.3 a	2.1	3.0	4.4	4.3	0.4	4.7	0.9	7.8	0.5	6.0	0.8	6.5	**	ns	***	ns	ns	ns	ns
ABA-GE (nmol g^{-1} DM)																									
E-L36	5.5	8.2	7.0	6.7	-	-	5.1 c	5.8 bc	9.0 a	7.6 b	-	-	-	-	-	-	-	-	***	ns	-	*	-	-	-
E-L37	6.2	12.3	9.4	9.2	9.1	9.5	5.7 c	6.8 c	13.1 a	11.5 b	6.1	6.4	12.2	12.5	9.5	9.4	8.8	9.6	***	ns	ns	*	ns	ns	ns
E-L38	8.6	16.2	13.1	11.7	7.9	16.9	8.2 c	8.9 c	17.9 a	14.5 b	5.0	12.2	10.8	21.6	8.4	17.7	7.4	16.0	***	ns	***	*	ns	ns	ns
7'OH-ABA (nmol g^{-1} DM)																									
E-L36	0.42	0.50	0.43	0.49	-	-	0.42	0.43	0.45	0.54	-	-	-	-	-	-	-	-	ns	ns	-	ns	-	-	-
E-L37	0.42	0.48	0.43	0.47	0.41	0.49	0.38	0.45	0.47	0.50	0.40	0.43	0.42	0.55	0.38	0.47	0.44	0.50	ns	ns	ns	ns	ns	ns	ns
E-L38	0.70	1.23	0.81	1.12	0.25	1.69	0.52	0.88	1.10	1.37	0.20 c	1.21 b	0.30 c	2.16 a	0.20 c	1.42 b	0.30 c	1.95 a	***	**	***	ns	***	*	ns

Table 4 *(Continued)*

	Main effects						*Two factor interactions*												*ANOVA*						
	Temperature (T)		Mycorrhization (M)		Irrigation (I)														T	M	I	T×M	T×I	M×I	T×M×I
	24	28	-M	+M	FI	LD	-M24	+M24	-M28	+M28	24FI	24LD	28FI	28LD	-MFI	-MLD	+MFI	+MLD							
PA (pmol g^{-1} DM)																									
E-L36	24.3	19.8	20.4	23.7	-	-	22.3	26.4	18.5	21.1	-	-	-	-	-	-	-	-	ns	ns	-	ns	-	-	-
E-L37	25.4	20.0	21.8	23.6	14.8	30.6	24.0	26.8	19.7	20.4	17.8	33.0	11.8	28.3	15.1	28.6	14.5	32.7	ns	ns	***	ns	ns	ns	***
E-L38	25.3	47.1	28.4	44.0	18.3	54.1	15.3	35.4	41.6	52.5	11.5	39.3	25.2	68.9	10.7	46.2	26.0	62.0	***	**	***	ns	ns	ns	***
DPA (nmol g^{-1} DM)																									
E-L36	0.12 b	0.29 a	0.19	0.22	-	-	0.10	0.15	0.29	0.30	-	-	-	-	-	-	-	-	***	ns	-	ns	-	-	-
E-L37	0.15	0.16	0.14	0.16	0.14	0.17	0.15	0.15	0.14	0.17	0.15	0.14	0.12	0.19	0.14	0.15	0.13	0.19	ns	ns	ns	ns	ns	ns	ns
E-L38	0.09 b	0.20 a	0.15	0.13	0.08 b	0.21 a	0.08 c	0.10 c	0.23 a	0.17 b	0.05	0.13	0.10	0.30	0.06	0.24	0.09	0.18	***	ns	***	*	ns	ns	*

Values represent means (n = 4) separated by Duncan's test (at P≤0.05). Within columns, means followed by different letters are significantly different as affected by the main factors temperature (24, 28), mycorrhization (+M, –M), irrigation (FI, LD) and their interactions. *P≤0.05; **P≤0.01; ***P≤0.001; ns, not significant (P>0.05). *ABA*: abscisic acid, *ABA-GE*: abscisic acid glucosylester; *7'OH-ABA*: 7-hydroxy-ABA; *DPA*: dihydrophaseic acid; *PA*: phaseic acid.

FIGURE LEGENDS

Figure 1. Experiment 1: evolution of total soluble solids (°Brix) recorded during berry ripening in fruit-bearing cuttings of Tempranillo (CL-1048) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M) and grown at 24/14°C (24) or 28/18°C (28) (day/night) temperatures. Values represent means ± SE (n = 4). A two-way ANOVA analysis was performed to evaluate the effects of temperature (T), mycorrhizal inoculation (M) and their interaction. ns, and * indicate non-significance or significance at 5% probability levels, respectively. When interaction between the main factors 'temperature, T' and 'mycorrhizal inoculation, M' was significant, different letters indicate significant differences according to Duncan test (P≤0.05).

Figure 2. Experiment 1: evolution of total anthocyanins recorded during berry ripening in fruit-bearing cuttings of Tempranillo (CL-1048) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M) and grown at 24/14°C (24) or 28/18°C (28) (day/night) temperatures during the berry ripening. Values represent means ± SE (n = 4). Within each phenological stage, a two-way ANOVA analysis was performed to evaluate the effects of temperature (T), mycorrhizal inoculation (M) and their interaction. ns, * and ** indicate non-significance or significance at 5% or at 1% probability levels, respectively. When the interaction between the main factors 'temperature, T' and 'mycorrhizal inoculation, M´ was significant, histograms with different letters indicate significant differences according to Duncan test (P≤0.05).

Figure 3. Experiment 1: evolution of berry ABA and its catabolites measured in fruit-bearing cuttings of Tempranillo (CL-1048) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M) and grown at 24/14°C (24) or 28/18°C (28) (day/night) temperatures during the berry ripening. Values represent means ± SE (n = 4). Within each phenological stage, when the interaction between the main factors 'temperature, T' and 'mycorrhizal inoculation, M´ was significant, histograms with different letters indicate significant differences according to

Duncan test (P≤0.05). *ABA*: abscisic acid, *ABA-GE*: abscisic acid glucosylester; *7'OH-ABA*: 7-hydroxy-ABA; *DPA*: dihydrophaseic acid; *PA*: phaseic acid.

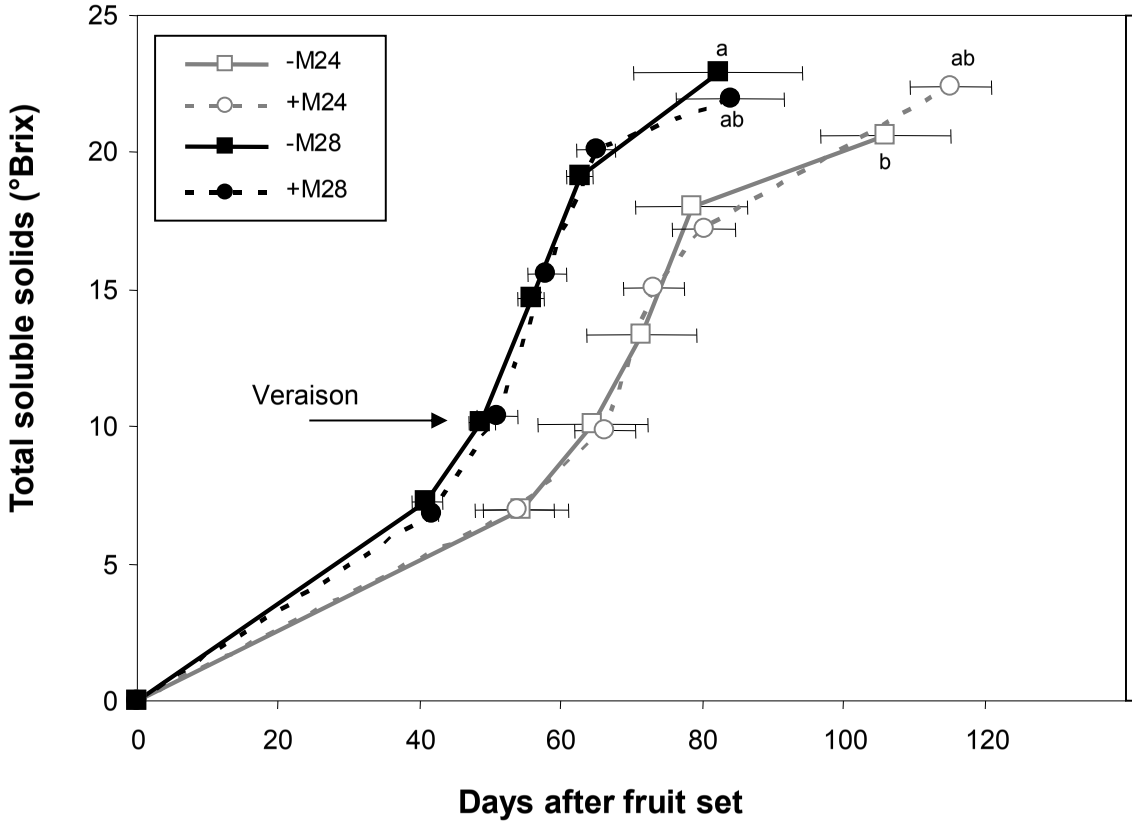
Figure 4. Experiment 2: evolution of total soluble solids (°Brix) recorded in fruit-bearing cuttings of Tempranillo (CL-1089) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M) and grown at 24/14°C (24) or 28/18°C (28) (day/night) temperatures and subjected to different irrigation regimes (FI, full irrigation; LD, late season deficit irrigation) during berry ripening. Values represent means ± SE (n = 4). Within each phenological stage, a three-way ANOVA analysis was performed to evaluate the effects of temperature (T), mycorrhizal inoculation (M), irrigation (I) and their interactions. ns indicate non-significance.

Figure 5. Experiment 2: evolution of total anthocyanins measured in fruit-bearing cuttings of Tempranillo (CL-1089) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M) and grown at 24/14°C (24) or 28/18°C (28) (day/night) temperatures and subjected to different irrigation regimes (FI, full irrigation; LD, late season deficit irrigation) during the berry ripening. Values represent means ± SE (n = 4). Within each phenological stage, a three-way ANOVA analysis was performed to evaluate the effects of temperature (T), mycorrhizal inoculation (M), irrigation (I) and their interactions. ** and *** indicate significance at 1% or at 0.1% probability levels, respectively. When the interaction between the main factors 'temperature, T', 'mycorrhizal inoculation, M´ and ´irrigation, I´ was significant, histograms with different letters indicate significant differences according to Duncan test (P≤0.05).

Figure 6. Experiment 2: evolution of berry ABA and its catabolites recorded in fruit bearing cuttings of Tempranillo (CL-1089) inoculated with arbuscular mycorrhizal fungi (+M) or uninoculated (-M) and grown at 24/14°C (24) or 28/18°C (28) (day/night) temperatures and subjected to different irrigation regimes (FI, full irrigation; LD, late season deficit irrigation) during the berry ripening. Values represent means ± SE (n = 4). Within each phenological stage, when the interaction between the main factors 'temperature, T', 'mycorrhizal inoculation, M´

and ´irrigation, I´ was significant, histograms with different letters indicate significant differences according to Duncan test (P≤0.05). *ABA*: abscisic acid, *ABA-GE*: abscisic acid glucosylester; *7'OH-ABA*: 7-hydroxy-ABA; *DPA*: dihydrophaseic acid; *PA*: phaseic acid.

Figure 1.



	Main effects				*ANOVA*
ºBrix	24	28	-M	+M	T × M
E-L34	7.0	7.1	7.1	6.9	ns
E-L35	10.0	10.3	10.2	10.1	ns
E-L36	14.2	15.2	13.8	15.3	ns
E-L37	17.5	19.6	18.6	18.4	ns
E-L38	21.5	22.5	21.8	22.2	*

	Main effects				*ANOVA*
Days	24	28	-M	+M	T × M
E-L34	54.3 a	41.4 b	47.8	47.9	ns
E-L35	65.4 a	49.9 b	56.6	58.6	ns
E-L36	72.4 a	56.9 b	63.6	65.6	ns
E-L37	79.4 a	63.9 b	70.6	72.6	ns
E-L38	110.6 a	83.1 b	94.1	99.6	ns

Figure 2.

	Main effects				*ANOVA*
	24	28	-M	+M	T × M
E-L34	0.54	0.98	0.80	0.73	**
E-L35	3.49	5.07	3.34 b	5.22 a	ns
E-L36	7.88	8.74	7.90	8.71	**
E-L37	20.37 b	32.55 a	29.39 a	23.53 b	ns
E-L38	43.76	31.13	31.57	43.32	*

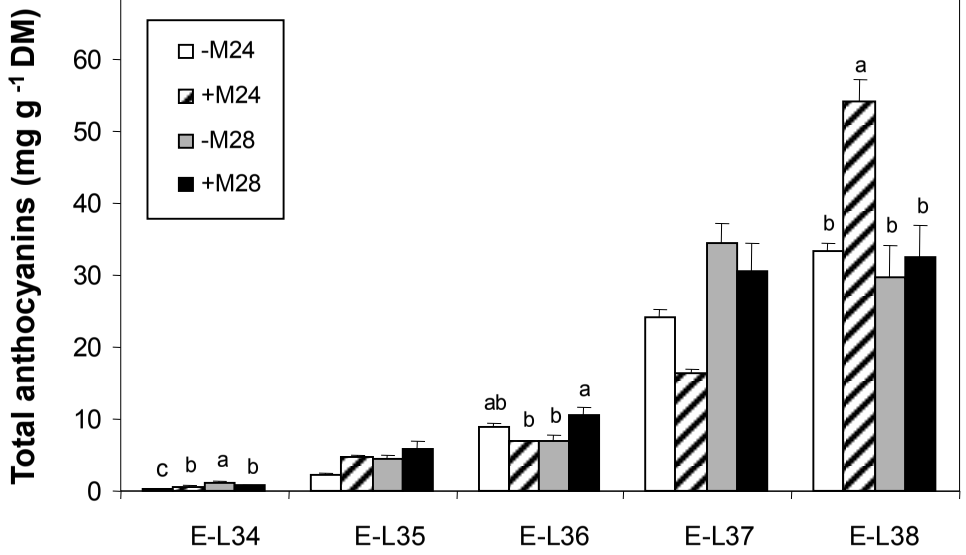


Figure 3.

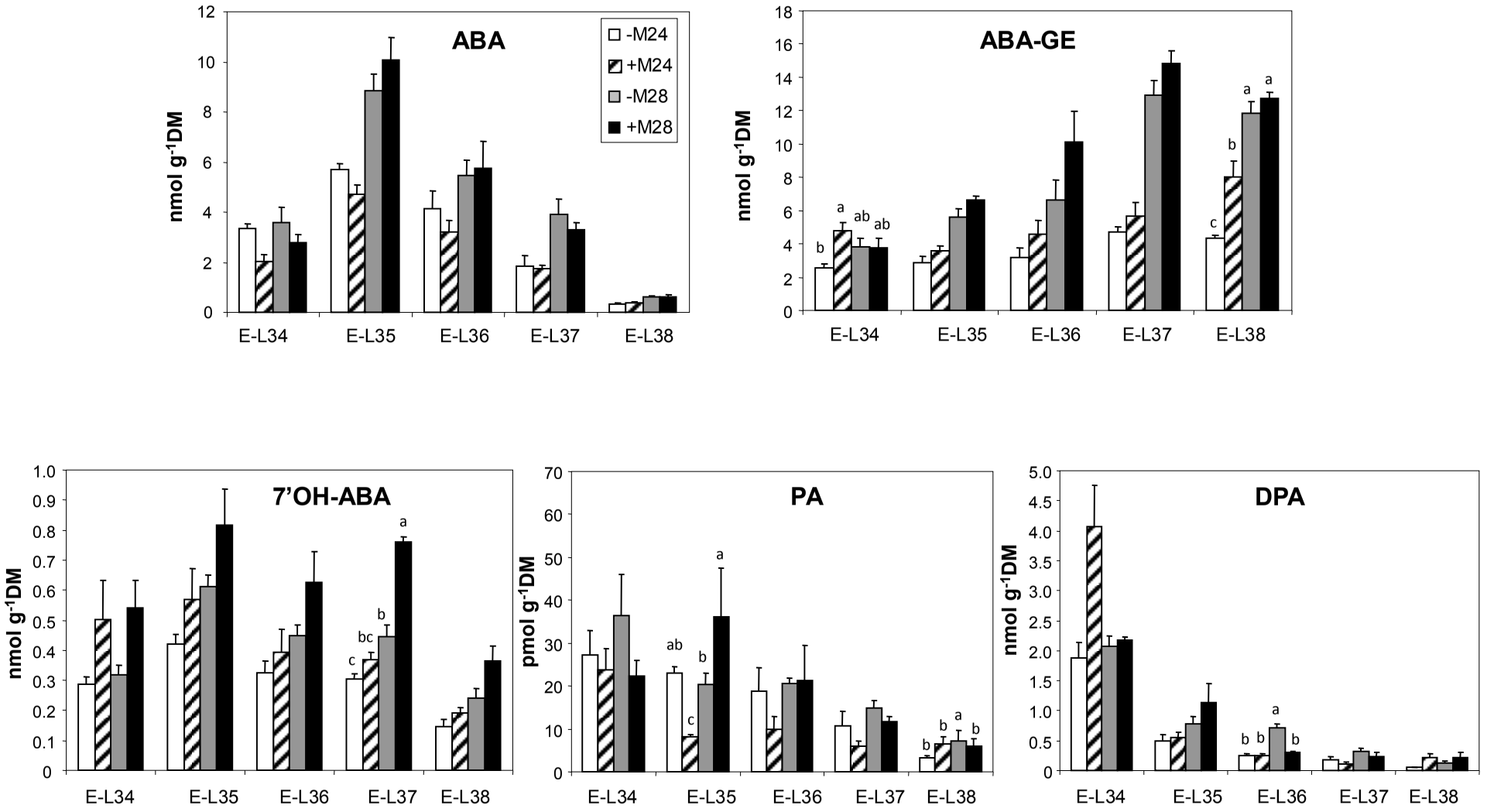
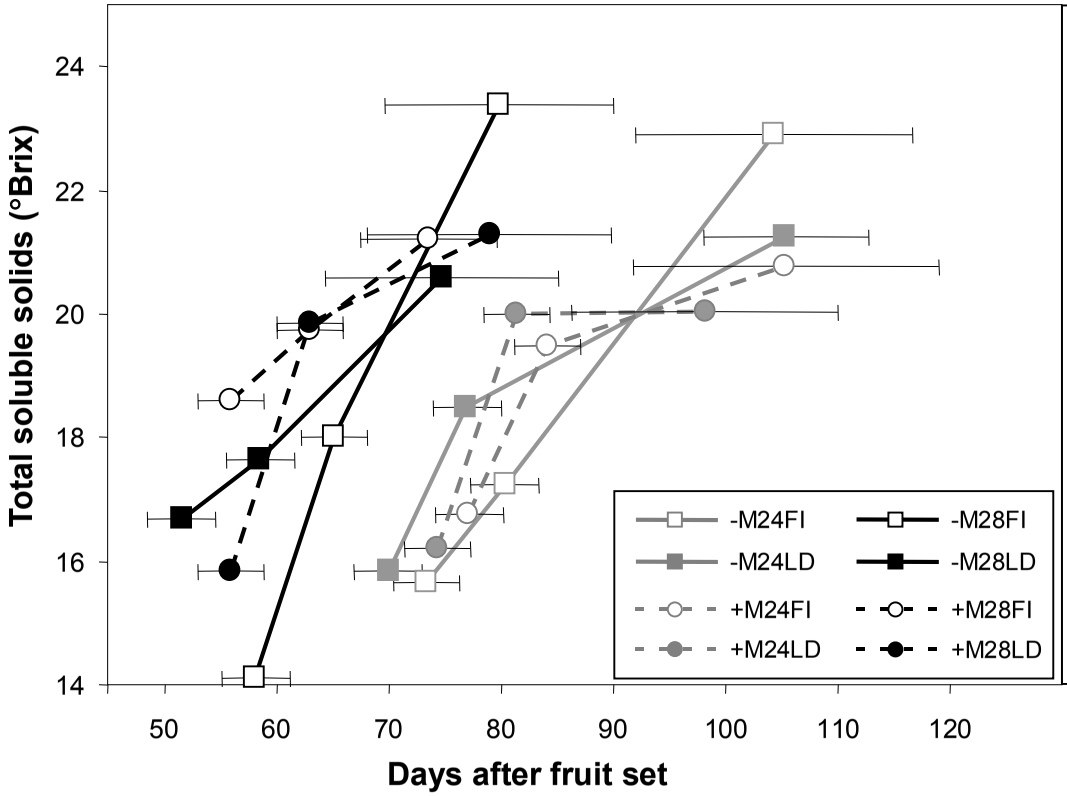

ABA
-M24
+M24
-M28
+M28
nmol g-1DM
E-L34
E-L35
E-L36
E-L37
E-L38
ABA-GE
7'OH-ABA
PA
pmol g-1DM
DPA

Figure 4.



	Main effects						*ANOVA*
°Brix	24	28	-M	+M	FI	LD	T×M×I
E-L36	16.1	16.3	15.6	16.9	-	-	ns
E-L37	18.8	18.8	17.9	19.8	18.6	19.0	ns
E-L38	21.2	21.6	22.0	20.8	22.1	20.8	ns

	Main effects						*ANOVA*
Days	24	28	-M	+M	FI	LD	T×M×I
E-L36	73.8 a	55.5 b	63.3	65.9	-	-	ns
E-L37	80.8 a	62.5 b	70.3	72.9	73.2	70.0	ns
E-L38	103.4 a	76.8 b	91.1	89.1	90.8	89.4	ns

Figure 5.

