



Universidad de Navarra

Facultad de Ciencias

ACLIMATACIÓN FOTOSINTÉTICA, PRODUCCIÓN Y CALIDAD DE PLANTAS DE VID (VITIS VINIFERA L.) DE LA VARIEDAD TEMPRANILLO TINTO Y BLANCO, CULTIVADAS EN DIFERENTES ESCENARIOS DE CAMBIO CLIMÁTICO

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VARIEDAD TEMPRANILLO TINTO Y BLANCO, CULTIVADAS EN DIFERENTES ESCENARIOS DE CAMBIO
CLIMÁTICO*

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Ambiental y autorizo su presentación ante el Tribunal que lo ha de juzgar.

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Abstract

Atmospheric CO₂ concentration has increased from preindustrial level of 280 μmol CO₂ mol⁻¹ air (ppm) to currently more than 400 ppm. Intergovernmental Panel on Climate Change foresees that atmospheric CO₂ concentration will continue increasing to reach at the end of this century 700 ppm (ranging from 421 to 936 ppm, depending on mitigation measures adopted). Due to its greenhouse effect, elevated atmospheric CO₂ concentration is leading to higher atmospheric temperatures, a phenomenon that is being accompanied by episodes of less water availability or drought periods. Grapevine (*Vitis vinifera* L.) is a plant species very sensitive to those environmental factors. Fruit-bearing cuttings of red and white Tempranillo were grown under elevated CO₂ (around 700 ppm, versus 400), high temperature (ambient temperature + 4°C, versus ambient) and water deficit (cyclic drought, versus well irrigated) in temperature gradient greenhouses located at the University of Navarra (Pamplona, Spain) for three consecutive growing seasons (years 2013, 2014 and 2015). Vegetative growth (total vegetative mass and leaf area) was significantly reduced by drought (consistent the three years) and was more associated to a worse substrate water status than to decreases in leaf water content. In spite of decreasing leaf water content in both cultivars, elevated CO₂ stimulated more vegetative (total vegetative mass) than reproductive (berry and rachis fresh weight) growth. The largest increases of elevated CO₂ were observed in leaf and root growth (dry mass accumulation) in white and red Tempranillo, respectively. There was a clear interaction between temperature and water availability, i.e., in full-irrigated grapevines elevated temperature increased leaf, petiole, stem and root dry weight (range 4-26%), whereas decreases (from 7 to 19%) were observed in plants exposed to drought. Yield was significantly reduced by drought and was year-dependent, being especially low in 2015 due to eventual heat shocks in the first week of July. The high temperatures of the heat shocks (above 35°C) induced berry burn and browning and finally loss of 50% of the berries in the bunches. The response of berry quality to climate change-related factors was highly variable and depended on the year. However, some general conclusions can be reached from the three years of experimentation. Thus, the simulated climate change scenarios affected to a greater extent the technological maturity parameters (primary metabolism) than the phenolic maturity (secondary metabolism). In particular, high temperature and drought significantly and consistently increased must pH, due to the decrease in malic acid. On

the contrary, elevated CO₂ decreased pH associated with significant increases in tartaric acid concentration. These changes in the acidic properties of the must affect its quality and potentially that of the resulting wine. Under elevated CO₂ concentration, grapevine photosynthesis increases, modulated by temperature and water availability. However, under prolonged exposure to elevated CO₂, grapevine down-regulates photosynthesis (also known as photosynthetic acclimation), decreasing photosynthetic capacity. Grapevine plants underwent photosynthetic acclimation after a long exposure to elevated CO₂ (700 ppm), regardless of temperature and water availability. Evidence comes from photosynthetic capacity decreases (net photosynthetic rates measured at 700 ppm), leaf starch accumulation, and increases in leaf carbon/nitrogen ratio. Photosynthetic acclimation was well correlated to leaf starch, but not to soluble sugars, accumulation. The white Tempranillo mutant has altered its response to prolonged exposure to elevated CO₂. For any given sink size (bunch, total vegetative or total plant dry matter) or any given leaf starch accumulation, white Tempranillo always had higher levels of photosynthetic acclimation than the red one (quantified by the extent of the increases in leaf carbon/nitrogen ratio). Data suggest that mutation in white Tempranillo has affected loci other than grape color.