	Main effects						*ANOVA*
	24	28	-M	+M	FI	LD	T×M×I
E-L36	19.05 a	12.90 b	14.04	17.90	-	-	-
E-L37	35.99	31.73	29.72	38.00	36.55	31.17	***
E-L38	37.56	45.02	38.52	44.06	40.10	42.48	**

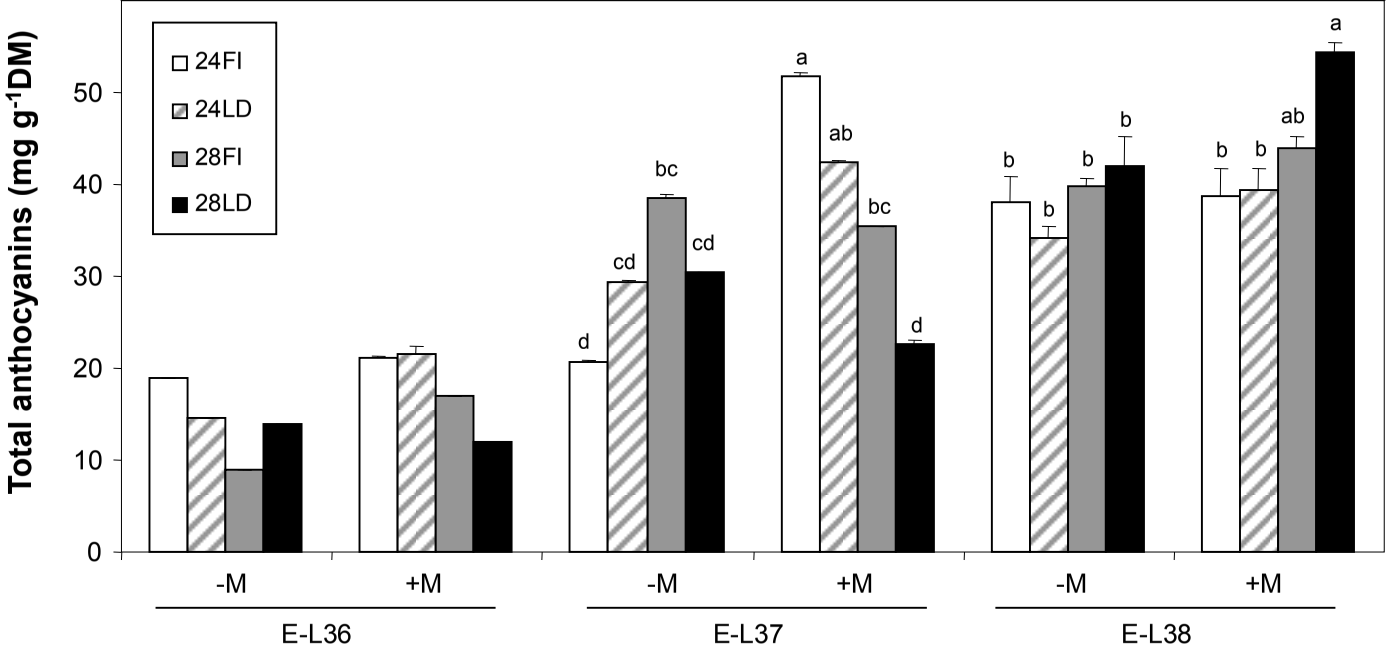
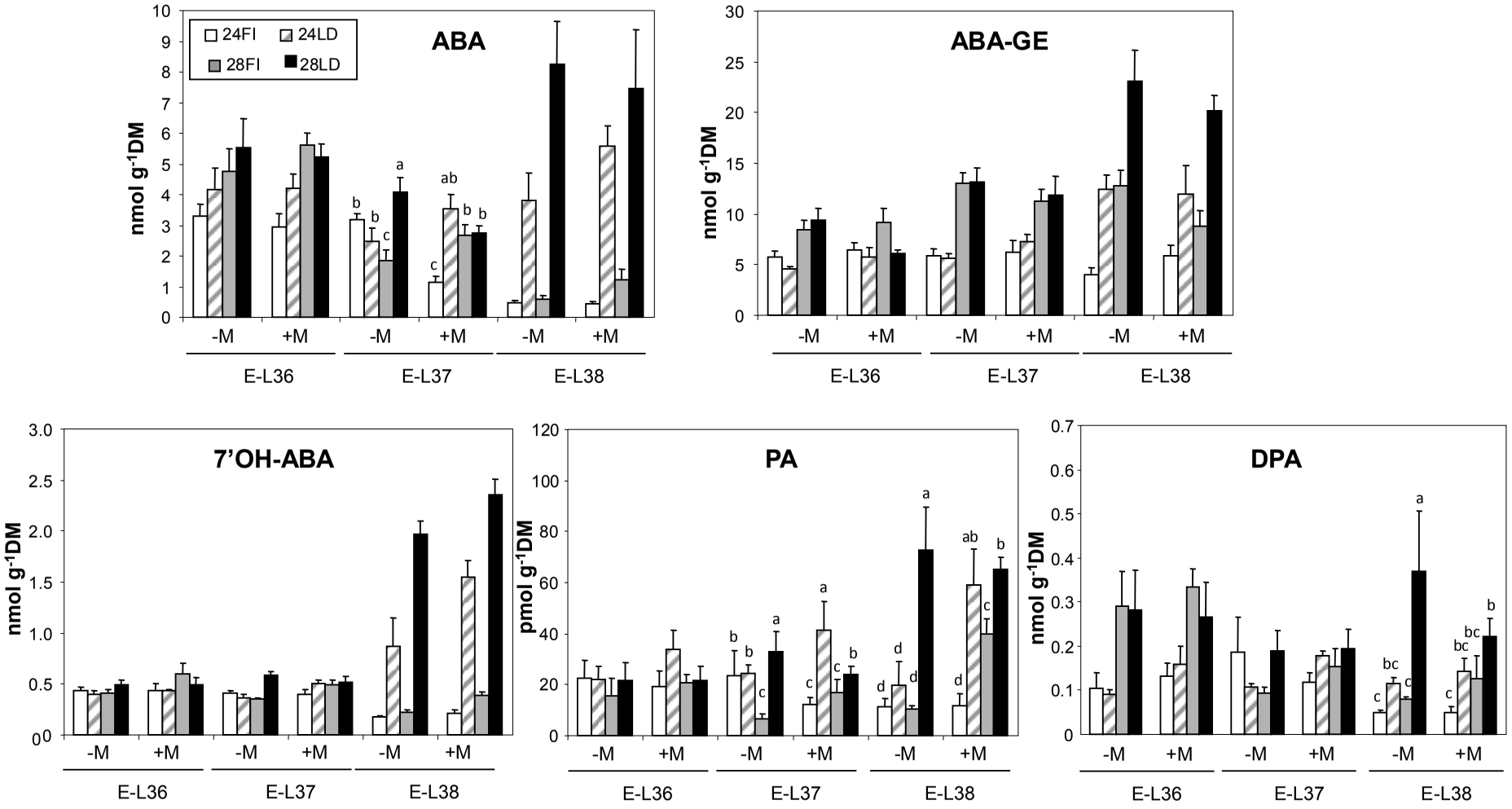


Figure 6.


ABA
24FI
24LD
28FI
28LD
nmol g-1DM
ABA-GE
7'OH-ABA
PA
pmol g-1DM
DPA
-M
+M
E-L36
E-L37
E-L38

Original research article

Mycorrhizal symbiosis affects ABA metabolism during berry ripening in Tempranillo (*Vitis vinifera* L.) grown under climate change scenarios

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Figure S1. Experiment 2: effect of irrigation treatments on pre-dawn leaf water potential (Ψ_{pd}) recorded at different stages of berry ripening.

Figure S2. Chromatographic profile of abscisic acid (ABA) and its catabolites in Tempranillo berries.

Table S1. Ionization source working parameters

Table S2. Masses of ABA, its catabolites, internal standards and their principal fragments.

Figure S1. Experiment 2: effect of irrigation treatments on pre-dawn leaf water potential (Ψ_{pd}) recorded at different stages of berry ripening in fruit-bearing cuttings of Tempranillo CL-1089 grown either at 24/14°C or 28/18°C (day/ night) temperature regimes. FI, full irrigation; LD, late season deficit irrigation. Values represent means ± SE (n = 4). Different letters indicate significant differences according to Duncan test (P≤0.05).

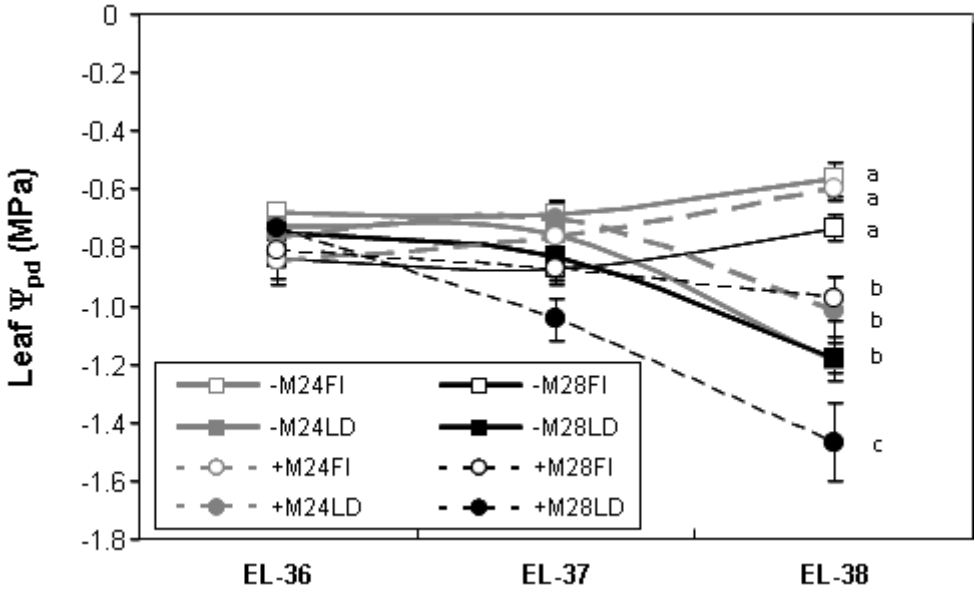
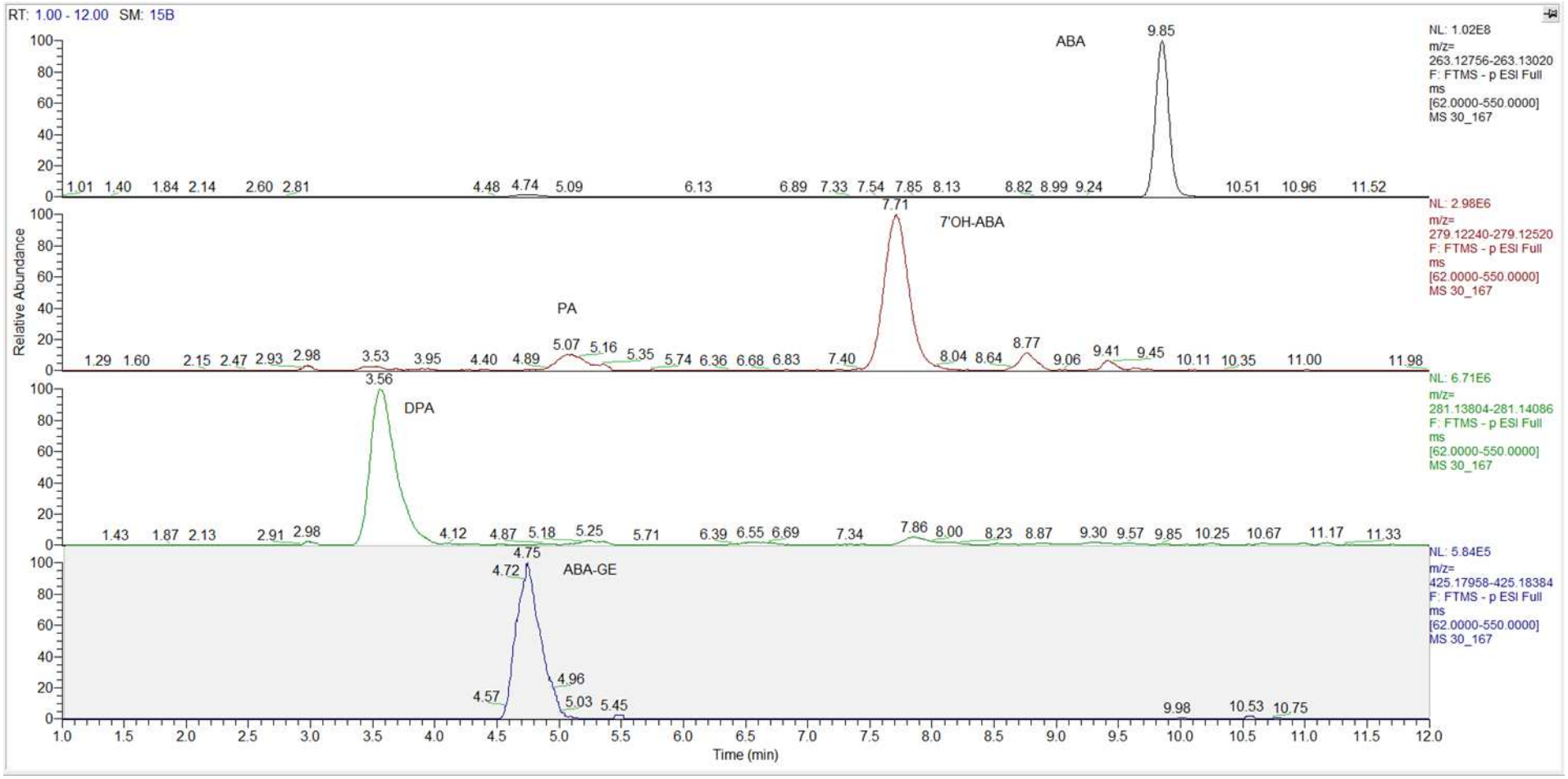


Figure S2. Chromatographic profile of abscisic acid (ABA) and its catabolites in Tempranillo berries.



ABA: abscisic acid, *ABA-GE*: abscisic acid glucosylester; *7'OH-ABA*: 7-hydroxy-ABA; *DPA*: dihydrophaseic acid; *PA*: phaseic acid.

Table S1: Ionization source working parameters

Instrumental parameters	Value
Sheath gas flow rate	44 au
Auxiliary gas flow rate	11 au
Sweep gas flow rate	1 au
Spray voltage	3.5 kV
Capillary temperature	340°C
S-lens RF level	50
Auxiliary gas heater temperature	300°C

au: arbitrary units.

Table S2: Masses of ABA, its catabolites, internal standards and their principal fragments.

Analyte	$[M-H]^{-1}$ phytohormone	$[M-H]^{-1}$ Fragment 1	$[M-H]^{-1}$ Fragment 2
ABA	263.12888	219.139	204.1155
ABA-GE	425.18171	263.12895	219.13907
7OH-ABA	279.1238	151.0765	217.12337
PA	279.1238	139.07651	205.12337
DPA	281.13945	237.1498	171.11803
$^{2}H_{4}$-ABA	267.15399	223.1642	208.1407
$^{2}H_{5}$-ABA-GE	430.21309	268.1604	224.17052
$^{2}H_{4}$-7´-OH-ABA	283.1489	154.0954	221.14859
$^{2}H_{3}$-PA	282.14263	142.09531	208.14224
$^{2}H_{3}$-DPA	284.15828	240.1686	174.13676

CAPÍTULO 5

Antioxidant properties of leaves from different accessions of grapevine (*Vitis vinifera* L.) cv. Tempranillo after applying biotic and/or environmental modulator factors.

(*Industrial Crops and Products* 76 (2015) 77-85.)

Antioxidant properties of leaves from different accessions of grapevine (*Vitis vinifera* L.) cv. Tempranillo after applying biotic and/or environmental modulator factors

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Abstract

Within climate change scenario, the maintenance of grape quality and wine characteristics will be the main concern for viticulture in the future years. However, changes in the composition of grapevine pruning wastes (i.e., leaves and stems) could be another interesting aspect as important antioxidant source for pharmaceutical industry due its richness in phenolic compounds beneficial for human health. To date, the effect of biotic and environmental factors in the accumulation of these compounds in leaves had received little attention. Therefore, the aims of study were: 1) to evaluate the effect of biotic (mycorrhizal inoculation) and environmental (temperature) factors, alone or combined, on phenolic composition and antioxidant activity of leaf extracts of grapevine, and 2) to determine whether such effects differed among accessions of the same cultivar of grapevine. The study was carried out using container-grown grapevines grown in greenhouses. Dormant *Vitis vinifera* (L.) cuttings of different accessions of Tempranillo were selected to get fruit-bearing cuttings. At transplanting, half of the plants of each accession were inoculated with the mycorrhizal inoculum and after fruit set, plants were exposed to two temperature regimes (24°C/14°C and 28°C/18°C (day/night)) to commercially berry ripe. Results showed that total phenolic content, antioxidant compounds like flavonols and anthocyanins, and antioxidant activity of leaves were improved with mycorrhizal inoculation under high temperature conditions. It was concluded that mycorrhizal inoculation of grapevines could contribute to preserve high level of antioxidant compounds of leaves in a future climate change scenario. However, the effects were strongly dependent of accession assayed, which indicated a significant intra-varietal diversity in the response of Tempranillo to biotic and environmental factors.

***Keywords**: Arbuscular mycorrhizal fungi, climate change, fruiting cuttings, grapevine wastes, intra-varietal diversity, phenolic compounds.*

Introduction

Phenolic compounds are the most commonly studied compounds because of their universal presence in high concentrations and their significant roles in plant cells and tissues. They include many secondary metabolites with multiple functions: i) as absorbing filters they reduce the penetration of UV and visible radiation (Teixeira et al., 2013); ii) as antioxidants they can scavenge reactive oxygen species (ROS) (Castellarin et al., 2012; Brunetti et al., 2013); iii) as regulators of soil processes they control recycling and nutrient availability for plants and soil microbes; and iv) as signal molecules they play a significant role in the interactions between plants and other organisms (Karabourniotis et al., 2014).

Grapevine (*Vitis vinifera* L.) phenolic compounds are divided in nonflavonoid and flavonoid compounds. Nonflavonoid compound are hydroxybenzoic acids, hydroxycinnamic acids, volatile phenols and stilbenes; while flavonoid compounds are flavones, flavonols, flavanones, flavan-3-ols and anthocyanins. Flavonoids constitute the major group of phenolic compounds in grapes and their composition is determined firstly by genetic factors but it could be changed during growth and season conditions (Teixeira et al., 2013; Niculcea et al., 2015) and they are important components in the determination of wine style and quality (Downey et al., 2006). Besides, phenolic compounds are of major interest for human nutrition and health (Ali et al., 2010; Brunetti et al., 2013). Several studies revealed that phenolic compounds of grapes exhibited a wide range of biological activities, including antioxidant, antifungal, antibacterial, antiviral and therapeutic properties (Cheynier, 2005; Pezzuto, 2008; Perron and Brumaghim, 2009; Jin et al., 2013). Other researches have shown that grape extracts exhibited anticancer properties (Sun et al., 2011; Zhou and Raffoul, 2012; Handoussa et al., 2013).

Phenolic compounds and other antioxidant molecules are especially abundant in grape berries (Kallithraka et al., 2009; Xu et al., 2010; Cramer et al., 2011; De Nisco et al., 2013) but other tissues as leaves were also rich in phenolic compounds and can be used for many applications such as therapeutic and food industries (Król et al., 2014; Eftekhari et al., 2012). In consequence, a possibility that emerge is reutilising the grape wastes from pruning and other viticultural activities as a potentially natural source of the well-known medicinal and antimicrobial phenolic compounds.

Phenolic metabolism and its accumulation of grapevine tissues can be strongly modified in response to biotic and environmental factors. Within biotic factors, arbuscular mycorrhizal fungi (AMF) are the most widespread and common root-fungus associations (Smith and Read, 2008) and have an increasingly important role in vineyard production systems (Likar et al., 2013). Thus, AMF inoculation of grapevines has been associated with increased growth (Linderman and Davis, 2001), drought tolerance (Nikolau et al., 2003), nutrient uptake

(Karagiannidis et al., 2007) and protection against diseases caused by pathogens (Nogales et al. 2009). However, there is little information about changes on phenolic composition in response to AMF inoculation in grapevines. Some studies have been reported that AMF inoculation did not modified berry composition (Karagiannidis et al., 2007) but a significant enhancement of phenolic content in leaves was found (Eftekhari et al., 2012).

Climate change refers to any change in climate over time, whether due to natural variability or as a result of human activity leading increased greenhouse gases emissions, which have a direct effect on air temperature (Webb et al., 2013). According to IPCC (2013) global temperatures average is predicted to increase between 1.8 and 4.0°C at the end the present century. Climate change will be particularly important for phenolic composition of grapevines, because heat, drought and light intensity are just some environmental stress factors that dramatically affect phenolic metabolism (Mira de Orduña, 2010; Teixeira et al., 2013). Associated with warming trends over the last few decades, early maturity of berries and changes in vegetative and reproductive growth, yield and berry attributes have been reported (Duchêne and Schneider, 2005; Petrie and Sadras, 2008; Webb et al., 2011).

Many studies have related clonal diversity among grapevine cultivars for a broad range of characteristics. Thus, precocity of the phenological cycle, yield, berry sugars and total acidity, berry phenolic composition and disease resistance (van Leeuwen et al., 2013) and response to environmental changes varied broadly among accessions (Mannini et al., 2010). However, to date, no attention had been paid on intra-varietal diversity on phenolic composition and its antioxidant potential in leaves. Therefore, the objectives of this study were: 1) to evaluate the effect of biotic (mycorrhizal inoculation) and environmental (temperature) factors, alone or combined, on phenolic composition and antioxidant activity of leaf extracts of grapevine, and 2) to determine whether such effects differed among accessions of the same cultivar of grapevine cv. Tempranillo. This variety is widely cultivated in northern and central Spain where it is the main variety in half of the Denominations of Origin. Potted vines were used to assure that all accessions experienced the same conditions of temperature and a controlled mycorrhizal inoculation.