Keywords: Grapevine, Red And White Tempranillo, Climate Change, Elevated CO₂, High Temperature, Drought, Photosynthetic Response, Acclimation, Anthocyanins, Malic And Tartaric Acid, Total Soluble Sugars, Total Polyphenol Index, Vegetative And Reproductive Growth, Grape Quality

Resumen

La concentración de CO₂ atmosférico ha aumentado desde el nivel preindustrial de 280 μmol de CO₂ mol⁻¹ aire (ppm) hasta actualmente más de 400 ppm. El Grupo Intergubernamental de Expertos sobre el Cambio Climático prevé que la concentración atmosférica de CO₂ continuará aumentando para llegar a finales de este siglo a 700 ppm (que van de 421 a 936 ppm, según las medidas de mitigación adoptadas). Debido a su efecto invernadero, la elevada concentración de CO₂ atmosférico está provocando mayores temperaturas atmosféricas, un fenómeno que se acompaña de episodios de menor disponibilidad de agua o períodos de sequía. La vid (*Vitis vinifera* L.) es una especie muy sensible a esos factores ambientales. Esquejes fructíferos de Tempranillo tinto y blanco se cultivaron bajo una concentración de CO₂ elevada (alrededor de 700 ppm, frente a 400), temperatura alta (temperatura ambiente + 4°C, frente a ambiente) y déficit de agua (sequía cíclica, frente a riego completo) en invernaderos con gradiente de temperatura localizados en la Universidad de Navarra (Pamplona, España) durante tres temporadas de crecimiento consecutivas (años 2013, 2014 y 2015). El crecimiento vegetativo (masa vegetativa total y área foliar) se redujo significativamente por la sequía (consistente los tres años) y estuvo más asociado al descenso de agua en el sustrato de crecimiento que a la disminución del contenido de agua en la hoja. A pesar de la disminución del contenido de agua de la hoja en ambos cultivares, el CO₂ elevado estimuló más el crecimiento vegetativo (masa vegetativa total) que el crecimiento reproductivo (peso fresco de bayas y de raspones). Los mayores aumentos debidos al CO₂ elevado se observaron en el crecimiento de hojas y raíces (acumulación de masa seca) en Tempranillo blanco y tinto, respectivamente. Hubo una clara interacción entre la temperatura y la disponibilidad de agua, es decir, en las vides con riego completo, la temperatura elevada aumentó el peso seco de hoja, pecíolo, tallo y raíz (rango 4-26%), mientras que las disminuciones (de 7 a 19%) se observaron en plantas expuestas a la sequía. El rendimiento se redujo significativamente por la sequía y fue dependiente del año, siendo especialmente bajo en 2015 debido a eventuales choques térmicos en la primera semana de julio. Las altas temperaturas de los choques térmicos (por encima de los 35°C) indujeron quemaduras y color pardo en las bayas y, finalmente, la pérdida del 50% de las bayas en los racimos. La respuesta de la calidad de la baya a los factores relacionados con el cambio climático fue variable y dependió del año. Sin embargo, se pueden llegar a algunas conclusiones generales a partir de los tres años de experimentación. Así, los escenarios de cambio climático simulados afectaron en mayor

medida los parámetros de madurez tecnológica (metabolismo primario) que la madurez fenólica (metabolismo secundario). En particular, la alta temperatura y la sequía aumentaron de manera significativa y consistente el pH del mosto, debido a la disminución del ácido málico. Por el contrario, el CO₂ elevado disminuyó el pH asociado con aumentos significativos en la concentración de ácido tartárico. Estos cambios en las propiedades ácidas del mosto afectan a su calidad y potencialmente a la del vino resultante. Con una elevada concentración de CO₂, aumenta la fotosíntesis de la vid, modulada por la temperatura y la disponibilidad de agua. Sin embargo, bajo exposición prolongada a CO₂ elevado, la vid regula a la baja la fotosíntesis (fenómeno también conocido como aclimatación fotosintética), disminuyendo la capacidad fotosintética. Las plantas de vid experimentaron aclimatación fotosintética después de una larga exposición al CO₂ elevado (700 ppm), independientemente de la temperatura y la disponibilidad de agua. La evidencia proviene de la disminución de la capacidad fotosintética (tasas fotosintéticas netas medidas a 700 ppm), acumulación de almidón en hoja y aumento de la relación carbono/nitrógeno de la hoja. La aclimatación fotosintética estuvo bien correlacionada con la acumulación de almidón en las hojas, pero no con los azúcares solubles. El Tempranillo blanco tiene alterada su respuesta a la exposición prolongada al CO₂ elevado. Para cualquier tamaño de sumidero dado (racimo, masa vegetativa total o masa total de la planta) o cualquier acumulación de almidón en hoja dado, el Tempranillo blanco siempre tuvo niveles más altos de aclimatación fotosintética que el tinto (cuantificado por el grado de aumento en la relación carbono/nitrógeno en hoja). Los datos sugieren que la mutación en Tempranillo blanco ha afectado a más loci que los implicados en el color de la baya.

Palabras clave: Vid, Tempranillo Tinto y Blanco, Cambio Climático, CO₂ Elevado, Alta Temperatura, Sequía, Respuesta Fotosintética, Aclimatación, Antocianinas, Ácido Málico y Tartárico, Azúcares Solubles Totales, Índice Total de Polifenoles, Crecimiento Vegetativo y Reproductor, Calidad De La Uva

Résumé

La concentration de CO₂ dans l'atmosphère a augmenté depuis la période pré-industrielle de 280 μmol de CO₂ mol⁻¹ d'air (ppm) à aujourd'hui plus de 400 ppm. Le Groupe d'experts intergouvernemental sur l'évolution du climat (GIEC) prévoit que la concentration de CO₂ dans l'atmosphère continuera d'augmenter pour atteindre 700 ppm (variant de 421 à 936 ppm, selon les mesures d'atténuation adoptées) à la fin de ce siècle. En raison de son effet de serre, la concentration élevée de CO₂ atmosphérique entraîne des températures atmosphériques plus élevées, un phénomène qui s'accompagne à d'épisodes de moins en mois de la disponibilité d'eau et/ou de périodes de sécheresse. La vigne (*Vitis vinifera* L.) est une espèce végétale très sensible à ces facteurs environnementaux. Les boutures fruitières de Tempranillo rouge et blanc ont été cultivées en présence de CO₂ élevé (environ 700 ppm contre 400), de températures élevées (température ambiante + 4°C contre température ambiante) et de déficit hydrique (sécheresse cyclique par rapport à bien irriguée) à l'Université de Navarre (Pampelune, Espagne) pendant trois saisons de croissance consécutives (années 2013, 2014 et 2015). La croissance végétative (masse végétative totale et surface foliaire) a été réduite de manière significative par la sécheresse (pendant les trois années) et a été plus associée à une dégradation de l'état du substrat d'eau plutôt qu'à une diminution de la teneur en eau des feuilles. Malgré la diminution de la teneur en eau des feuilles chez les deux cultivars, le CO₂ élevé a stimulé plus la croissance végétative (masse végétative totale) plutôt que la croissance reproductive (poids frais des baies et du rachis). Les plus fortes augmentations de CO₂ élevé ont été observées dans la croissance des feuilles et des racines (accumulation de masse sèche) dans le Tempranillo blanc et rouge, respectivement. Il y avait une interaction claire entre la température et la disponibilité d'eau, c'est à dire que dans les vignes pleines irriguées, la température augmentait le poids sec des feuilles, des pétioles, des racines et des racines (allant de 4% au 26%), tandis que les diminutions (de 7% à 19%) sur de plantes exposées à l'influence de la sécheresse. Le rendement a été réduit de manière significative par la sécheresse et aussi dépendant de l'année, particulièrement au bas en 2015 en raison des chocs thermiques pendant la première semaine de juillet. Les températures élevées des chocs thermiques (au-dessus de 35°C) ont induit la brûlure et le brunissement des baies et la perte de 50% des baies dans les grappes. La réponse de la qualité des baies aux facteurs liés au changement climatique était très variable et en relation avec l'année.