Material and methods

Biological material and growth conditions

Dormant *Vitis vinifera* (L.) cuttings of different accessions of Tempranillo with 400-500 mm long were selected in the winter of 2013 in different villages located in Rioja Alavesa (Denomination of Origin Rioja, North of Spain). A brief description of selected accessions is

presented in Table 1. Cuttings of each accession were selected to get fruit-bearing cuttings according to steps originally outlined by Mullins (1966) with some modifications described in Ollat et al. (1998) and Antolín et al. (2010). Previous research demonstrated that the fruiting cuttings technique is a useful model for grapevine physiology studies (Antolín et al., 2010) that allows the development of vegetative and reproductive organs similar to the vineyard grapevines but under fully controlled environmental conditions (Dai et al., 2013). Briefly, rooting was made in a heat-bed (27°C) kept in a cool room (4°C). One month later, the cutting were planted 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 glasshouses. Peat (Floragard, Vilassar de Mar, Barcelona, Spain) had a pH of 5.2-6.0, 70-150 mg L^{-1} of nitrogen, 80-180 mg L^{-1} P_2O_5 and 140-220 mg L^{-1} K_2O and it was previously sterilized at 100°C for 1 h on three consecutive days.

At transplanting, half of the plants were inoculated with the mycorrhizal inoculum 'GLOMYGEL® vid, olivo, frutales' (Mycovitro S.L., Pinos Puente, Granada, Spain) (+M plants). The concentrated commercial inoculum derived from an *in vitro* culture of arbuscular mycorrhizal fungi (AMF): *Rhizophagus intraradices* (Schenck and Smith) Walker & Schüβler comb. nov. (Krüger et al., 2012). It contained around 2,000 mycorrhizal propagules (inert pieces of roots colonized by AMF, spores and vegetative mycelium) per mL of inoculum. In order to facilitate its application, the concentrated commercial inoculum was diluted with distillate water until obtaining a resultant mycorrhizal inoculum with around 250 propagules per mL. Each +M plant received 8 mL of the diluted mycorrhizal inoculum close to the roots thus making a total of 2,000 propagules. A filtrate was added to plants that did not receive the mycorrhizal inoculum (-M plants) in an attempt to restore other soil free-living microorganisms accompanying AMF. The filtrate was obtained by passing diluted mycorrhizal inoculum through a layer of 15-20 µm filter papers (Whatman, GE Healthcare, UK) and each –M plant received 8 mL of filtrate close to the roots. The selection of *in vitro*-produced inoculum of *R. intraradices* was based on two expected benefits: (1) easy application of the product and (2) low colonization of onion roots by contaminant fungi (Vimard et al., 1999).

Plants were transferred to greenhouses, which was adapted to simulate climate change conditions as described recently in Morales et al. (2014). 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} at the level of the inflorescence. Humidity and temperature were controlled using M22W2HT4X transmitters (Rotronic Instrument Corp., Hauppauge, USA). PPFD was monitored with a LI-190SZ quantum

sensor (LI-COR, Lincoln, USA). Under these conditions, bud-break took place after one week. Careful control of vegetative growth before flowering improves the partitioning of stored carbon towards the roots and the reproductive structures. Thus, only a single flowering stem was allowed to develop on each plant during growth. Plants were irrigated with the nutrient solution detailed by Ollat et al. (1998). The electric conductivity (EC) of the nutrient solution adjusted to pH 5.5 was 1.46 ± 0.15 mS cm^{-1}; after applying the nutrient solution to grapevines the EC of the substrate was 2.35 ± 0.10 mS cm^{-1} for non-inoculated plants and 0.18 ± 0.04 mS cm^{-1} for plants inoculated with *R. intraradices*, as determined with a conductivity meter 524 Crison (Crison Instruments S.A., Alella, Spain).

Experimental design

Fruit-bearing cuttings (4–6 plants per accession) were exposed to two temperature regimes at fruitset (Eichhorn and Lorenz (E–L) fruit stage 27) (Coombe, 1995): 24°C/14°C (day/night) and 28°C/18°C (day/night). The 24°C/14°C temperature regime was selected according average temperatures registered in Rioja Alavesa (1997-2000) (AEMET, Spain) during growing season. The 28°C/18°C temperature regime was selected according to predictions of a rise of 4.0°C at the end the present century (IPCC, 2013). Both temperature regimes were maintained to harvest that corresponded to commercially ripe berries (approximately 22°Brix) (E-L 38 stage). Leaves from each treatment (40-50 leaves per biological replicate) were frozen at -80°C for further analysis.

Plant growth, mycorrhizal colonization and mycorrhizal efficiency index (MEI)

Leaf area was measured with a portable area meter (model LI-3000, Li-Cor, Lincoln, Nebraska, USA). A good correlation (R = 0.97) was obtained between the length of the main vein of the leaf and leaf area using several leaves of each accession. Total leaf area of each plant was calculated after measuring the length of the main vein in all leaves and applying the following formula: leaf area = -4.98 + (2.54 x vein length) + 0.90 x vein $length^2$).

Root samples were cleared and stained (Phillips and Hayman, 1970) and mycorrhizal colonization was determined by examining 1 cm root segments (n = 45 per pot) under the microscope. Extension (E), incidence (I) and intensity (Int) of mycorrhizal colonization were calculated for each pot as described by Baslam et al. (2014). The E of mycorrhizal colonization was firstly determined for every root segment and it was calculated as the product between value of mycorrhizal colonization in width (W) and value of mycorrhizal colonization in length (L). Values of mycorrhizal colonization in width (W) and length (L) were ascribed according a scale in which 0 meant complete absence of fungal structures and 10 meant that fungal

structures occupied the full length or width of the root segment. Afterwards, total E per pot was calculated as E = ∑ (W×L)/n, where 'n' was the total number of root segments observed under the microscope (n = 45 per pot) and it was expressed as a percentage. Incidence (I) of mycorrhizal colonization per pot was calculated as the ratio between number of root segments with fungal structures (arbuscules, vesicles and/or hyphae) and total number of root segments observed under the microscope (n = 45 per pot). Finally, the intensity (Int) of mycorrhizal colonization per pot was calculated as the product between E and I (Int = E × I) and results were expressed as percentage of infection (Hayman et al., 1976).

The mycorrhizal efficiency index (MEI) was estimated according to Bagyaraj (1994) as: MEI = (leaf DM of inoculated plant – leaf DM of non-inoculated plant) × 100/leaf DM of inoculated plant where DM means dry matter. Determination of MEI allows assessment of the improvement provided by inoculation of plants with a mycorrhizal fungus.

Phenolic compounds determinations

The content of epidermal levels of flavonols and anthocyanins in leaves were evaluated at harvest, coinciding with berry ripening. Determinations were estimated *in situ* by using a handheld, non-destructive fluorescence-based proximal sensor Multiplex3^{TM} (Force-A, Orsay, France) as described by Agati et al. (2013). It has four LED-matrix light sources: 373 nm (Ultraviolet, UV), 470 nm (Blue, B), 516 nm (Green, G) and 635 nm (Red, R) and three synchronised detectors for fluorescence recording: yellow (YF), red (RF) and far-red (FRF). The fluorescence ratios linked to polyphenol content were based on FRF_UV (FRF excited by UV light), FRF_R (FRF excited by red light), FRF_G (FRF excited by green light) and RF_R (RF excited by red light). The FERARI (fluorescence excitation ratio anthocyanin relative index) index has been inversely correlated with the anthocyanin content in grapes (Baluja et al., 2012). The definition of a fluorescence index for flavonols is based in that they attenuate UV radiation because they attain maximum absorption at 350 nm. The FLAV index is obtained by comparing the fluorescence signals excited by UV and by FRF_R and has been directly correlated with the flavonol content in grapes (Agati et al., 2013). Thus, the fluorescence indices used in this work were defined as:

FERARI = log (5000/ FRF_R)

FLAV = log (FRF_R/FRF_UV).

Total soluble phenolic compounds were extracted according to Chapuis-Lardy et al. (2002) with some modifications. Samples of leaves (0.2 g fresh weight) were pulverized in liquid nitrogen, mixed with 10 mL of 80% methanol, and homogenized at room temperature for 1 min. After filtration, 0.5 mL of each sample was mixed with 10 mL of distilled water. Total

phenolic content was determined from aqueous solutions by spectrophotometric analysis at 760 nm with Folin-Ciocalteau reagent (Waterman and Mole, 1994). Results were expressed as mg of gallic acid per g of DM.

Antioxidant activity

Total antioxidant activity of leaf extracts was evaluated by using the free radical scavenging activity (α, α-diphenil-β-picrylhydrazyl (DPPH) assay) (Brand-Williams et al., 1995). Leaf extracts were the same used for determining the content of total soluble phenolic compounds. The free radical scavenging activity using the free radical DPPH• was evaluated by measuring the variation in absorbance at 515 nm after 30 min of reaction in parafilm-sealed glass cuvettes (to avoid methanol evaporation) at 25°C (Espín et al., 2000). The reaction was started by adding 20 µL of the corresponding sample to the cuvette containing 80 µM (methanol solution) (980 µL) of the free radical (DPPH•) (Llorach et al., 2004). The final volume of the assay was 1 mL. Reaction was followed with a spectrophotometer (Jasco V-630, Analytical Instruments, Easton, MD, USA). Calibration curve was made using gallic acid as standard. Results were expressed as µg of gallic acid per g of DM.

Statistical analysis

Data were subjected to a two-factor analysis of variance (ANOVA), and variance was related to the main treatments (temperature, T, and mycorrhizal inoculation, M) and to the interaction between them (T x M) within each grape accession. In the case of mycorrhizal efficiency index (MEI) the main treatments were temperature (T) and accession (A). Means ± standard errors (SE) were calculated and, when the F ratio was significant ($P \leq 0.05$), a Duncan test was applied by using the Statistical Package for the Social Sciences (SPSS) (SPSS Inc., Chicago, IL, USA) version 21.0 for Windows XP. All values shown in the figures are means ± SE. The significance of regression equations was also evaluated using this program.

Results and discussion

Mycorrhizal colonization, phenology and plant growth

In the present study, the percentage of mycorrhizal colonization in grapevine roots never exceeded 15% regardless plants were cultivated under either 24°/14°C or 28°/18°C regimes. These values are clearly lower than those measured by Eftekhari et al. (2012) in *V. vinifera* inoculated with different species of arbuscular mycorrhizal fungi, including *R. intraradices* (70% of root colonization). Several factors could have been implied: (1) differences in root

colonization may be at least partially due to the different type of inocula applied to grapevine in both studies: while Eftekhari et al. (2012) used a commercial inoculum that contained spores, mycelium and root fragments of clover colonized by mycorrhizal fungi, in our study the main component of the mycorrhizal inoculum was spores of *R. intraradices*. Bettoni et al. (2014), working with onion, also found low colonization percentages when using monoxenically-produced spores of *R. intraradices* as mycorrhizal inoculum; (2) percentages of mycorrhizal colonization are highly dependent on the grape variety (Eftekhari et al., 2012); and (3) as in the grape fruiting cuttings model several leaves are removed to allow fruit development, the total leaf area may have resulted insufficient to provide enough photoassimilates for satisfying the demand of the main sinks in plants inoculated with *R. intraradices* (fungal structures in roots and fruits) in detriment of the spread of mycorrhizal colonization. Such limitation would have resulted in a reduced improvement of vegetative growth brought about by inoculation of plants with *R. intraradices* (low or negative MEI, Figure 1). The application of nutrient solution can also influence the percentage of mycorrhizal colonization. In a recent assay performed by Navarro et al. (2012) with carnation plants inoculated with a very similar in vitro-produced commercial inoculum (Glomygel Garden, *R. intraradices*, from MYCOVITRO) than that used in our study (GLOMYGEL® vid, olivo, frutales, *R. intraradices*, from MYCOVITRO), the highest mycorrhizal colonization (26%) was reached in plants irrigated with fresh water, whereas increases of salinity in the irrigation water caused decreased levels of mycorrhizal colonization. In our study, however, such effect is expected to be inappreciable due to the low EC of the substrate where inoculated grapevine plants were cultivated.

Although it was established a monophyletic origin with low genetic variability for Tempranillo variety (Cervera et al., 2002), accessions selected in this work have significant diversity for some agronomic traits as yield or the length of phenological cycle (Table 1). Under our experimental conditions, a rise of temperature from 24°C/14°C to 28°C/18°C resulted in accelerated phenology (i.e., number of days from fruit set to harvest) that was more pronounced in long-cycle accessions (Table 2). Such observations show a consistent trend towards earlier flowering, veraison and harvest commonly observed in previous studies (Duchêne and Schneider, 2005; Petrie and Sadras, 2008). The combination of temperature and AMF inoculation contributed significantly to length cycle reduction in CL-1089 and CL-8 accessions. On the other hand, vegetative plant growth estimated from leaf area was decreased mainly by temperature in most of Tempranillo accessions and no significant differences between +M and –M plants was detected (Table 2). Our results were in contrast with previous reports that showed increased vegetative growth in mycorrhizal grapevines

(Karagiannidis et al., 2007; Camprubí et al., 2008). Such discrepancies could be explained because in our experimental model, mycorrhizal fungi could have imposed an additional sink to bunch, which have limited vegetative development of plants (Mortimer *et al.* 2005). Calculation of MEI enables showing that positive effects of AMF inoculation disappeared at 28°C/18°C, suggesting that increased temperature diminished the effectiveness of mycorrhizal symbiosis for improving vegetative growth (Figure 1). Our results agree with those reported by Bunn et al. (2009), which reduction of plant growth in high temperatures allocated more carbon to fungal structures. In our case, this effect was strongly dependent of accession as showed by significant interaction of between temperature and accession ($P<0.001$) (Figure 1).

Phenolic compounds

The concentration of total soluble phenolic compounds was strongly modified by temperature and AMF inoculation but the extent of effects was accession-dependent (Figure 2). Thus, the combination of elevated temperature and AMF inoculation significantly improved phenolic content of leaves in short-cycle accessions. This positive effect of AMF inoculation of leaf phenolic content was also reported by other authors and was attributed mainly to improved concentrations of flavonols (Eftekhari et al., 2012). By contrast, AMF inoculation of plants grown at 28°C/18°C did no lead to increased phenolic content in long-cycle accessions (Figure 2). The effect of AMF on the content of total phenols is contradictory in the literature. For instance, Toussaint et al. (2007) showed the potential of AMF to enhance the production of phenolic compounds in sweet basil leaves, but Geneva et al. (2010) observed decreased concentrations of total phenols in leaves of *Salvia officinalis* associated with *R. intraradices*.

It was well established that FLAV index and FERARI index measured though Multiplex3^{TM} sensor is a good indicative of flavonol and anthocyanin contents of tissues (Baluja et al., 2012; Agati et al., 2013). Our data revealed that the temperature was the main factor modulating flavonol content of leaves as showed by two-factor ANOVA (Table 3). Although at 28°C/18°C temperature regime AMF inoculation reduced flavonol content in some accessions (CL-1089 and CL-280), the combination of elevated temperature and AMF inoculation improved flavonol content in a half of accessions analysed (CL-260, CL-1048 and CL-843). Our results agree partially with those other authors that showed that leaves of mycorrhizal grapevines have higher flavonol content than non-mycorrhizal plants (Eftekhari et al., 2012); however, our data revealed that the positive effect of AMF inoculation varied in function of accession studied and could be mediated by interaction with elevated temperature. These findings open new perspectives for reuse of grapevine wastes because some recent researches have shown that flavonols present in

vegetative organs of grapevines inhibited different cancer cell growth (Apostolou et al., 2013; Sahpazidou et al., 2014).

The FERARI index is indicative of the anthocyanin content of leaves (Table 3). In general, temperature was the main factor modulating anthocyanin content of grapevine leaves. Besides, at 28°C/18°C, AMF inoculation improved anthocyanin content in all short-cycle accessions but this effect was not observed in long-cycle accessions. It is well documented that high temperature results in the reduction of anthocyanin content of berries that could be caused by chemical and/or enzymatic degradation (Mori et al., 2007, Cohen et al., 2008); our data contrast with these findings, but no data was yet reported analyzing the effects of elevated temperatures in grapevine leaves. However, the beneficial role of AMF inoculation on anthocyanin content detected in the present work was also showed in strawberry (Lingua et al., 2013) and lettuce (Baslam et al., 2011, 2013; Baslam and Goicoechea, 2012).

Antioxidant properties

Similarly to total phenolic content, the antioxidant activity evaluated by using the DPPH assay changed according to temperature and AMF inoculation in an accession-dependent way (Figure 3). In general, results suggest a role of AMF inoculation in improvement of antioxidant activity of foliage of grapevine grown at 28°C/18°C temperature regime. Thus, the combination of elevated temperature and AMF inoculation stimulated antioxidant activity in all short-cycle accessions but no changes were observed in long-cycle accessions. In consequence, two-factor ANOVA indicated significant interactions between temperature and AMF for most of accessions.

A correlation analysis was done among the phenolic compounds and the antioxidant capacity for all Tempranillo accessions (Figure 4). Combining all measurements data revealed that improved antioxidant activity from leaf extracts was significantly correlated with higher phenolic content ($R = 0.58$, $P < 0.001$) (data not shown). This result is in agreement with other reports in the literature (Kallithraka et al., 2009; Xu et al., 2010; Król et al., 2014). However, other authors did not find any correlation between both parameters, indicating that antioxidant potential was due to specific phenols (Apostolou et al., 2013; Sahpazidou et al., 2014). Such contrasting results could be attributed to the different grapevine varieties used for obtaining the extracts and that were subjected to different environmental conditions during growth. This idea was supported by the present study that showed different relationships between total antioxidant activity and phenolic content of leaf extracts from accessions grown in the same controlled conditions (Figure 4). These correlations were significant in CL-260, CL-1048, CL-1089 and CL-843 accessions but were no significant in CL-8 and CL-280.

It would be interesting to consider if the observed improvement of phenolic content and antioxidant activity by AMF inoculation at moderate high temperatures used in this study will be loss at higher temperatures. Indeed, some research showed decreases in mycorrhizal activity with warm temperatures and increases in carbon allocation to fungi which can lead to increased respiration (Martin and Stutz, 2004; Hawkes et al., 2008; Mohan et al., 2014) or to increased growth of the extraradical mycelium (Heinemeyer et al. 2006). In consequence, the advantages to the host plant could disappear by high carbon cost resulting in low available carbon for phenolic biosynthesis. However, this could not implicate necessarily that antioxidant activity were diminished because compounds other than phenolics (i.e., ascorbic acid, glutathione, alkaloids, tocopherol and carotenoids) also contribute to antioxidant activity (Apel and Hirt, 2004; Noctor, 2006). In our study, this possibility may be applied to the accessions in which the total antioxidant capacity was not significantly correlated with the phenolic content in leaves (CL-8, CL-280) (Figure 4). In addition, it has been reported that AMF may protect plants against temperatures up to 30°C and even 40°C by different ways including increasing antioxidant activity and antioxidant compounds as ascorbic acid (Zhu et al., 2011; Aktar Maya and Matsubara, 2013). However, the effects can depend on plant-fungus combinations (Baslam et al., 2011; 2013; Aktar Maya and Matsubara, 2013).

Conclusions

This study provides evidence of intra-varietal-dependent response of phenolic composition and antioxidant properties of leaf extracts to mycorrhizal inoculation under elevated temperatures applied during berry ripening in Tempranillo grapevines. The most striking result that emerges from data was that antioxidant compounds were improved with mycorrhizal inoculation at 28°C/18°C temperature conditions, which showed higher levels of total soluble phenolics, flavonols and anthocyanins even when mycorrhizal colonization of grapevine roots did not achieve high rates. This increase of antioxidant compounds content was highly correlated with enhanced total antioxidant activity. The combination of the modulator factors mycorrhizal inoculation and elevated temperatures could exert an additive effect in improving phenolic synthesis and antioxidant power of grapevine leaf wastes for pharmaceutical uses. In addition, some accessions showed a particularly interesting combination of attributes for human health applications. To our knowledge, this is the first study reporting intra-varietal diversity of phenolic content and antioxidant activity in leaves of Tempranillo grapevines as well as the effect of the interaction between mycorrhizal inoculation and temperature on the accumulation of secondary metabolites in leaves of grapevine.

Conflict of interest

Authors have no conflict of interest to declare.

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Table 1. Tempranillo accessions used in this study (Rioja Alavesa, Spain).

Number of accession	Agronomic traits	City of origin (region)
CL-260	Short cycle-Low yield	San Vicente de la Sonsierra (La Rioja)
CL-1048	Short cycle-Medium yield	Laguardia (Álava)
CL-1089	Short cycle-High yield	Bargota (Navarra)
CL-8	Long cycle-Low yield	Sorzano (La Rioja)
CL-280	Long cycle-Medium yield	Villalba de Rioja (La Rioja)
CL-843	Long cycle-High yield	Oyón (Álava)

Table 2. Phenological cycle expressed as days from fruit set (onset of temperature treatments) to harvest (commercially ripe berries) 5 and leaf growth recorded from fruiting cuttings of Tempranillo accessions inoculated (+M) or non-inoculated (-M) with arbuscular mycorrhizal fungi and grown either at 24°C/14°C or 28°C/18°C temperature regimes.

Treatment	Phenology (days)						Leaf area (m^2 $plant^{-1}$)					
	Short cycle			Long cycle			Short cycle			Long cycle		
	CL-260	CL-1048	CL-1089	CL-8	CL-280	CL-843	CL-260	CL-1048	CL-1089	CL-8	CL-280	CL-843
-M24	105.7 a	111.6 a	100.9 ab	104.5 a	110.0 a	102.8 a	0.59 ab	0.86 a	0.80 a	0.72 a	0.82 a	0.83 a
+M24	99.9 ab	107.5 a	103.3 a	99.4 ab	114.8 a	105.0 a	0.70 a	0.66 ab	0.55 b	0.58 ab	0.81 a	0.58 ab
-M28	87.6 b	90.9 b	87.7 b	93.0 ab	94.5 b	90.6 b	0.34 b	0.42 b	0.45 b	0.37 b	0.43 b	0.51 b
+M28	94.1 ab	91.4 b	88.7 b	87.8 b	92.1 b	91.1 b	0.37 b	0.50 b	0.46 b	0.48 ab	0.41 b	0.38 b
Temperature (T)	*	*	**	**	***	***	**	***	**	*	***	*
Mycorrhization (M)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
TxM	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Values represent means (n = 4-6). Within each column and accession, means followed by different letter are significantly different ($p<0.05$) according to Duncan test. ns, *, ** and *** indicate non-significance or significance at 5%, 1% and 0.1% probability levels, respectively.

Table 3. Flavonol content (FLAV index) and anthocyanin content (FERARI index) measured with the Multiplex3TM in leaves from fruiting cuttings of Tempranillo accessions inoculated (+M) or non-inoculated (−M) with arbuscular mycorrhizal fungi and grown either at 24°C/14°C or 28°C/18°C temperature regimes.

Treatment	FLAV						FERARI					
	Short cycle			Long cycle			Short cycle			Long cycle		
	CL-260	CL-1048	CL-1089	CL-8	CL-280	CL-843	CL-260	CL-1048	CL-1089	CL-8	CL-280	CL-843
-M24	0.01 c	0.01 c	0.01 b	0.01 b	0.04 b	0.01 b	0.80 b	0.96 b	0.76 c	0.84 ab	0.78 b	0.80 a
+M24	0.04 bc	0.15 b	0.01 b	0.01 b	0.03 b	0.01 b	0.88 ab	0.89 b	0.81 b	0.90 a	0.74 b	0.85 a
-M28	0.17 a	0.25 a	0.33 a	0.12 a	0.28 a	0.22 a	0.79 b	0.81 b	0.94 a	0.85 ab	0.88 a	0.84 a
+M28	0.27 a	0.30 a	0.05 b	0.20 a	0.02 b	0.20 a	0.97 a	1.01 a	0.96 a	0.76 b	0.74 b	0.85 a
Temperature (T)	***	***	***	***	*	***	ns	***	***	*	ns	ns
Mycorrhization (M)	ns	**	**	ns	**	ns	***	ns	*	ns	**	ns
TxM	ns	ns	*	ns	ns	ns	ns	*	ns	*	ns	ns

Values represent means (n = 4-6). Within each column and accession, means followed by different letter are significantly different ($p<0.05$) according to Duncan test. ns, *, ** and *** indicate non-significance or significance at 5%, 1% and 0.1% probability levels, respectively.

Figure legends

Figure 1. Mycorrhizal efficiency index (MEI) from fruiting cuttings of Tempranillo accessions grown either at 24°C/14°C or 28°C/18°C temperature regimes during berry growth and ripening. Values represent means ± S.E. (n=4-6). Within each accession, different letters indicate significant differences ($p<0.05$) between treatments according to Duncan test. One-way ANOVA analysis to evaluate the temperature (T), accession (A) and interaction (TxA) effects was performed. *** indicate significance at 0.1% probability level.

Figure 2. Total phenols of leaves from fruiting cuttings of Tempranillo accessions inoculated (+M) or non-inoculated (-M) with arbuscular mycorrhizal fungi and grown either at 12 24°C/14°C or 28°C/18°C temperature regimes during berry ripening. Values represent 13 means ± S.E. (n=4-6). Within each accession, different letters indicate significant differences ($p<0.05$) between treatments according to Duncan test. Two-way ANOVA analysis to evaluate the temperature (T), presence of mycorrhizal fungi (M) and interaction (TxM) effects was performed. ns, *, ** and *** indicate non-significance or significance at 5%, 1% and 0.1% probability levels, respectively.

Figure 3. Total antioxidant activity of leaves from fruiting cuttings of Tempranillo accessions inoculated (+M) or non-inoculated (-M) with arbuscular mycorrhizal fungi and grown either at 24°C/14°C or 28°C/18°C temperature regimes during berry ripening. Values represent means ± S.E. (n=4-6). Within each accession, different letters indicate significant differences ($p<0.05$) between treatments according to Duncan test. Two-way ANOVA analysis to evaluate the temperature (T), presence of mycorrhizal fungi (M) and interaction (TxM) effects was performed. ns, *, ** and *** indicate non-significance or significance at 5%, 1% and 0.1% probability levels, respectively.

Figure 4. Relationships between antioxidant activity and total phenolic content of leaves from fruiting cuttings of Tempranillo. For each accession, straight lines correspond to the regression lines fitted for the joint data of all treatments. ns indicates non significance.

Figure 1.

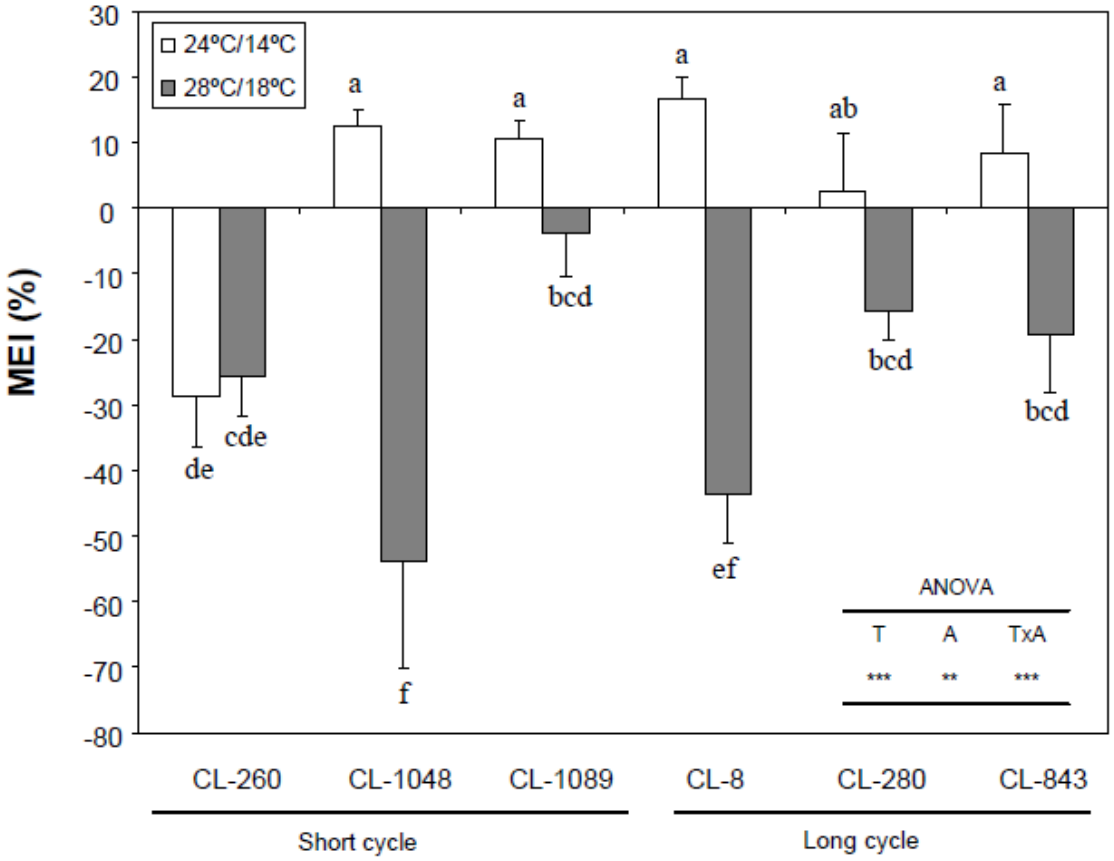

24ºC/14ºC
28ºC/18ºC
MEI (%)
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
a
a
a
ab
a
de
cde
f
bcd
ef
bcd
bcd
ANOVA
T
A
TxA
***
**
***
CL-260
CL-1048
CL-1089
CL-8
CL-280
CL-843
Short cycle
Long cycle

Figure 2.

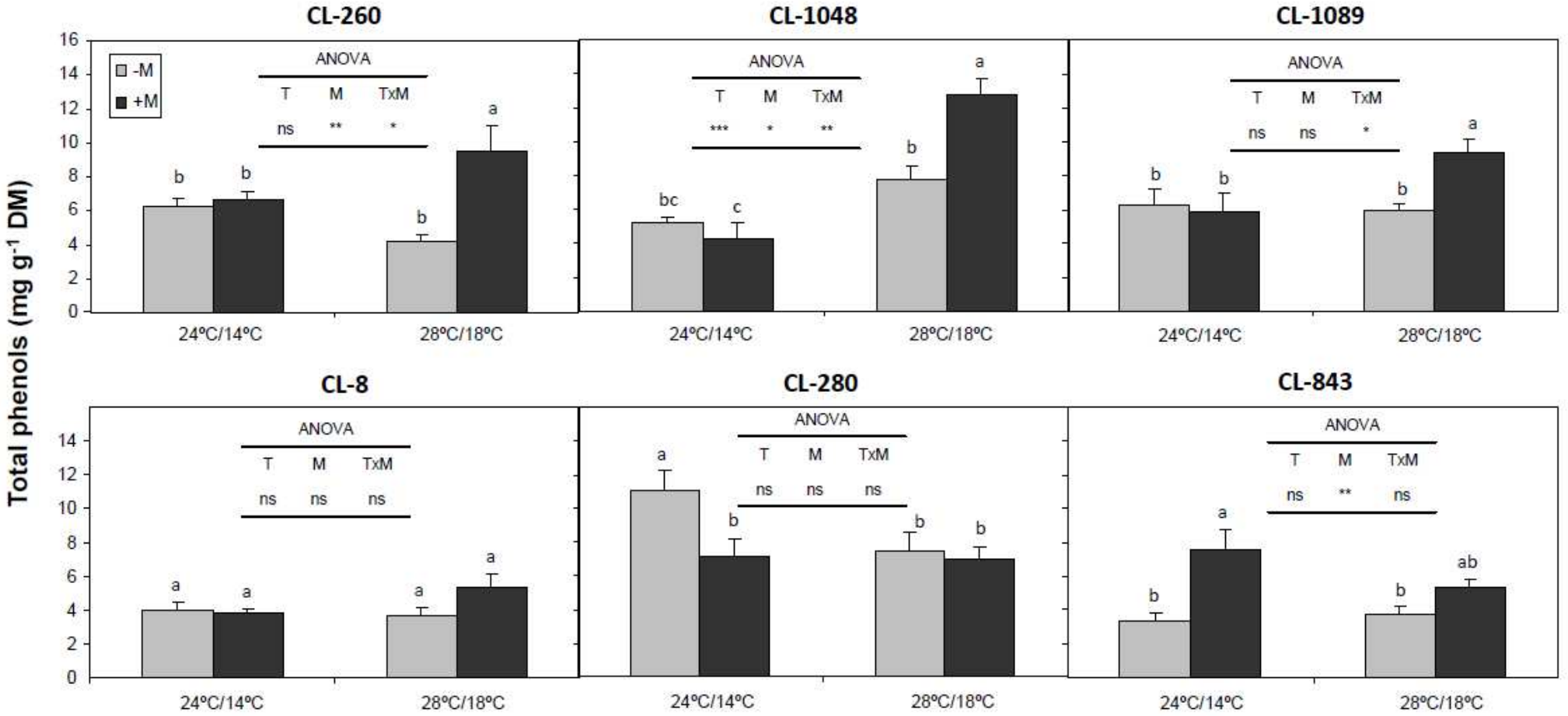

CL-260
CL-1048
CL-1089
CL-8
CL-280
CL-843
-M
+M
ANOVA
T M TxM
ns ** *
*** * **
ns ns *
ns ns ns
ns ** ns
Total phenols (mg g-1 DM)
24ºC/14ºC
28ºC/18ºC

Figure 3.

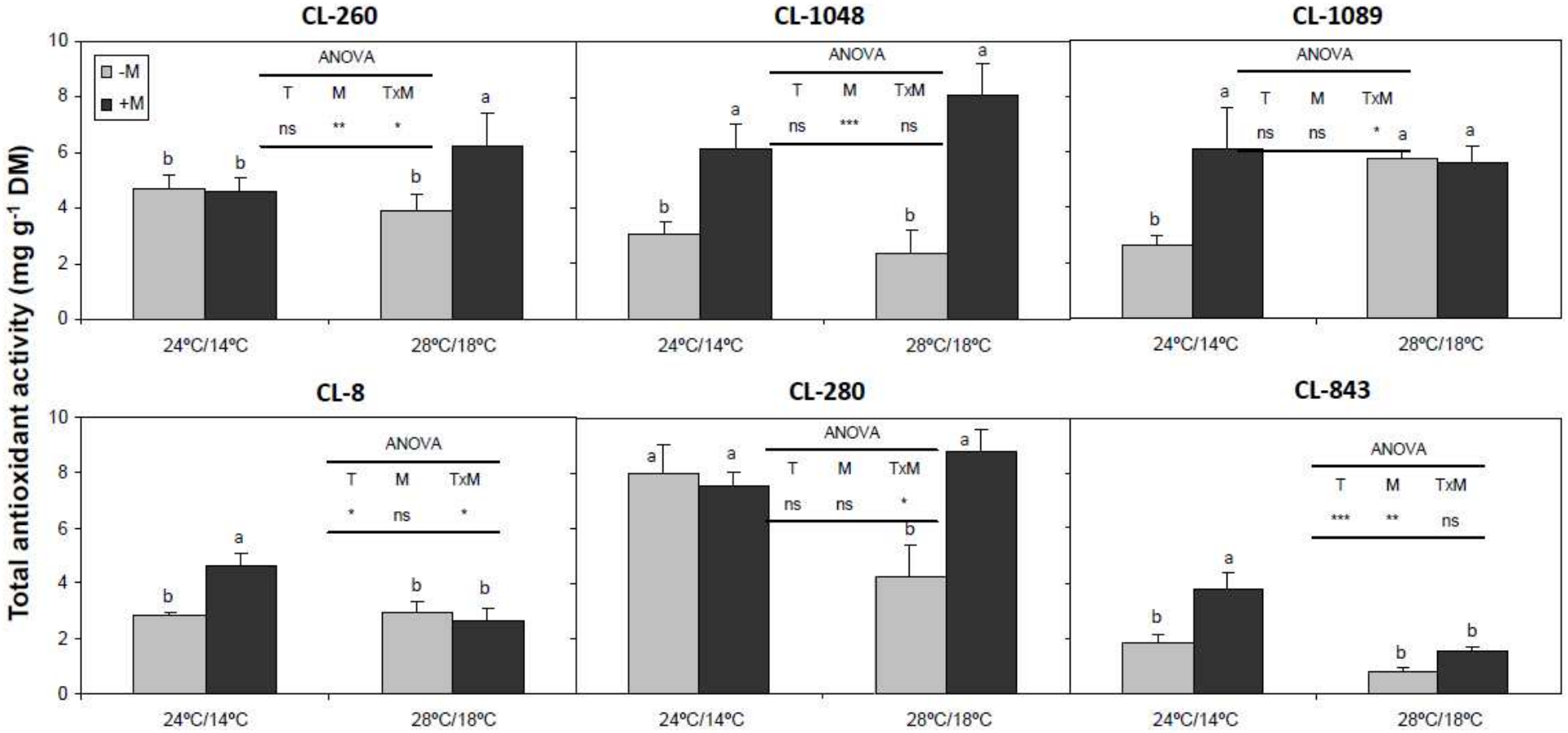
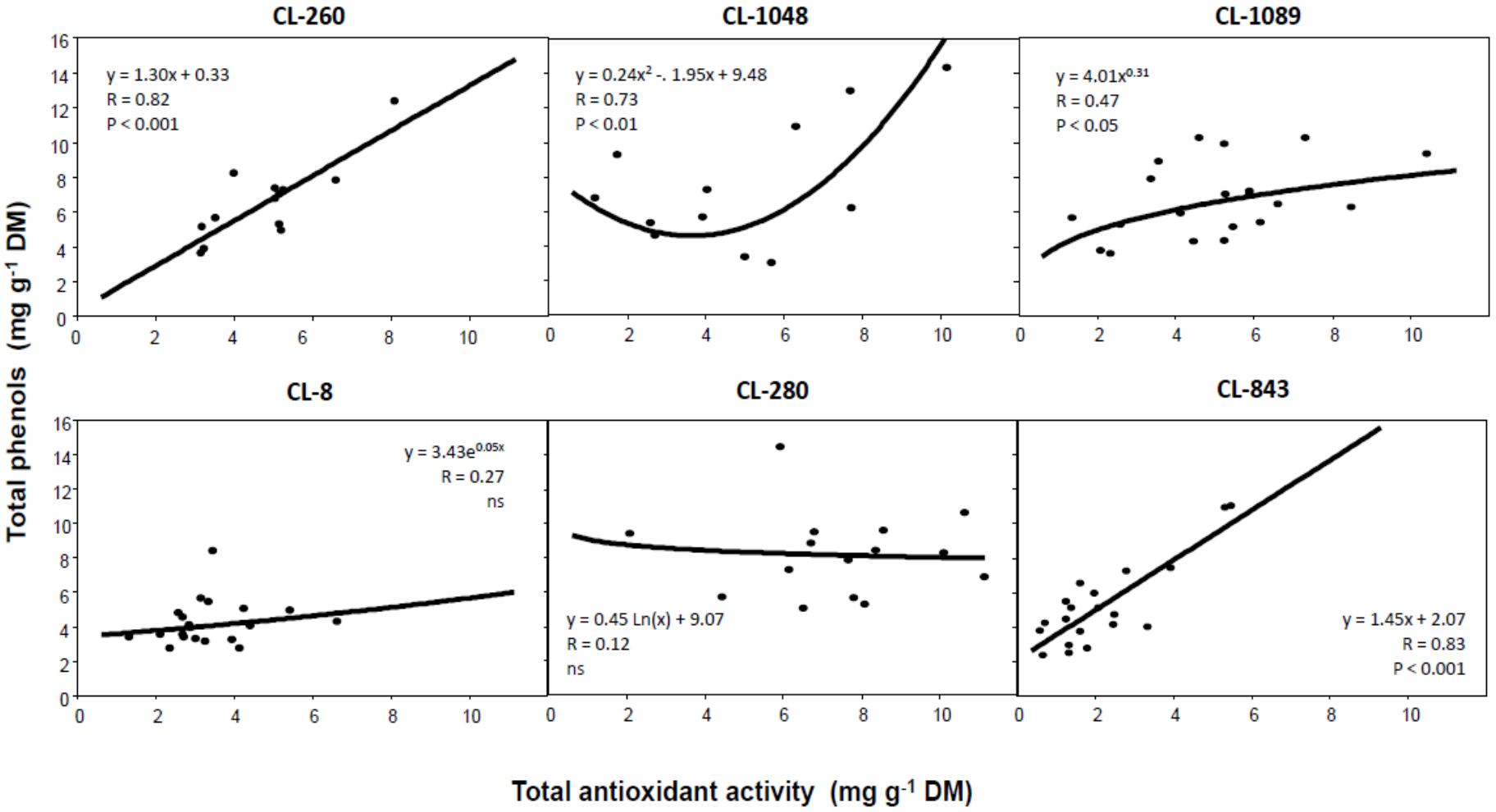

CL-260
CL-1048
CL-1089
CL-8
CL-280
CL-843
-M
+M
ANOVA
T M TxM
ns ** *
ns *** ns
ns ns *
* ns *
ns ns *
*** ** ns
Total antioxidant activity (mg g-1 DM)
24ºC/14ºC
28ºC/18ºC
0
2
4
6
8
10

Figure 4.


CL-260
y = 1.30x + 0.33
R = 0.82
P < 0.001
CL-1048
y = 0.24x² -. 1.95x + 9.48
R = 0.73
P < 0.01
CL-1089
y = 4.01x^0.31
R = 0.47
P < 0.05
CL-8
y = 3.43e^0.05x
R = 0.27
ns
CL-280
y = 0.45 Ln(x) + 9.07
R = 0.12
ns
CL-843
y = 1.45x + 2.07
R = 0.83
P < 0.001
Total phenols (mg g-1 DM)
Total antioxidant activity (mg g-1 DM)

CAPÍTULO 6

Nutritional properties of Tempranillo grapevine leaves are affected by clonal diversity, mycorrhizal symbiosis and air temperature regime. Submitted to *Plant Physiology and Biochemistry* (under review)

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

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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. 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 different applications for leaves 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; DM = dry matter; -M = non-mycorrhizal plants; +M = mycorrhizal plants; Pro = proline; T = temperature; TAC = total antioxidant capacity; TSP = total soluble proteins; TSS = total soluble sugars

1. Introduction

The 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) or as an ingredient for the preparation of dishes for human consumption, especially in rural regions of some Mediterranean areas, such as Turkey, Greece and Middle East countries (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. 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 the potential application of these vegetative residuals in the human diet.

2. Materials and methods

2.1. Biological material

Three-node segments of *Vitis 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). 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 (*Septglomus deserticola*, *Funneliformis mosseae, Rhizoglomus intraradices, Rhizoglomus clarum* and *Glomus aggregatum*), containing 100 spores per g of inoculum and a mixture of rhizobacteria belonging to the genera *Bacillus* and *Paenibacillus* (2 x 10^6 cfu g^{-1}). 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). 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 mycorrhizal colonization

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 plate intersection method (Giovannetti and Mosse, 1980).

2.4. Fluorimetric sensor measurements in leaves through berry ripening

The evolution of epidermal levels of chlorophylls, nitrogen, 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 dry matter, DM) 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 DM 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 DM.

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 normality of the data with the Kolmogorov-Smirnov normality test due to the small sample size (n= 4), data were subjected to a two-way analysis of variance (ANOVA) within each clone. 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≤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. Mycorrhizal colonization at fruit harvest (E-L38)

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 1).

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 epidermal 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 1). 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 1), 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 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 1). 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 (*Septglomus deserticola*, *Funneliformis mosseae, Rhizoglomus intraradices, Rhizoglomus clarum* and *Glomus 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 2). 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 2). 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. 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 2). 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 2; 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} DM) was more than double than that in leaves of CL-1089 (below 20 mg g^{-1} DM) (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 1), 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} DM) was found in leaves of non-mycorrhizal plants (-M24) belonging to CL-1048 (Table 3). However, this concentration was reduced by half (29.14 mg g^{-1} DM) 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} DM at 24/14°C till 26.34 mg g^{-1} DM 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} DM 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} DM (Table 3). 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 3). 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 3), 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 3), 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 3). By contrast, the association of grapevine with AMF clearly induced the accumulation of these phenolic compounds in leaves from CL-1089 (Table 3). 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).

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 3). 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 33.48 % of the total variance and it clearly separated temperature treatments (Fig. 4A), some minerals (Ca, Mg, N, P, Mn and Cu) 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 18.48% 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

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 would provide an additional improvement of the nutritional value of leaves because it can induce the accumulation of flavonols in these three clones.

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Table 1. Evolution of epidermal level of chlorophylls (SFR_G), nitrogen balance index (NBI_1), anthocyanins (ANTH_RG) and 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

Table 1 *(Continued)*

		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

Table 1 *(Continued)*

		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 = 4-6) 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 2. Mineral composition 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} DM)	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} DM)	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

Table 2 *(Continued)*

		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} DM)	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} DM)	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	**

Table 2 *(Continued)*

		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} DM)	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} DM)	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 = 4-6) 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 ($P > 0.05$).

Table 3. 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} DM)	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} DM)		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} DM)	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} DM)		1.67	2.85	1.94	2.60	2.26	2.27	1.80 b	2.73 a	ns

Table 3 *(Continued)*

		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} DM)	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} DM)		1.62	1.90	2.24	2.51	1.76 b	2.37 a	1.93	2.20	ns

Values represent means (n = 4-6) 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 ($P > 0.05$).

Figure legends

Figure 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 = 4). 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.

Figure 2. Total soluble proteins (TSP) (mg g^{-1} DM) (A), proline (Pro) (µmol g^{-1} DM) (B), total soluble sugars (TSS) (mg g^{-1} DM) (C), and starch (mg g^{-1} DM) (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 = 4). 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 ≤ 0.05) according to Duncan's test. DM = dry matter.

Figure 3. Total chlorophylls (a+b) (mg g^{-1} DM) (A) and total carotenoids (mg g^{-1} DM) (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 = 4). 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 ≤ 0.05) according to Duncan's test. DM = dry matter.

Figure 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 (means of 48 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.

Figure 1.

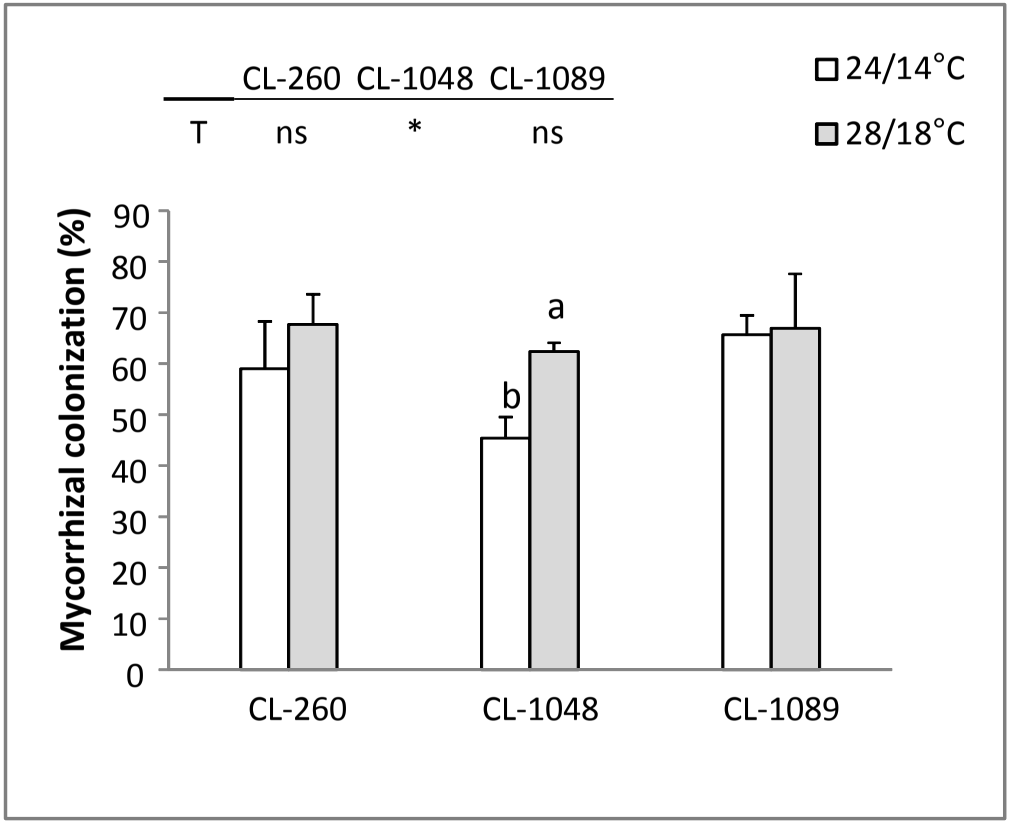
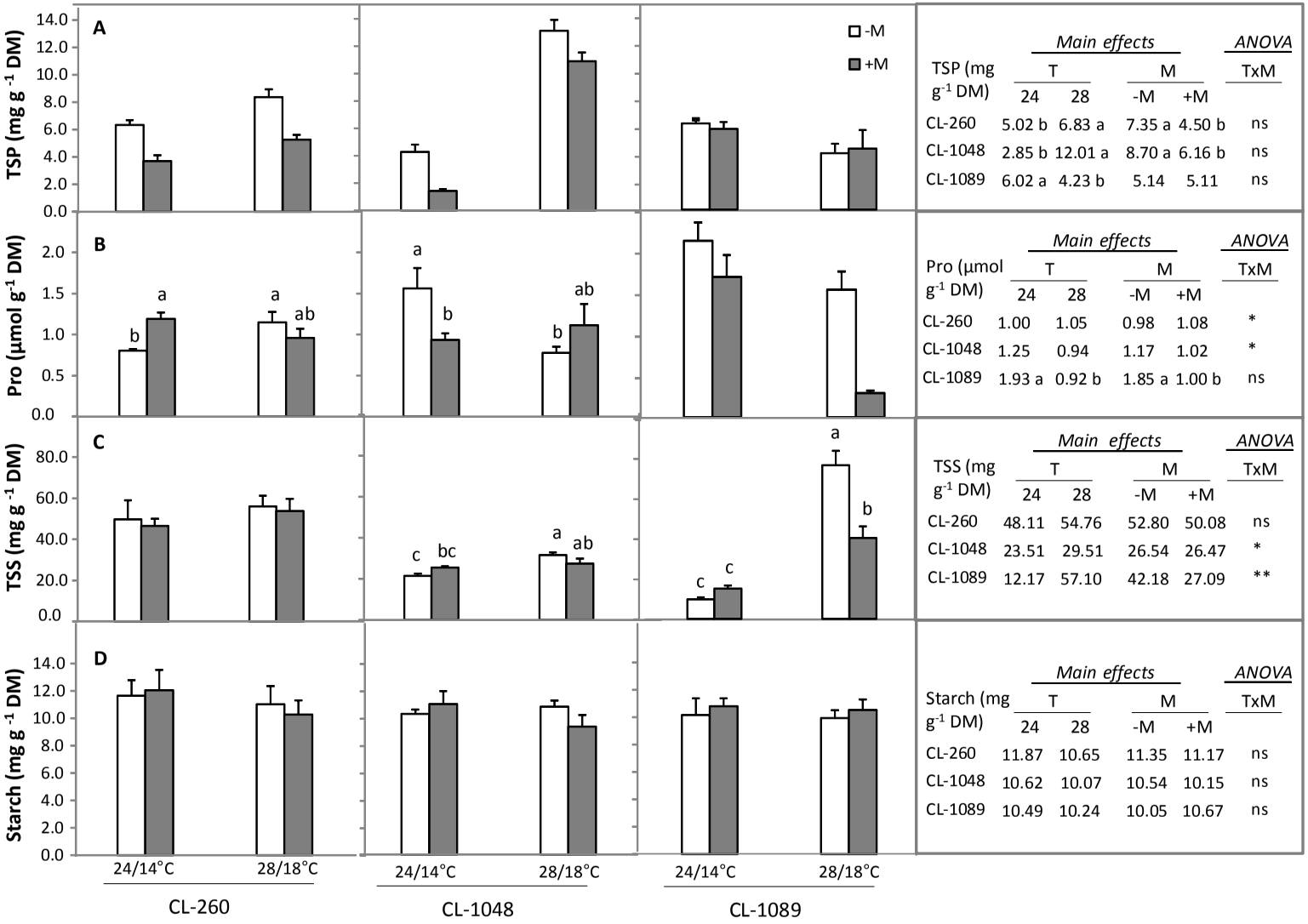

CL-260 CL-1048 CL-1089
T ns * ns
24/14°C
28/18°C
Mycorrhizal colonization (%)
90
80
70
60
50
40
30
20
10
0
a
b
CL-260
CL-1048
CL-1089

Figure 2.



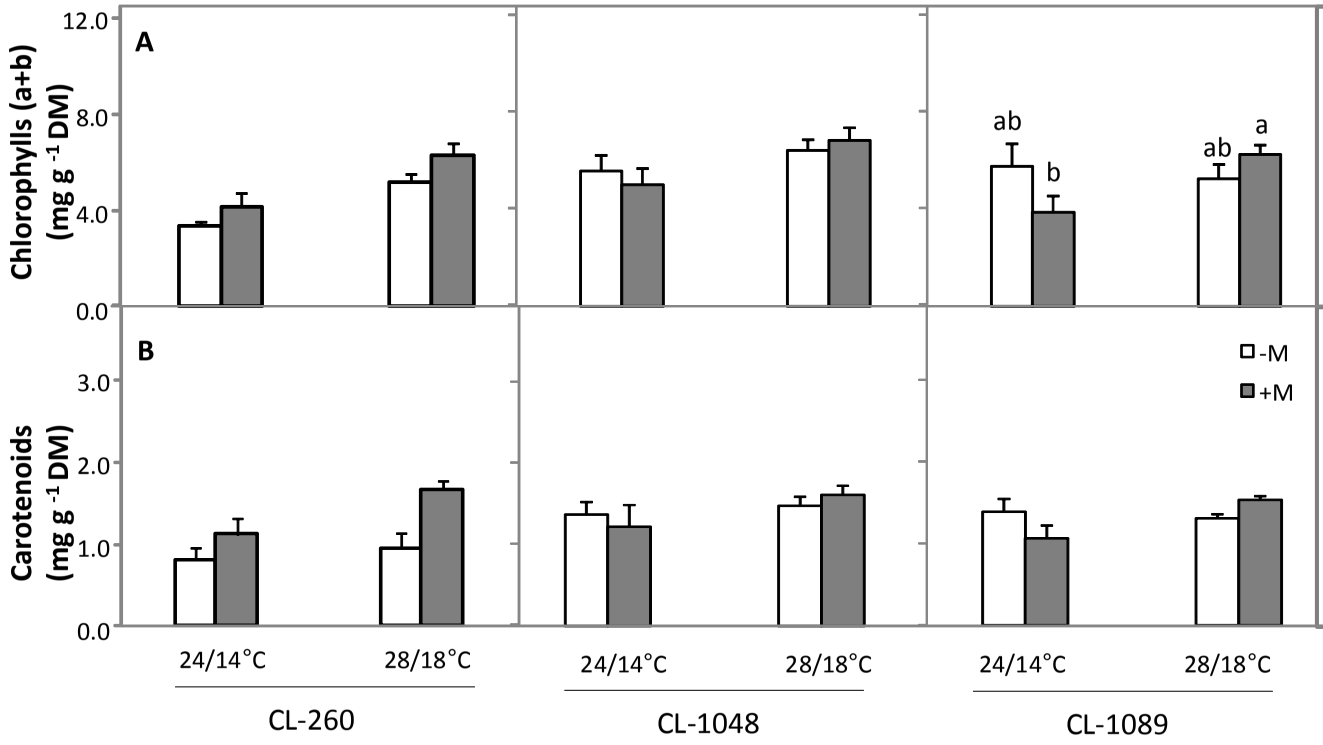
TSP (mg g^{-1} DM)	Main effects T 24	T 28	M -M	M +M	ANOVA TxM
CL-260	5.02 b	6.83 a	7.35 a	4.50 b	ns
CL-1048	2.85 b	12.01 a	8.70 a	6.16 b	ns
CL-1089	6.02 a	4.23 b	5.14	5.11	ns

Pro (µmol g^{-1} DM)	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} DM)	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} DM)	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

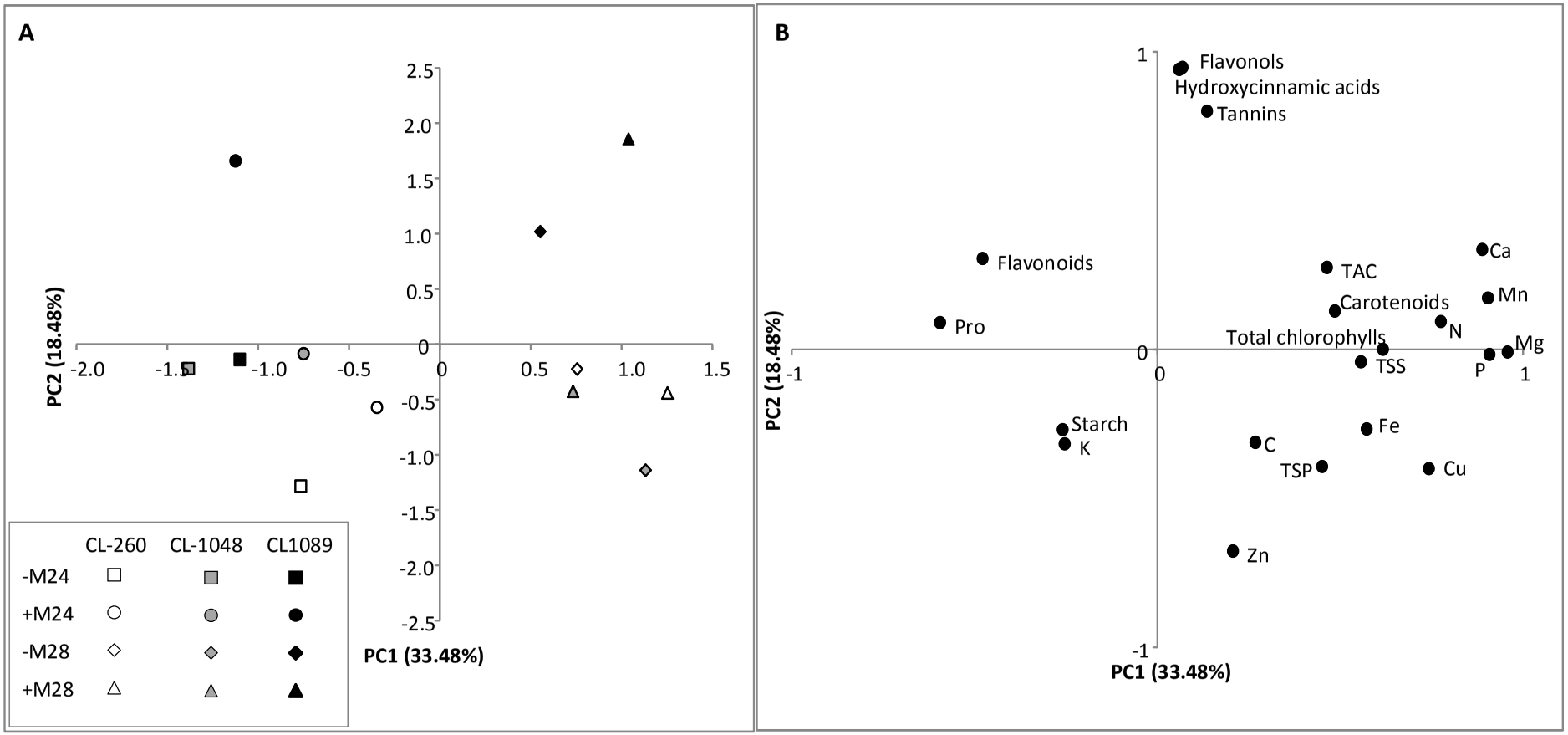
Figure 3.



Chlorophylls (mg g^{-1} DM)	*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} DM)	*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 (18.48%)
PC1 (33.48%)
CL-260
CL-1048
CL1089
-M24
+M24
-M28
+M28
B
Flavonols
Hydroxycinnamic acids
Tannins
Flavonoids
Pro
TAC
Carotenoids
Total chlorophylls
Ca
Mn
N
Mg
TSS
P
Starch
K
C
Fe
TSP
Cu
Zn
PC2 (18.48%)
PC1 (33.48%)

CAPÍTULO 7

Potential biomedical reuse of vegetative residuals from mycorrhized grapevines subjected to warming.

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Potential biomedical reuse of vegetative residuals from mycorrhized grapevines subjected to warming

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Abstract

Grapevine leaves are by-products widely discarded in open fields despite their known antioxidant properties and potential reuse as organic fertilizers or for human or animal nutrition. This study tests the cytotoxicity of leaf extracts from Tempranillo grapevine against four human cancer cell lines and assesses the effect of air temperature and mycorrhizal association on the cytotoxic activity. Leaf extracts were obtained from three clones (CL-260, CL-1048, CL-8) of *Vitis vinifera* L. cv. Tempranillo cultivated at either ambient (24/14°C) or elevated (28/18°C) day/night temperatures, and inoculated (+M) or not (-M) with arbuscular mycorrhizal fungi (AMF). MTT assays were performed to analyze the cytotoxicity against colon (HT-29) and breast (MCF-7) adenocarcinomas, lung carcinoma (HTB-54), and lymphoblastic leukemia (CCRF-CEM). The increase of four degrees in the air temperature enhanced the cytotoxicity of leaf extracts from CL-260 against HT-29, CCRF-CEM and HTB-54 and that from CL-8 against MCF-7. Mycorrhization improved the cytotoxicity of leaf extracts from CL-1048 against HT-29, CCRF-CEM, HTB-54 and MCF-7. The cytotoxic activities of extracts from CL-260 against HTB-54 and from CL-1048 against HT-29 were correlated, respectively, with total phenols and total antioxidant capacity. Levels of phenols and antioxidant activity, however, did not completely explain the cytotoxicity of Tempranillo leaf extracts. We conclude that the predicted increase in the air temperature for the future climate change and the association of grapevines with AMF may enhance the cytotoxicity of leaves, which strengthens the potential application of these agricultural residuals for biomedical purposes. The clonal diversity in the response to AMF and air temperature, however, highlights the importance of choosing the most adequate Tempranillo clone for a concrete environmental scenario.

Keywords: arbuscular mycorrhizal fungi, climate change, cytotoxicity, DPPH assay, vegetative by-products, MTT assay, phenols, *Vitis vinifera*

1. Introduction

The agricultural sector produces huge amounts of wastes known as 'vegetative agricultural residuals (VAR)' with high disposal costs imposed on the producers (Raviv et al., 2018). Grapes are one of the most important crops in Europe with a production of 23.7 million tons in 2016 (Eurostat, 2017) and their cultivation generates high volumes of by-products such as stems and leaves, usually discarded in the field. Only a small portion of these vegetative residuals are used as animal feed and/or in the production of natural organic fertilizers (Arvanitoyannis et al., 2006), although their use as feedstock of an economic feasible waste management may transform them into a valuable resource (Raviv et al., 2018). Only in some Mediterranean countries grapevine leaves are consumed in the human diet (Abed et al., 2015) in spite of having demulcent, laxative, refrigerant, stomachic, diuretic and cooling effects (El-Hawary et al., 2012). Moreover, grapevine leaves have shown antihyperglycaemic, antioxidant, anti-inflammatory, analgesic and antipyretic activities (Orhan et al., 2006; Aouey et al., 2016). Other *in vitro* studies have demonstrated effective cytotoxic activity of grapevine leaves against breast (Esfahanian et al., 2013), leukemia (Handoussa et al., 2013) and lung (Abed et al., 2015) cancer cells. Many of these properties have been attributed to the phenolic compounds accumulated in grapevine leaves (Handoussa et al., 2013; Aouey et al., 2016; Lima et al., 2016). Phenolic metabolism in tissues can be strongly modified in response to biotic and environmental factors. Esfahanian et al. (2013) observed increased cytotoxic activity for leaf extracts obtained from virus infected grapevines and suggested that this effect could be due to the high levels of polyphenols in the infected leaves. Within biotic factors, special attention should be paid to the arbuscular mycorrhizal fungi (AMF), which normally colonize grapevine roots in the field (Balestrini et al., 2010). Mycorrhizal symbiosis can stimulate key genes of the phenylpropanoid biosynthesis in grapevine (Bruisson et al., 2016) thus inducing the accumulation of phenolic compounds (Eftekhari et al., 2012). Phenylalanine ammonia-lyase

(PAL) catalyzes the deamination of the aminoacid phenylalanine to give cinnamic acid, which is the first step in the phenylpropanoid pathway. *PAL* gene expression is responsive to biotic and abiotic environmental stimuli, including extreme temperatures (Dixon and Paiva, 1995). Global temperature average is predicted to increase between 1.8 and 4.0°C by the end of the present century (IPCC, 2014), which presumably will influence the synthesis and levels of phenolic substances in plant tissues. Torres et al. (2015) found that both phenolic content and antioxidant activity of grapevine leaves were stimulated by the combination of elevated temperatures and mycorrhizal inoculation, although results were highly dependent on the intra-varietal diversity of grapevine.

Taking all together, our hypothesis was that leaves of grapevines associated with AMF and/or grown under elevated temperatures may have improved cytotoxic potential against cancer cells by means of an enhanced accumulation of phenolic compounds, which would increase their interest to be reused for pharmacological and biomedical purposes.

2. Materials and methods

2.1. Biological material and growth conditions

Dormant *Vitis vinifera* (L.) cuttings of Tempranillo with 400-500 mm long were obtained in the winter of 2014 from an experimental vineyard of the Institute of Sciences of Vine and Wine (Logroño, Spain) (Denomination of Origin Rioja, North of Spain). Three clones (CL-260, CL-1048, and CL-8) with different agronomic traits (Table 1) and whose leaves showed increased phenolic content and antioxidant activity when subjected to elevated temperature and/or mycorrhizal inoculation (Torres et al., 2015) were chosen for the study.

Fruit-bearing cuttings were obtained according to steps originally outlined by Mullins (1966) and modified by Ollat et al. (1998) and Antolín et al. (2010). Rooting was made in a

heat-bed (27°C) kept in a cool room (4°C). One month later, the cuttings were planted in 6.5 L plastic pots containing vermiculite-sand-peat (2.5:2.5:1, v:v:v). Peat (Floragard, Vilassar de Mar, Barcelona, Spain) had a pH of 5.2-6.0, 70-150 mg L^{-1} of N, 80-180 mg L^{-1} P_2O_5 and 140-220 mg L^{-1} K_2O and it was previously sterilized at 100°C for 1 h on three consecutive days.

At transplanting, half of the plants (+M plants) were inoculated with the mycorrhizal inoculum 'GLOMYGEL® vid, olivo, frutales' (Mycovitro S.L., Pinos Puente, Granada, Spain). GLOMYGEL® inoculum derived from an *in vitro* culture of *Rhizophagus intraradices* (Schenck and Smith) Walker & Schüβler comb. nov., and contained inert pieces of roots colonized by AMF, spores and vegetative mycelium as propagules. It was diluted with distilled water in order to have an inoculum with around 250 propagules per mL. Each +M plant received 8 mL of the diluted inoculum close to the roots thus making a total of 2,000 propagules. A similar volume of a filtrate was added to plants that did not receive the mycorrhizal inoculum (-M plants) in an attempt to restore other soil free-living microorganisms accompanying AMF. The filtrate was obtained by passing diluted mycorrhizal inoculum through a layer of 15-20 µm filter paper with particle retention of 2.5 µm (Whatman 42, GE Healthcare Life Sciences, Little Chalfont, UK).

Plants were transferred to glasshouses adapted to simulate climate change conditions (Morales et al., 2014). Initial growth conditions (day/night temperatures and relative humidity, photosynthetic photon flux density, photoperiod, irrigation and fertilization) were those explained by Torres et al. (2015).

2.2. Experimental design

At fruit set (Eichhorn and Lorenz (E–L) fruit stage 27) (Coombe, 1995), 20 fruit-bearing cuttings per clone with 4-5 fully expanded leaves were exposed to two temperature regimes: 24/14°C or 28/18°C (day/night). The 24/14°C temperature regime was selected according to

the average temperatures registered in La Rioja (1971-2000) (AEMET, Spain) during grapevine growing season. The 28/18°C temperature regime was selected according to predictions of a rise of 4.0°C by the end the present century (IPCC, 2014). Both temperature regimes were maintained to harvest that corresponded to commercially ripe berries (approximately 22°Brix) (E-L 38 stage). Harvested leaves were frozen at -80°C for further analysis. To avoid excessive soil warming sides of pots were covered with a reflecting material (Passioura, 2006).

2.3. Mycorrhizal colonization

Root samples were cleared and stained (Phillips and Hayman, 1970) and mycorrhizal colonization was determined by examining 1 cm root segments (n = 45 per pot) under the microscope. Extension, incidence and intensity of mycorrhizal colonization were calculated as described by Torres et al. (2015) and results were expressed as percentage of infection (Hayman et al., 1976).

2.4. Preparation of foliar extracts

Extracts were obtained according to Esfahanian et al. (2013) with some modifications. Leaves were dried in oven at 70°C and then powdered in liquid nitrogen. Afterwards, 15 g of dry matter (DM) were placed in a stopped conical flask and macerated with 250 mL of 98% (v/v) methanol (Panreac, Spain) at room temperature for three days with stirring. After evaporation, a new extraction was performed with 100 mL of 98% (v/v) methanol for one day. Then, solvent was filtered and evaporated in a vacuum rotary evaporator (Heidolph OB2000, Gemini BV Laboratory, Apeldoorn, Netherlands) at 45°C. Residue was placed in the freeze-dryer (VirTis BenchTop 2K, SP Industries Inc., Warminster, PA, USA) until complete dryness. The crude extract was stored at 4°C in darkness.

2.5. Determination of phenolic compounds and antioxidant activity

Phenolic compounds and antioxidant activity were determined in 100 mg of crude leaf extracts dissolved in 1 mL of 98% methanol and sonicated for 15 min. All determinations were made in triplicate. Absorbance was 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 per gram of extract DM.

2.5.1. Determination of phenolic compounds

Total phenols (TP), flavonols, anthocyanins, and hydroxycinnamic acids were determined by the method of Lima et al. (2016) adapted to grapevine leaves. Briefly, 0.5 mL of diluted extracts was added to the same volume of 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 280, 320, 360 and 520 nm, using gallic acid, caffeic acid, quercetin and malvidin as standards for TP, hydroxycinnamic acids, flavonols, and anthocyanins, respectively. 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 methanol) 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.

2.5.2. Determination of total reducing capacity (TRC) and total antioxidant capacity (TAC) in foliar extracts

Total reducing capacity (TRC) was spectrophotometrically determined at 760 nm with

Folin-Ciocalteau reagent (Waterman and Mole, 1994) using gallic acid as a standard.

Total antioxidant capacity (TAC) was evaluated by the free radical scavenging activity (*α,* *α*-diphenil-*β*-picrylhydrazyl, DPPH) assay (Brand-Williams et al., 1995). The scavenging activity using the free radical DPPH• was evaluated by measuring the variation in absorbance at 515 nm after 30 min of reaction in parafilm-sealed glass cuvettes at 25°C (Espín et al., 2000). The reaction was started by adding 20 µL of each sample to a cuvette containing 80 µM methanol (980 µL) of DPPH• (Llorach et al., 2004) until a final volume of 1 mL. Gallic acid was used as a standard.

2.6. In vitro *cytotoxic assays*

Extracts were obtained by dissolving 25 mg of leaf DM in 1 mL of dimethyl sulfoxide (DMSO), followed by sonication. Then extracts were screened for its cytotoxic activity against five human tumor cell lines provided by the American Type Culture Collection (ATCC, Manassas, VA) and the European Collection of Cell Cultures (ECACC, Porton Down, Salisbury, UK): lymphoblastic leukemia (CCRF-CEM), colon carcinoma (HT-29), lung carcinoma (HTB-54), and breast adenocarcinoma (MCF-7). Moreover, one non-malignant mammary gland derived (184B5) cell line was used to determine the selectivity of Tempranillo leaf extracts. The cell lines CCRF-CEM, HT-29, and HTB-54 were grown in RPMI-1640 medium (Life Technologies, Barcelona, Spain) supplemented with 10% fetal calf serum, 2 mM *L*-glutamine, 100 units mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 10 mM HEPES buffer (pH 7.4). The cell line MCF-7 was grown in EMEM medium (Clonetics™, Lonza Biologics Porriño, S.L., Barcelona, Spain) supplemented with 10% fetal calf serum, 2 mM *L*-glutamine, 100 units mL^{-1} penicillin and 100 µg mL^{-1} streptomycin. The 184B5 cell line was cultured in Hams F-12/DMEM (50:50) supplemented as described by Li et al. (2007).

Cytotoxic effect of leaf extracts was tested at five different concentrations ranging between 15 and 250 µg mL^{-1} by consecutive dilutions with the respective culture medium. Cells were seeded in 96-well plates at a density of 10^4 cells per well and incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. Cytotoxicity assays were performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] method (Encío et al., 2005) with little modifications. Results were obtained from at least 3 independent experiments performed in quadruplicate and the half maximal inhibitory concentration (IC_{50}) was calculated. The selectivity index (SI) for breast adenocarcinoma cells was calculated as the ratio between IC_{50} (184B5) and IC_{50} (MCF-7) values.

2.7. Statistical analyses

Statistics was carried out using the Statistical Package for the Social Sciences (SPSS) (SPSS Inc., Chicago, IL, USA) version 21.0 for Windows. Data were subjected to Kolmogorov-Smirnov normality test due to the small sample size. Data followed a normal distribution; therefore a two-way analysis of variance (ANOVA) within each clone was applied. The test was performed to assess the main effect of the factors temperature (T) (24°C/14°C, 24, and 28°C/18°C, 28) and mycorrhizal inoculation (M) (uninoculated, -M and inoculated, +M) and the interaction between them (T × M). The main factors for data concerning mycorrhizal colonization were temperature (T), and clone (CL); the interaction between both factors (T × CL) was also studied. Means ± standard errors (SE) were calculated and, when the F ratio was significant ($P \leq 0.05$), a Duncan test was applied. Within each cancer cell line and clone, Pearson's analyses were performed to test the effect of TP, TRC and TAC of foliar extracts on their cytotoxic activity (IC_{50}). Significant levels were always set at $P \leq 0.05$.

3. Results and discussion

3.1. Mycorrhizal colonization

Hyphae and fungal vesicles were visible, indicating that the mycorrhizal association was not incipient but mature (Fig. 1A). Colonization rates (which ranged from 10 to 15%) did not significantly differ between clones and were not strongly influenced by the air temperature (Fig. 1B).

3.2. Phenolic compounds and antioxidant activity (TRC and TAC)

Table 2 shows data on phenols, TRC and TAC in Tempranillo foliar extracts. Mycorrhizal symbiosis can promote the accumulation of phenols in leaves of crops such as lettuce (Baslam and Goicoechea, 2012) and grapevine (Eftekhari et al., 2012; Torres et al., 2015), this effect being dependent on the variety, cultivar or clone and also on the incidence of environmental factors such as drought (Baslam and Goicoechea, 2012) or temperature (Torres et al., 2015). In CL-260, mycorrhization increased hydroxycinnamic acids and anthocyanins in leaves, although this effect disappeared under elevated temperatures (Table 2). Warming (28/18°C) and mycorrhizal inoculation (+M) increased, respectively, the levels of flavonols and anthocyanins in leaves of CL-1048. In these clones (CL-260 and CL-1048) the applied biotic (AMF) or abiotic (high temperatures) factors did not strongly affect the foliar accumulation of flavan-3-ols and TP. Contrariwise, the amounts of hydroxycinnamic acids, flavonols, anthocyanins and TP in leaves of CL-8 were reduced by the incidence, alone or in combination, of high temperatures and mycorrhization.

The values of TRC or TAC did not significantly varied in any grapevine clone after applying elevated temperatures and/or AMF inoculation (Table 2). In line with these results, working

with grapevine stem extracts, Apostolou et al. (2013) observed that none of the eighteen identified individual polyphenols was clearly correlated with DPPH radical scavenging activity.

3.3. Cytotoxicity of leaf extracts

Vegetative by-products of vineyards, such as stems and leaves, have been tested for their anticarcinogenic activity, results being very promising for limiting the growth of cells from several types of cancer. While the application of stem extracts from Greek varieties of *V. vinifera* inhibited the growth of liver, cervical, colon, breast, renal and thyroid (Rice-Evans et al., 1996; Sahpazidou et al., 2014) cancer cells, foliar extracts from a variety cultivated in arid regions of Palestine were effective against lung cancer (Abed et al., 2015). In all these studies, plant material was collected from grapevines grown in field. In our study, the cultivation of plants under controlled conditions allows to assess if some abiotic (temperature) or biotic (mycorrhization) factors can modulate the cytotoxic potentiality of grapevine foliar extracts. Results, expressed as IC_{50}, are shown in Table 3.

The cell line CCRF-CEM (lymphoblastic leukemia) was the most sensitive to the application of foliar extracts regardless the clone of Tempranillo and the abiotic or biotic factors applied to grapevines. In this case, IC_{50} values ranged from 22.16 µg mL^{-1} in +M24 from CL-1048 to 45.68 µg mL^{-1} in +M24 from CL-260. These IC_{50} values are clearly lower than those obtained by Abed et al. (2015) working with foliar extracts against lung cancerous cells and to IC_{50} values measured after adding stem extracts to breast, colon, renal and thyroid cancer cells (Sahpazidou et al., 2014). The application of abiotic and biotic factors modulated the cytotoxicity of foliar extracts against lymphoblastic leukemia cells. The study of the main effects (T and M) showed that the IC_{50} value in CL-260 was significantly decreased by elevated temperatures (42.02 µg mL^{-1} at 24/14°C and 24.77 µg mL^{-1} at 28/18°C) and increased by mycorrhizal inoculation (29.13 µg mL^{-1} in –M plants and 37.66 µg mL^{-1} in +M plants). In

contrast, IC_{50} for CCRF-CEM cell line in CL-1048 was lower when applied extracts collected from mycorrhizal (+M, 23.17 µg mL^{-1}) than from non-mycorrhizal (–M, 33.48 µg mL^{-1}) grapevines. In CL-8, both factors interacted significantly (T × M, $P \leq 0.001$), so that mycorrhization only increased IC_{50} value when plants were cultivated at 24/14°C.

The higher values of IC_{50} demonstrate that the cytotoxic effect of foliar extracts from Tempranillo against colon adenocarcinoma (HT-29), lung carcinoma (HTB-54) and breast adenocarcinoma (MCF-7) cell lines was lower than that found against lymphoblastic leukemia (CCRF-CEM). Those values, however, were sometimes reduced by the application of abiotic or biotic factors. In CL-260, elevated temperatures decreased IC_{50} against HT-29 and HTB-54 cell lines and mycorrhizal inoculation enhanced the cytotoxicity of foliar extracts from CL-1048 against HT-29, HTB-54 and MCF-7 cell lines. In CL-8, IC_{50} diminished against MCF-7 cell line when applied high temperatures and against HTB-54 when combined elevated temperatures and mycorrhizal inoculation.

An anticancer compound should be, in first instance, safe for normal cells. The selectivity index (SI) reflects the differential cytotoxicity of a given compound against tumor and normal cells. In our study the SI was performed against the breast cancer cell line (Table 4). Leaf extracts from CL-260 were the most selective, showing values of SI above 2 when obtained from –M plants cultivated at ambient (24/14°C) temperatures, which indicates good selective toxicity (Badisa et al., 2009). Elevated air temperatures decreased the SI of foliar extracts from CL-1048 and the combination of high temperatures and mycorrhizal inoculation reduced the SI in CL-260.

3.4. Correlation between TP, TRC, TAC and cytotoxicity

In order to assess the possible mechanisms underlying the cytotoxic activity of the Tempranillo leaves, Pearson correlation analyses were carried out between TP, TRC, TAC and cytotoxicity for every human cancer cell line and Tempranillo clone (Figs. 2, 3 and 4).

In CL-260 (Fig. 2), negative correlation between TP and IC_{50} was found for three cancer cell lines, correlations being significant in MCF-7 and HTB-54. Working with MCF-7 breast cancer cell line, Lin et al. (2006) concluded that resveratrol (also detected in the phenolic profile of Tempranillo leaf extracts, Fig. S1) can interact with the αVα3 integrin receptor in these cells and induce apoptosis. Furthermore, Nagappan et al. (2016) demonstrated that flavonoids from *Citrus platymamma* caused apoptosis and G2/M arrest of lung cancer cells by activating caspase-3. Xingyu et al. (2016) concluded that quercetin (a flavonol identified in high concentration in Tempranillo leaf extracts, Fig. S1) may suppress lung cancer by inhibiting the aurora B kinase, an enzyme which promotes tumorigenesis and progression.

On the other hand, the higher cytotoxicity of foliar extracts from CL-1048 against HT-29 may be due to antioxidant activities (TRC and TAC) (Fig. 3). It has been recently shown that extracts of white tea (*Camellia sinensis*) with high antioxidant activity inhibited HT-29 colon cancer cells by the death receptor and mitochondrial apoptotic pathways as demonstrated by increased expression levels of caspases-3/7, -8, and -9 (Hajiaghaalipour et al., 2015).

Finally, although negative correlation coefficients were found between TP, TRC, TAC and IC_{50} for almost all cancer cell lines in CL-8 (Fig. 4), none of them were significant. The lack of a significant correlation between TP and IC_{50} suggests that the cytotoxic activity of the leaf extract from CL-8 may be the resulting synergic action of many compounds, some of them different from phenols.

4. Conclusions

Our results have shown that the cytotoxicity of leaf extracts from Tempranillo against several cancer cell lines was higher than that detected when used other grapevine varieties. To our knowledge this is the first study in which cytotoxicity is evaluated after applying biotic and abiotic factors under controlled conditions and simulating the predicted global warming.

Elevated temperatures and mycorrhizal inoculation, separately or in combination, can enhance the cytotoxic activity of grapevine leaves, which strengthen the potential application of these by-products for biomedical purposes. There were, however, important differences among Tempranillo clones that should be taken into account for a hypothetical use of their vegetative residuals. The mycorrhization of CL-1048 would be an interesting resource for improving the cytotoxic effect of its foliar extracts against several human cancer types. Leaves from CL-260 appear as an interesting product for pharmacological purposes under the future warming conditions and foliar extracts from CL-8 associated with AMF and grown under elevated temperatures may be a promising tool against lung carcinoma cells.

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Table 1. Summary of the agronomic characteristics of Tempranillo clones used in this study.

Clone	Agronomic traits	City of origin (Region)
CL-260	Short cycle-Low yield	San Vicente de la Sonsierra (La Rioja)
CL-1048	Short cycle-Medium yield	Laguardia (Álava)
CL-8	Long cycle-Low yield	Sorzano (La Rioja)

Table 2. Phenols, total reducing capacity (TRC) and total antioxidant capacity (TAC) in leaf extracts 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).

		Treatments				*Main effects*				*ANOVA*
		-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
						24	28	-M	+M	TxM
Hydroxycinnamic acids (mg g^{-1} DM)	CL-260	18.10 b	24.07 a	19.86 b	17.39 b	21.08	18.63	18.98	20.73	*
	CL-1048	14.25	16.97	18.30	15.58	15.61	16.94	16.27	16.27	ns
	CL-8	18.33	12.05	11.21	11.36	15.19 a	11.29 b	14.77	11.70	ns
Flavonols (mg g^{-1} DM)	CL-260	13.61	17.36	15.31	17.51	15.49	16.41	14.46	17.44	ns
	CL-1048	8.60	10.24	11.33	12.43	9.42 b	11.88 a	9.97	11.33	ns
	CL-8	21.02 a	7.66 b	9.51 b	8.72 b	14.34	9.11	15.27	8.19	**
Anthocyanins (mg g^{-1} DM)	CL-260	17.13 c	23.11 a	20.77 b	18.75 c	20.12	19.76	18.95	20.93	***
	CL-1048	7.01	11.83	7.60	13.38	9.42	10.49	7.30 b	12.60 a	ns
	CL-8	18.20 a	6.71 b	9.38 b	9.07 b	12.45	9.22	13.79	7.89	**
Flavan-3-ols (mg g^{-1} DM)	CL-260	0.35	0.30	0.34	0.31	0.33	0.32	0.35	0.30	ns
	CL-1048	0.27	0.28	0.28	0.35	0.28	0.31	0.27	0.31	ns
	CL-8	0.36 a	0.31 b	0.25 c	0.32 ab	0.33	0.29	0.31	0.31	***
Total phenols (mg g^{-1} DM)	CL-260	53.39	55.77	59.02	57.48	54.58	58.25	56.21	56.63	ns
	CL-1048	30.12	39.31	37.50	41.74	34.72	39.62	33.81	40.53	ns
	CL-8	57.91 a	26.72 b	30.35 b	31.06 b	42.31	30.69	44.13	28.88	*
TRC (mg g^{-1} DM)	CL-260	1.48	1.41	1.60	1.49	1.45	1.55	1.54	1.45	ns
	CL-1048	1.62	1.46	1.66	1.57	1.54	1.62	1.64	1.52	ns
	CL-8	1.46	1.33	1.35	1.28	1.40	1.32	1.41	1.31	ns
TAC (mg g^{-1} DM)	CL-260	9.70	10.27	9.69	10.01	9.99	9.85	9.70	10.14	ns
	CL-1048	8.72	8.03	9.42	10.14	8.38	9.78	9.07	9.09	ns
	CL-8	8.39	8.15	7.79	8.87	8.27	8.33	8.09	8.51	ns

Values represent means (n = 3) separated by Duncan's test (at $P \leq 0.05$). Within each clone, means followed by different letters are significantly different as affected by the main factors temperature (T, 24, 28), mycorrhizal inoculation (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. Half maximal inhibitory concentration that reduces by 50% the growth of treated cells (IC_{50}) for the leaf extracts from fruit-bearing cuttings of Tempranillo clones inoculated (+M) or not (-M) with arbuscular mycorrhizal fungi, and grown at 24/14°C or 28/18°C day/night temperatures against HT-29 (colon adenocarcinoma), CCRF-CEM (lymphoblastic leukemia), HTB-54 (lung carcinoma) and MCF-7 (breast adenocarcinoma) cell lines after 48 h of treatment.

		IC_{50} (µg mL^{-1})								
		Treatments				*Main effects*				*ANOVA*
		-M24	+M24	-M28	+M28	Temperature (T)		Mycorrhizal inoculation (M)		
Cell line	Clone					24	28	-M	+M	T × M
HT-29	CL-260	153.75	140.97	95.11	95.36	147.36 a	95.23 b	124.43	118.16	ns
	CL-1048	155.21	138.97	175.11	129.20	147.09	152.15	165.16 a	134.08 b	ns
	CL-8	90.24 c	126.24 a	110.91 b	108.18 b	108.24	109.54	100.57	117.21	***
CCRF-CEM	CL-260	38.03	45.68	20.98	28.90	42.02 a	24.77 b	29.13 b	37.66 a	ns
	CL-1048	34.73	22.16	32.22	24.17	28.44	28.20	33.48 a	23.17 b	ns
	CL-8	23.43 b	39.90 a	37.72 a	25.30 b	31.67	31.51	30.58	32.6	***
HTB-54	CL-260	137.53	153.52	111.28	110.94	145.52 a	111.11 b	124.40	132.23	ns
	CL-1048	128.53	91.81	117.95	99.83	110.17	108.89	123.24 a	95.82 b	ns
	CL-8	120.16 b	166.53 a	113.45 b	94.84 c	143.34	104.14	116.81	130.68	***
MCF-7	CL-260	118.36	107.38	83.78	100.37	112.87	90.89	101.07	104.38	ns
	CL-1048	163.62	156.07	169.68	152.88	159.68	159.6	166.17 a	154.48 b	ns
	CL-8	133.36	148.57	118.29	114.03	139.76 a	116.07 b	125.83	127.85	ns

Values represent means (n = 12) separated by Duncan's test (at $P \leq 0.05$). Within cell line and clone, means followed by different letters are significantly different as affected by the main factors temperature (T, 24, 28), mycorrhizal inoculation (M, +M, -M) and their interaction (T × M). *** $P \leq 0.001$; ns, not significant ($P > 0.05$).

Table 4. Selectivity index (SI) for breast adenocarcinoma (MCF-7) cells of leaf extracts from fruit-bearing cuttings of Tempranillo clones inoculated (+M) or not (-M) with arbuscular mycorrhizal fungi, and grown at 24/14°C or 28/18°C day/night temperatures.

	CL-260	CL-1048	CL-8
Treatments			
-M24	2.16 a	1.64	1.06 b
+M24	1.63 b	1.61	0.96 b
-M28	1.18 b	1.49	1.18 a
+M28	1.57 b	1.43	0.82 c
Main effects			
Temperature (T)			
24	1.90	1.63 a	1.01
28	1.38	1.46 b	1.00
Mycorrhizal inoculation (M)			
-M	1.67	1.56	1.12
+M	1.60	1.52	0.89
ANOVA			
T × M	*	ns	**

Values represent means (n = 12) separated by Duncan's test (at $P \leq 0.05$). Within clones, means followed by different letters are significantly different as affected by the main factors temperature (T, 24, 28), mycorrhizal inoculation (M, +M, -M), and their interaction (T × M). * $P \leq 0.05$; ** $P \leq 0.01$; ns, not significant ($P > 0.05$).

Figure legends

Fig. 1. Mycorrhizal colonization of Tempranillo fruiting cuttings roots. Microscopic image (× 100) (A) of roots belonging to Tempranillo fruiting cuttings inoculated (+M) with arbuscular mycorrhizal fungi (AMF) and intensity of mycorrhizal colonization (%) (B) of roots from fruit-bearing cuttings of Tempranillo clones (CL) grown at normal (24/14°C) (white histograms) or elevated (28/18°C) (black histograms) day/night temperatures at berry ripening. Values of mycorrhizal colonization are means (n = 4-6) ± SE. Means not followed by different letters are not significantly different as affected by the main factors temperature (T, 24, 28), clone (CL, CL-260, CL-1048, CL-8) and their interaction (T × CL). ns, not significant ($P > 0.05$).

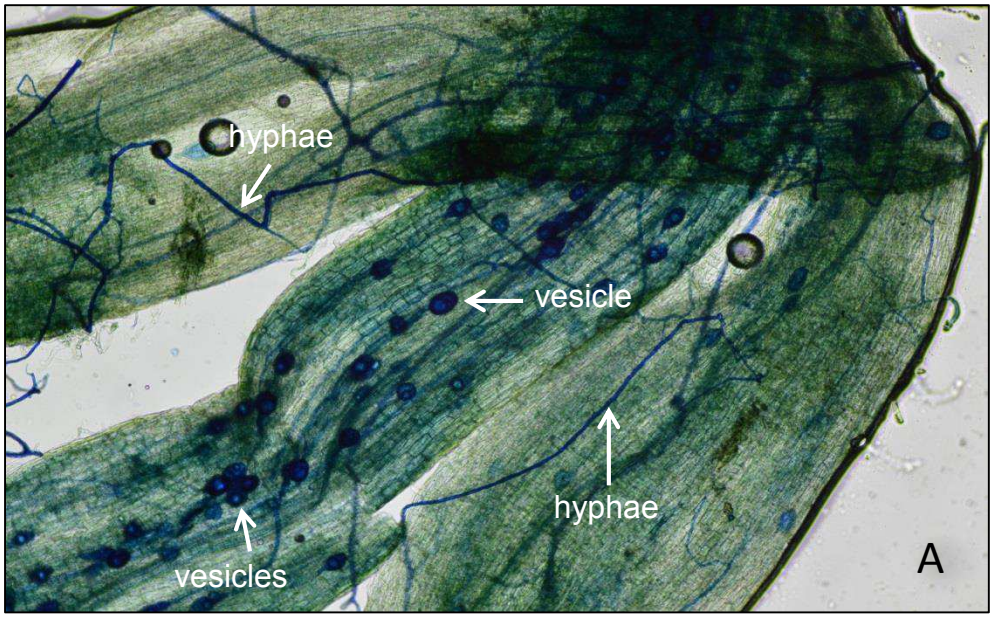
Fig. 2. Correlation between total phenols (TP, squares), total reducing capacity (TRC, circles), antioxidant capacity (TAC, triangles) and cytotoxic activity (IC_{50}) in leaf extracts from Tempranillo CL-260 inoculated (+M) or not (-M) with arbuscular mycorrhizal fungi (AMF) and grown either at 24/14°C or 28/18°C day/night air temperatures. Relationships were tested against four cancer cell lines (HT-29, CCRF-CEM, HTB-54 and MCF-7). For each cell line, straight lines correspond to the regression lines fitted for the joint data of all treatments. ns indicates not significant ($P > 0.05$); * $P \leq 0.05$.

Fig. 3. Correlation between total phenols (TP, squares), total reducing capacity (TRC, circles), antioxidant capacity (TAC, triangles) and cytotoxic activity (IC_{50}) in leaf extracts from Tempranillo CL-1048 inoculated (+M) or not (-M) with arbuscular mycorrhizal fungi (AMF) and grown either at 24/14°C or 28/18°C day/night air temperatures. Relationships were tested against four cancer cell lines (HT-29, CCRF-CEM, HTB-54 and MCF-7). For each cell line, straight lines correspond to the regression lines fitted for the joint data of all treatments. ns indicates not significant ($P > 0.05$); * $P \leq 0.05$.

Fig. 4. Correlation between total phenols (TP, squares), total reducing capacity (TRC, circles), antioxidant capacity (TAC, triangles) and cytotoxic activity (IC_{50}) in leaf extracts from Tempranillo CL-8 inoculated (+M) or not (-M) with arbuscular mycorrhizal fungi (AMF) and grown either at 24/14°C or 28/18°C day/night air temperatures. Relationships were tested against four cancer cell lines (HT-29, CCRF-CEM, HTB-54 and MCF-

7). For each cell line, straight lines correspond to the regression lines fitted for the joint data of all treatments. ns indicates not significant ($P > 0.05$).

Figure 1.


hyphae
vesicle
hyphae
vesicles
A

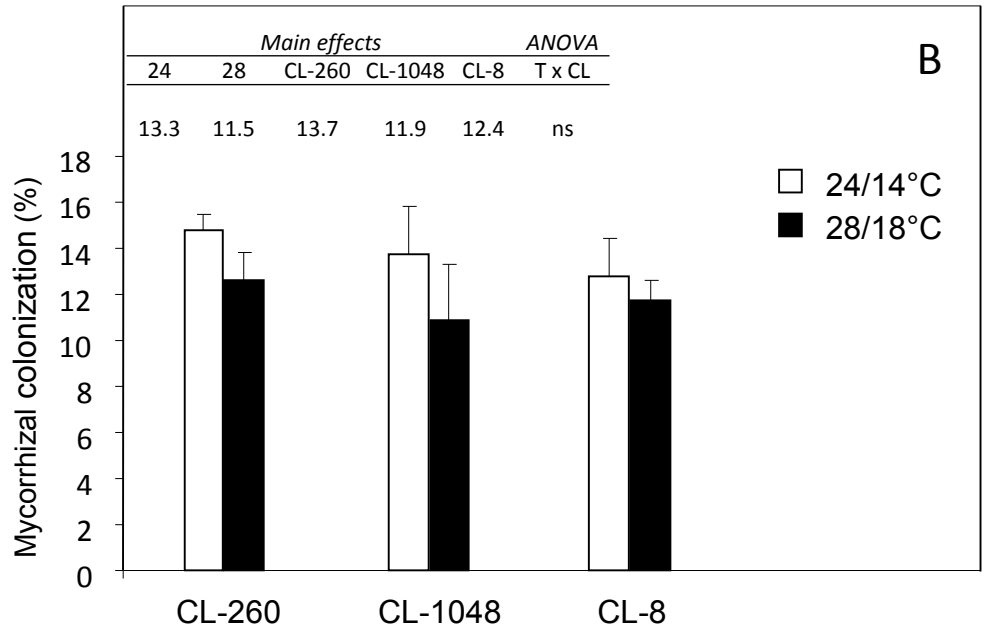

Main effects
ANOVA
24
28
CL-260
CL-1048
CL-8
T x CL
13.3
11.5
13.7
11.9
12.4
ns
B
24/14°C
28/18°C
Mycorrhizal colonization (%)
0
2
4
6
8
10
12
14
16
18
CL-260
CL-1048
CL-8

Figure 2.

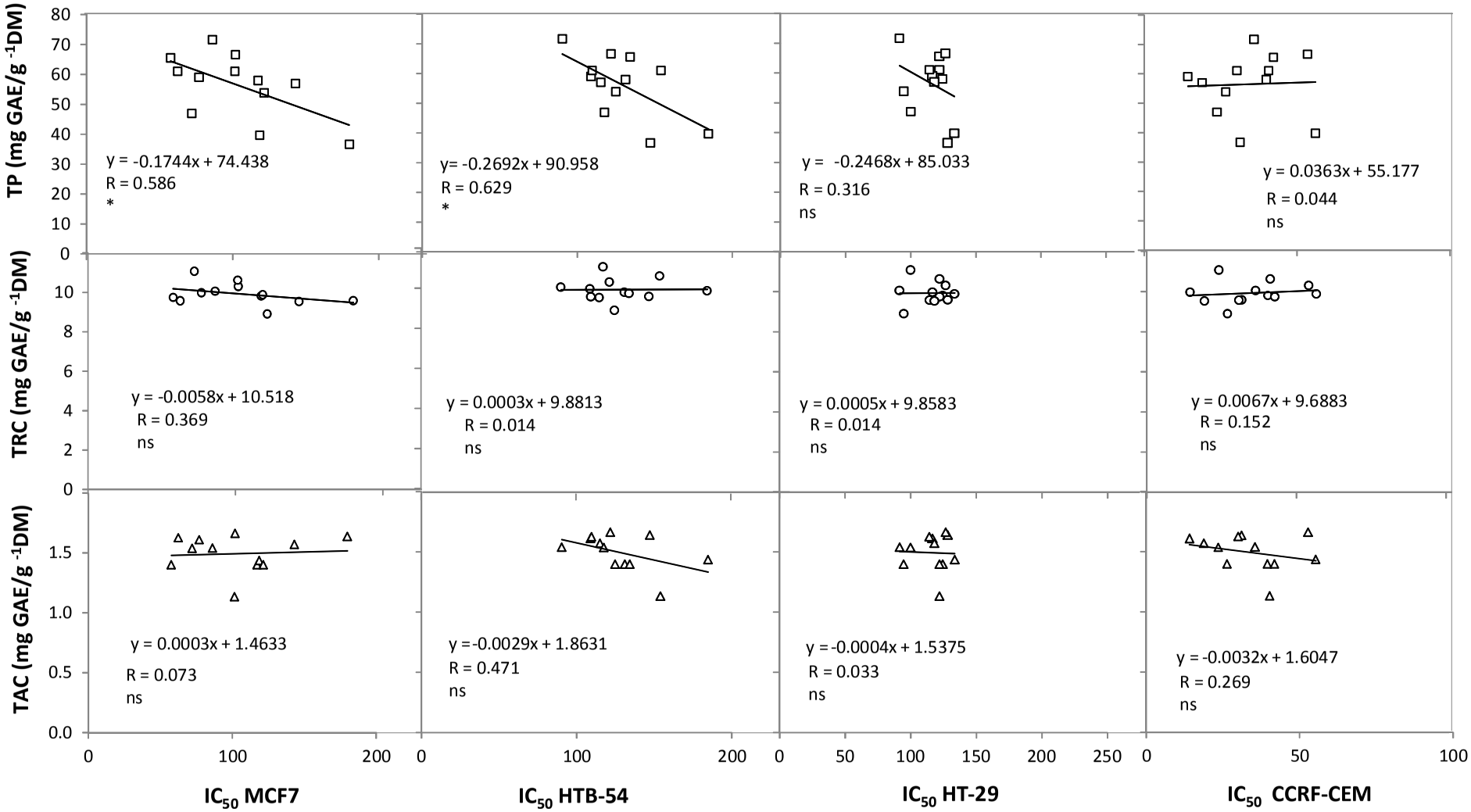

TP (mg GAE/g -1DM)
TRC (mg GAE/g -1DM)
TAC (mg GAE/g -1DM)
y = -0.1744x + 74.438
R = 0.586
*
y= -0.2692x + 90.958
R = 0.629
*
y = -0.2468x + 85.033
R = 0.316
ns
y = 0.0363x + 55.177
R = 0.044
ns
y = -0.0058x + 10.518
R = 0.369
ns
y = 0.0003x + 9.8813
R = 0.014
ns
y = 0.0005x + 9.8583
R = 0.014
ns
y = 0.0067x + 9.6883
R = 0.152
ns
y = 0.0003x + 1.4633
R = 0.073
ns
y = -0.0029x + 1.8631
R = 0.471
ns
y = -0.0004x + 1.5375
R = 0.033
ns
y = -0.0032x + 1.6047
R = 0.269
ns
IC50 MCF7
IC50 HTB-54
IC50 HT-29
IC50 CCRF-CEM

Figure 3.

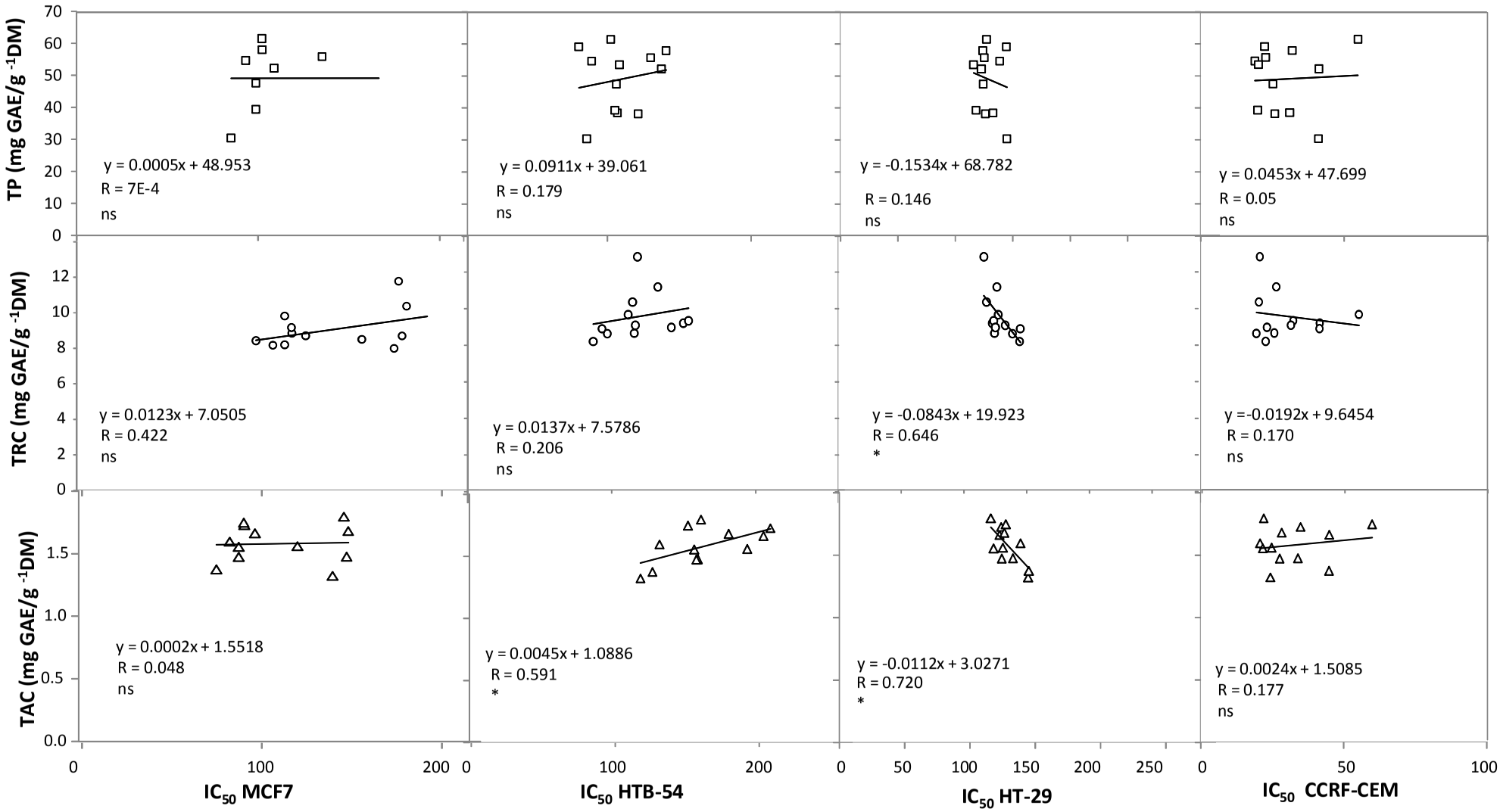
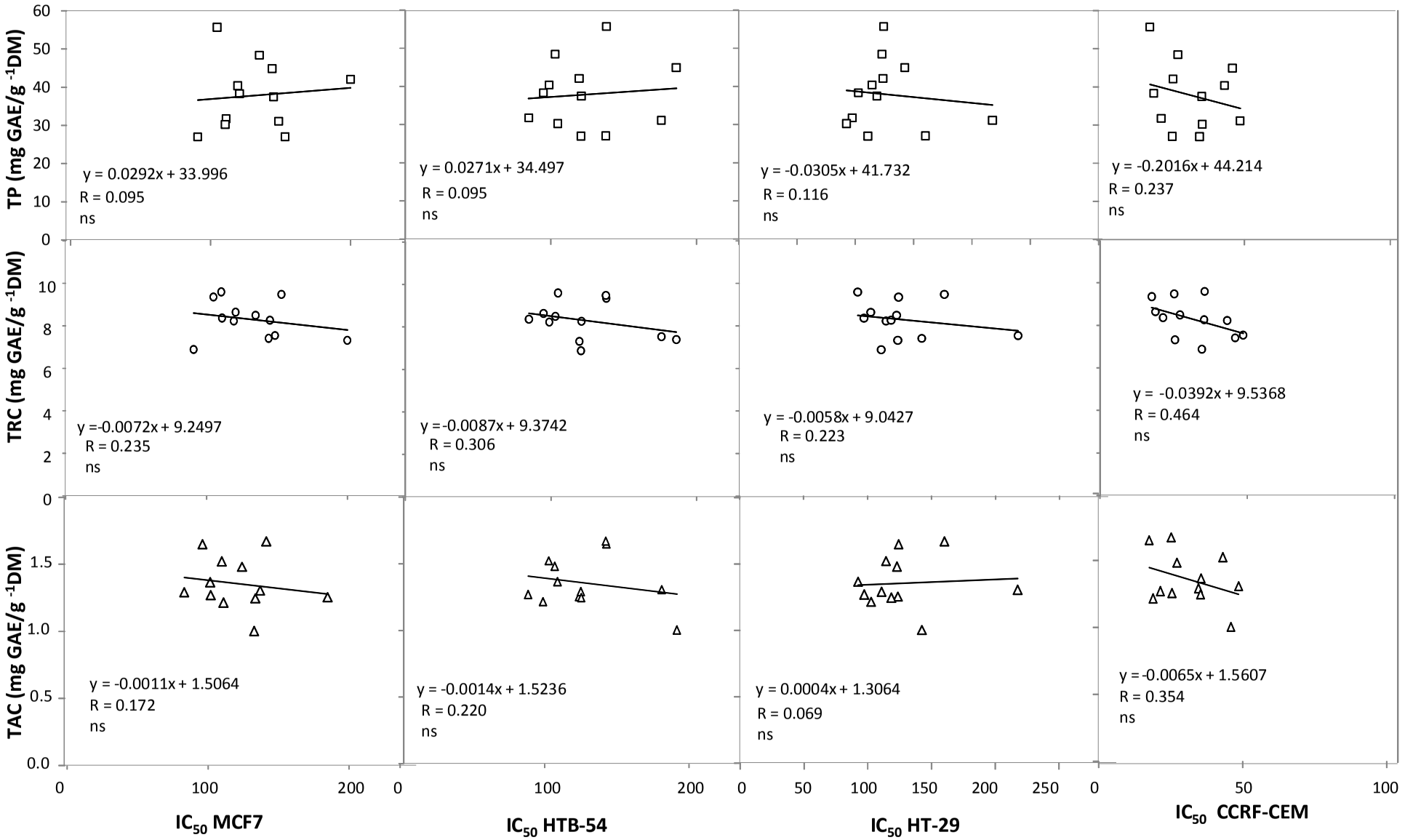

TP (mg GAE/g -1DM)
y = 0.0005x + 48.953
R = 7E-4
ns
y = 0.0911x + 39.061
R = 0.179
ns
y = -0.1534x + 68.782
R = 0.146
ns
y = 0.0453x + 47.699
R = 0.05
ns
TRC (mg GAE/g -1DM)
y = 0.0123x + 7.0505
R = 0.422
ns
y = 0.0137x + 7.5786
R = 0.206
ns
y = -0.0843x + 19.923
R = 0.646
*
y =-0.0192x + 9.6454
R = 0.170
ns
TAC (mg GAE/g -1DM)
y = 0.0002x + 1.5518
R = 0.048
ns
y = 0.0045x + 1.0886
R = 0.591
*
y = -0.0112x + 3.0271
R = 0.720
*
y = 0.0024x + 1.5085
R = 0.177
ns
IC50 MCF7
IC50 HTB-54
IC50 HT-29
IC50 CCRF-CEM

Figure 4.


TP (mg GAE/g -1DM)
TRC (mg GAE/g -1DM)
TAC (mg GAE/g -1DM)
y = 0.0292x + 33.996
R = 0.095
ns
y = 0.0271x + 34.497
R = 0.095
ns
y = -0.0305x + 41.732
R = 0.116
ns
y = -0.2016x + 44.214
R = 0.237
ns
y =-0.0072x + 9.2497
R = 0.235
ns
y =-0.0087x + 9.3742
R = 0.306
ns
y = -0.0058x + 9.0427
R = 0.223
ns
y = -0.0392x + 9.5368
R = 0.464
ns
y = -0.0011x + 1.5064
R = 0.172
ns
y = -0.0014x + 1.5236
R = 0.220
ns
y = 0.0004x + 1.3064
R = 0.069
ns
y = -0.0065x + 1.5607
R = 0.354
ns
IC50 MCF7
IC50 HTB-54
IC50 HT-29
IC50 CCRF-CEM

Original research article

Potential biomedical reuse of vegetative residuals from mycorrhized grapevines subjected to warming

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Supplementary methods. (Poly)phenolic compounds in foliar extracts from Tempranillo clones

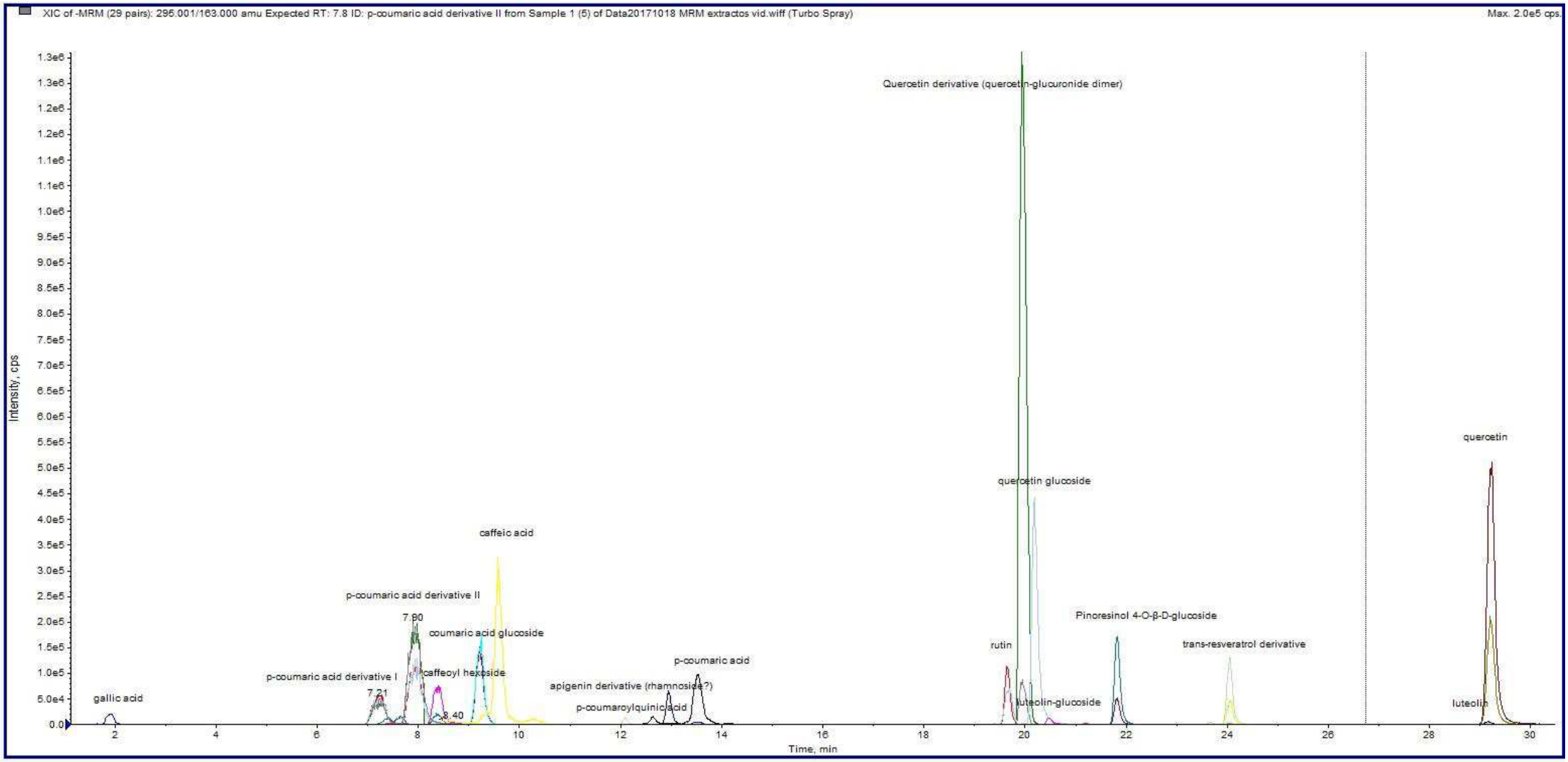
Supplementary Figure (Fig. S1). Profile of hydroxycinnamic and hydroxybenzoic acids, flavonols and stilbenes in Tempranillo grapevine leaf extracts.

Supplementary methods

(Poly)phenolic compounds in foliar extracts of Tempranillo grapevine

Foliar extracts were obtained according to Esfahanian et al. (2013) with some modifications. Afterwards, 1 mL of methanol/acidified water (0.1% formic acid) (80:20 v/v) was added to 100 mg of each lyophilized extract, sonicated for 15 min, filtered through a 0.2 µm syringe filter and stored at 20°C until analyzed. Qualitative analysis of (poly)phenolic compounds were carried out using an HPLC unit model 1200 (Agilent Technologies, Palo Alto, CA, USA) equipped with a triple quadrupole linear ion trap mass spectrometer (3200 Q-TRAP, AB SCIEX). The column used was a CORTECS® C18 (3 x 75 mm, 2.7 µm) from Waters. A preliminary analysis was carried out in a full scan MS^2, scanning from *m*/*z* of 100 to 1000, and a consecutively selective product ion mode analysis. Finally, for the identification of the phenolic compounds, ion multiple reaction monitoring (MRM) mode was used. For HPLC separation, mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was acetonitrile. Separations were carried out with an injection volume of 4 µL, column oven temperature of 30°C and elution flow rate of 0.35 mL min^{-1}. The mobile phases comprised a program of 0–1.20 min, 5% B; 1.20–8.80 min, 5–11.4% B; 8.80-10 min, 11.4–11% B; 10–30 min, 11-28% B; 30-32 min, 28-100% B and then return to 5% B in 2 min and maintained isocratic until the end of the analysis (38 min) to re-equilibrate the column. The MS functioned in negative ionization mode, with the turbo heater maintained at 500°C and IonSpray voltage set at -3500. Nitrogen was used as nebulizing, turbo heater and curtain gas and was set at the pressure of 40, 50 and 35 psi, respectively. Chromatograms and spectral data were acquired using Analyst software 1.6.3 (AB SCIEX).

Fig. S1. Profile of hydroxycinnamic and hydroxybenzoic acids, flavonols and stilbenes in Tempranillo grapevine leaf extracts.



DISCUSIÓN GENERAL

Grapevine (*Vitis vinifera* L.) is an important perennial crop worldwide, covering several regions where rising temperatures have been recorded during the last century and will be accentuated in the future (IPCC, 2014). South Mediterranean Europe, an important wine production area, is characterized by climate variability and stressful environments, so that the projected climate change scenario is expected to have detrimental impact on its viticulture suitability (Fraga et al., 2016). Consequently, winemaking sector is aware of adaptation measures may be required for maintaining the current varietal distribution. Among the potential measures that could be adopted appear appropriate clonal selections due to the broad variability within varieties, an adequated management of irrigation strategies in order to save water and the implementation of measures to promote the symbiosis with AMF in vineyards.

Therefore, in this PhD Thesis a characterization of the intra-varietal response of grapevine cv. Tempranillo to elevated air temperature and deficit irrigation, applied alone or in combination, as well as, an analysis of the potential benefits of mycorrhizal symbiosis on plant metabolism, and thus fruit quality, under the future stressful conditions have been assessed. Experiments were performed on fruit-bearing cuttings from different clones of *V. vinifera* cv. Tempranillo grown under controlled conditions in greenhouses. Potted vines were used to ensure that all clones experienced the same conditions, to facilitate the imposition of a similar water deficit procedure and to control mycorrhizal inoculation. Fruit bearing cutting technique has been validated in field as a valuable model to study grapevine phenology, physiology and also as a useful tool to study predicted climate change conditions (Morales et al., 2016). Nevertheless, working with potted vines in greenhouses could have some drawbacks such as abrupt changes in temperature cycles, lack of wind, limited soil volume or warming of roots above air temperature (Bonada and Sadras, 2015), which were prevented as possible throughout the experiments of this work. In spite of these limitations, this model allowed avoiding, in many cases, the year-to-year variations recorded in field-grown grapevines, and therefore obtaining similar results within each clone when environmental or biotic factors were applied. However, conclusions obtained along this dissertation require that extrapolations to field-grown grapevines should be made with caution.

Grapevine phenology is driven by temperature and the warming trends are shortening the phenological cycles. Likewise, they are impairing the balance between phenolic and technological maturity leading to more alcoholic wines due to the increased sugar content and the decreased acidity and anthocyanins in berries in detriment of wine quality (Teixeira et al., 2013). In dry regions of Mediterranean Europe, irrigation of vineyards is being expanded to mitigate the effects of warming and more stressful environment where water scarcity may constrain grapevine production. Therefore, deficit irrigation has emerged as a potential

strategy to guarantee viticulture suitability under these conditions (Costa et al., 2016). Smart irrigation strategies such as the timing of water deficit could be applied to guarantee a good balance between vegetative growth and berry quality, as well as the economic viability and environmental sustainability of viticulture (see Chaves et al., 2010, for further details). Under this scenario, our first goal was to evaluate the impact of pre- and post-veraison deficit irrigation on berry metabolism of two Tempranillo clones (CL) subjected to elevated temperatures. The clonal variability within cv. Tempranillo resulted in different responses to deficit irrigation under warm temperatures due to changes in berry skin metabolism. Thus, although temperature and irrigation strategy modified the profile of amino acids, anthocyanins and flavonols, CL-1089 stood out as quite tolerant to warm temperatures. In line with the detrimental effects of climate change previously described, elevated temperature accounted for increased levels of hexoses while early deficit (ED) irrigation diminished the antioxidant properties of must by means of losses of anthocyanins and flavonols at 24/14°C. This result evidenced that late deficit (LD) irrigation performed better than ED, although such differences were clearly attenuated at warmer temperatures. Given these results, a reasonable strategy of adaptation to climate change might be the optimization of timing of water deficit and the appropriate selection of clones.

The symbiosis between arbuscular mycorrhizal fungi (AMF) and plants is widespread with 80% of plants colonized (Smith and Read, 2008), including grapevines. In fact, AMF are known to increase grapevine nutrient uptake, growth and resistance to abiotic or biotic stresses (see Trouvelot et al., 2015, for further details). Therefore, our second purpose was to determine whether mycorrhizal inoculation can improve berry antioxidant properties in three Tempranillo clones and thus ameliorate the deleterious effect of elevated air temperature on fruit quality. Again, the clonal diversity within cv. Tempranillo resulted in different abilities to respond to AMF inoculation under warming temperatures. Thus, the protective role of AMF on berry quality was particularly evident in CL-1048, in which fruit quality and antioxidant properties were maintained or even improved under elevated temperatures. These results highlighted the importance of adopting measures to protect the indigenous cohorts of AMF in vineyards.

These benefits derived from the association of Tempranillo with AMF did not occur in all studied clones (i.e. the tolerance of the CL-1089 to elevated temperatures decreased, in terms of berry quality, when inoculated with AMF). On the other hand, AMF symbiosis was known to alleviate grapevine water stress by enhancing water uptake or drought tolerance (Trouvelot et al., 2015). However, it was unknown if this potential benefit would be maintained under future constraints. Consequently, our third aim was to characterize the quality and antioxidant

properties of fruits in three clones of Tempranillo when subjected to the combination of different water deficit irrigations (ED and LD) and AMF inoculation under elevated temperatures. Results confirmed that AMF inoculation may improve the beneficial effect of LD under warming conditions, where the loss of anthocyanins detected in the non-mycorrhizal plants did not occur when plants were inoculated with AMF, especially in CL-1089. Thereby, the implementation of measures to promote the association of grapevines with AMF may optimize the irrigation strategy effects under warming conditions.

Berry ripening and consequently berry quality (mainly anthocyanins) are very dependent on abscisic acid (ABA) metabolism. Thus, ABA plays a major role on the ripening process because it peaks around veraison, the stage where many ripening related processes, such as sugar accumulation, berry softening or colour development, take place (Fortes et al., 2015; Pilati et al., 2017). However, recent researches have pointed out that compounds derived from ABA catabolism/conjugation could also be involved in berry ripening (Wheeler et al., 2009; Castellarín et al., 2016) and that they may be affected by heat and drought (Zarrouk et al., 2016). Moreover, it is known that ABA is involved in the establishment and functioning of the mycorrhizal symbiosis (Pozo et al., 2015). Taking all into account, we hypothesized that ABA metabolism could be implied in the ability of AMF for improving berry quality under global warming scenarios in CL-1048 and CL-1089. Our data indicated that the ABA catabolism/conjugation along berry development was modulated by AMF inoculation and by climate change conditions. Under elevated temperatures, plants inoculated with AMF had higher ABA glucose ester (ABA-GE), 7´-hydroxy-ABA (7´OH-ABA) and lower phaseic acid (PA) content than uninoculated plants. Besides, an important role of 7´OH-ABA in the fruit quality of Tempranillo berries was evidenced. Under the most stressful conditions (elevated temperatures and deficit irrigation), berry quality (namely, anthocyanins) was improved by AMF inoculation, which also led to increased ABA hydroxylation at the position 7' in detriment of position 8', providing an explanation to the ability of AMF for improving berry quality under global warming scenarios.

A complementary aspect developed in this PhD thesis was the suitability of grapevine wastes for their reutilization in the future climate change scenario. With few exceptions in some Mediterranean countries, grapevine leaves are usually discarded as wastes of the grapes processing industry. However, they are rich in phenolics and antioxidant compounds that help plants to cope with abiotic stresses, being these secondary compounds increased by the association of grapevines with AMF (Eftekhari et al., 2012). In the last years, there is an increasing interest in the study of these wastes due to the possibility of taking profit from

them for nutritional or biomedical industries (Fernandes et al., 2013; Lima et al., 2017). However, it remains unclear if the composition and properties of leaves could be maintained under global warming conditions. Taking all into account, we aimed to evaluate the effect of AMF inoculation and elevated temperatures on phenolic composition and antioxidant activity of leaf extracts from the cultivar Tempranillo. A first screening of Tempranillo clones highlighted the different intra-varietal responses to elevated temperatures and AMF inoculation with regard to phenolic composition and antioxidant properties. In some clones, AMF inoculation enhanced total soluble phenolic, flavonol and anthocyanin content and the antioxidant capacity when grown under warm temperatures. Given these results, our last objective was to assess the potential application of these vegetative wastes in the human diet or for pharmacological and biomedical purposes. The nutritional value of leaves from Tempranillo was improved under the projected warming climate. Thus, elevated temperatures increased the contents of several minerals and those of soluble sugars, photosynthetic pigments and soluble proteins. Mycorrhizal symbiosis enhanced the accumulation of flavonols and hydroxycinnamic acids. However, the intra-varietal diversity in such responses suggests different applications for each studied clone. On the other hand, Tempranillo foliar extracts were more active against some human cancer cell lines than other extracts from different varieties of grapevine (Esfahanian et al., 2013; Handoussa et al., 2013; Abed et al., 2015). Besides, cytotoxic activity was enhanced by AMF inoculation, elevated temperatures or their combination. Preliminary data obtained in this PhD Thesis strengthen the potential application of these agricultural wastes for pharmaceutical and biomedical purposes.

Altogether, this dissertation demonstrates the broad diversity of responses to biotic (AMF) or environmental (high temperature or water deficit irrigation strategies) factors within the cultivar Tempranillo, which ultimately determines the metabolism, hormonal balance, quality of berries and leaf composition. Moreover, it has been highlighted the importance of protecting the mycorrhizal fungal communities in vineyards in order to promote their association with grapevines and thus, optimize the effects of a late water deficit irrigation under future warming conditions.

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CONCLUSIONES GENERALES

Conclusions

1. The responses to deficit irrigation under warm temperatures were explained by clonal-specific changes in berry skin metabolism of Tempranillo. Under our experimental conditions, early deficit (ED) irrigation performed worse than late deficit (LD) at 24/14°C regarding anthocyanin and flavonol levels and berry traits, even though such differences were clearly attenuated at 28/18°C.
2. The extent of alteration in primary metabolism due to temperature was higher than in secondary metabolism, which was mainly affected by deficit irrigation. Temperature increased levels of hexoses which could enhance the content of alcohol in wine, whereas ED at 24/14°C decreased anthocyanin and flavonol levels, which probably diminished the antioxidant properties.
3. Temperature and irrigation modified the amino acid, anthocyanin and flavonol metabolite profiles, with CL-1089 appearing to be more tolerant than CL-843 to elevated temperature. These results suggested that the optimization of timing of water deficit, as well as the proper clonal selection might guarantee the adaptation of grapevines to warming trends in South Mediterranean Europe.
4. Clonal diversity of Tempranillo also resulted in different abilities to respond to arbuscular mycorrhizal fungi (AMF) inoculation under elevated temperature. Thus, the protective role of AMF in avoiding warming effects on berry quality was particularly evident in CL-1048.
5. The association of CL-1048 with AMF allowed maintaining or improving berry quality and antioxidant properties in spite of the earlier ripening. Thus, CL-1048 associated with AMF may be an interesting alternative to typical clones of Tempranillo used to make wine within future climate-change scenario. These results highlighted the relevance of adopting measures to protect the indigenous cohorts of AMF in vineyards.
6. The association of grapevines with AMF could impair the detrimental effect of water restriction and elevated temperatures. Thereby, AMF inoculation may improve the potential benefits of LD at 28/18°C, due to the loss of anthocyanin detected in the non-mycorrhizal plants never occurred in those inoculated with AMF, however, this benefit differed between Tempranillo clones. Results emphasized the importance of implementing measures to promote the association of grapevines with AMF which may contribute to optimize effects of irrigation strategy on berry properties under elevated temperatures.
7. Throughout berry ripening, the catabolism and conjugation of abscisic acid (ABA) was affected by AMF inoculation and climate change conditions evidencing the important role of 7´OH-ABA in anthocyanin accumulation of Tempranillo berries. Thus, plants inoculated

with AMF had higher ABA-GE and 7´OH-ABA and lower PA content than uninoculated plants at 28/18°C.

8. Under the most stressful climate change conditions (elevated temperature and deficit irrigation), AMF inoculation improved anthocyanins by means of increasing ABA hydroxylation at the position 7' in detriment of position 8'.
9. Intra-varietal-diversity of Tempranillo clones was also showed in their responses to AMF inoculation and elevated temperature with regard to phenolic composition and antioxidant properties of leaf extracts. Thus, in some clones grown at 28/18°C, AMF inoculation improved antioxidant power of leaf wastes by enhancing total soluble phenolics, flavonols and anthocyanins.
10. Under future warming conditions, the nutritional value of leaves from Tempranillo may be improved. Elevated temperatures induced the accumulation of several minerals, soluble sugars, photosynthetic pigments and soluble proteins, while AMF inoculation increased the accumulation of flavonols and hydroxycinnamic acids. However, the intra-varietal diversity in such responses suggested different applications of leaves from each studied clone when included in the human diet.
11. Foliar extracts of Tempranillo exhibited high cytotoxic activity against some cancer cell lines, which was modulated by mycorrhizal inoculation and elevated temperature. Preliminary data obtained in this PhD Thesis suggested that both, biotic and abiotic factors may enhance the cytotoxic properties of grapevine leaves, which strengthened the potential application of these agricultural wastes for pharmaceutical and biomedical purposes.