Cependant, des conclusions générales peuvent être tirées après trois années d'expérimentation. Ainsi, les scénarios simulés de changement climatique affectent dans une plus large mesure les paramètres de maturité technologique (métabolisme primaire) que la maturité phénolique (métabolisme secondaire). En particulier, la température élevée et la sécheresse augmentent de manière significative et constante le pH du moût, dû à la diminution de l'acide malique. Au contraire, une concentration de CO₂ élevée a diminué le pH associé à des augmentations significatives de la concentration d'acide tartrique. Ces changements dans les propriétés acides du moût affectent sa qualité et potentiellement celle du vin obtenu. Sous une concentration de CO₂ élevée, la photosynthèse de la vigne augmente, modulée par la température et la disponibilité d'eau. Cependant, en cas d'exposition prolongée à un taux élevé de CO₂, la vigne régule la photosynthèse (aussi appelée comme acclimatation photosynthétique), ce qui diminue la capacité photosynthétique. Les plants de vigne ont été soumis à une acclimatation photosynthétique après une longue exposition à un taux élevé de CO₂ (700 ppm), indépendamment de la température et de la disponibilité d'eau. La preuve est fournie de la diminution de la capacité photosynthétique (taux de photosynthèse net mesuré à 700 ppm), de l'accumulation d'amidon foliaire et de l'augmentation du ratio carbone / azote foliaire. L'acclimatation photosynthétique était bien corrélée à l'amidon des feuilles, mais pas aux sucres solubles, à l'accumulation. Le mutant blanc Tempranillo a modifié sa réponse à une exposition prolongée à un taux élevé de CO₂. Le Tempranillo blanc a toujours eu des niveaux d'acclimatation photosynthétique plus élevés que le rouge (quantifié par l'ampleur des augmentations du rapport carbone / azote foliaire). Les données suggèrent que la mutation dans le Tempranillo blanc a affecté les loci autres que la couleur du raisin.

Mots clés: Vigne, Rouge Et Blanc Tempranillo, Changement Climatique, CO₂ Élevé, Température Élevée, Sécheresse, Réponse Photosynthétique, Acclimatation, Anthocyanes, Acide Malique Et Tartrique, Total Des Sucres Solubles, Indice Polyphénol Total, Croissance Végétative Et Reproductive, Qualité Du Raisin

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INTRODUCCIÓN GENERAL

1. Grapevine

Grapevines (*Vitis*) are one of the most economically important fruit crops (FAO, 1997). The members of *Vitaceae* family, both *Muscadinia* (2n=40) and *Vitis* (or Euvitis; 2n=38) genera, include the grapevine species. Genus *Muscadinia* contains only 3 species. However, genus *Vitis* comprises 60-70 species (Keller, 2010). *Vitis vinifera* L. subsp. *vinifera* (referred to as *V. vinifera* hereafter) is the most extensively grown grape species, but its yield was historically restricted due to its sensitivity to pests, diseases, and abiotic stress factors such as temperature (Keller, 2010). Grapevine is a species with a large biodiversity (Moore, 1991; Chen et al., 2007). Grapevines species are widespread in the eastern and southwestern US, Mexico, Asia, China, Japan and Java (Indonesia), and middle Asia and Europe (Fig. 1).



Figure 1. Four main native geographic distribution of the genus *Vitis* (grey shading) and geographic regions of some of the important *Vitis* species' origin (Wan et al., 2013).

1.1 Tempranillo grapevine

V. vinifera L. cv. Tempranillo is an extensively cultivated native red grape variety to northern and central Spain. Contrary to most other Mediterranean varieties, Tempranillo (as a characteristic of Northern varieties) has a short vegetative cycle, and large hairy

five up to seven lobulated leaves as well uniform fruit set, and soft dark-blue berries with thick skin (Cervera et al., 2002; Chomé et al., 2003).

“White Tempranillo” is a new white berry somatic variant of Tempranillo that resulted from a spontaneous, natural mutation of red Tempranillo. It was discovered in an old red Tempranillo vineyard in 1988 in Murillo de Río Leza, La Rioja, Spain (García-Escudero et al., 2011). White Tempranillo has deletions and chromosomal reorganizations, affecting both linkage groups 2 and 5. The complex color locus in linkage group 2 has lost the functional alleles at both *MybA1*, which was located in the berry skin and has the ability to induce the anthocyanin-producing cells in the skin tissues (Kobayashi et al., 2005), and *MybA2*, losing berry pigmentation and affecting also the transmission of the corresponding chromosome (Fernández et al., 2013). White Tempranillo berries are green-yellow and the leaf has the typical characteristics of red Tempranillo, but smaller, more swelling in the beam and higher density of erect hairs on nerves in the back. The cluster is medium sized and loose, and berry is slightly flattened (Martínez et al., 2006). This mutation allowed the development of the new cultivar White Tempranillo, with very good features for elaboration of white wines for both young wine making as well as that intended for barrel aging process (Vicente et al., 2001).

1.2 Grapevine development

As a perennial plant, biotic and abiotic factors influence grapevine’s longevity. Several authors have described its annual developmental stages (Baillod and Baggiolini, 1993; Coombe, 1995).

1.2.1. Grapevine phenology

The modified E-L system (**Fig. 2**) describes grapevine phenology (Coombe, 1995).

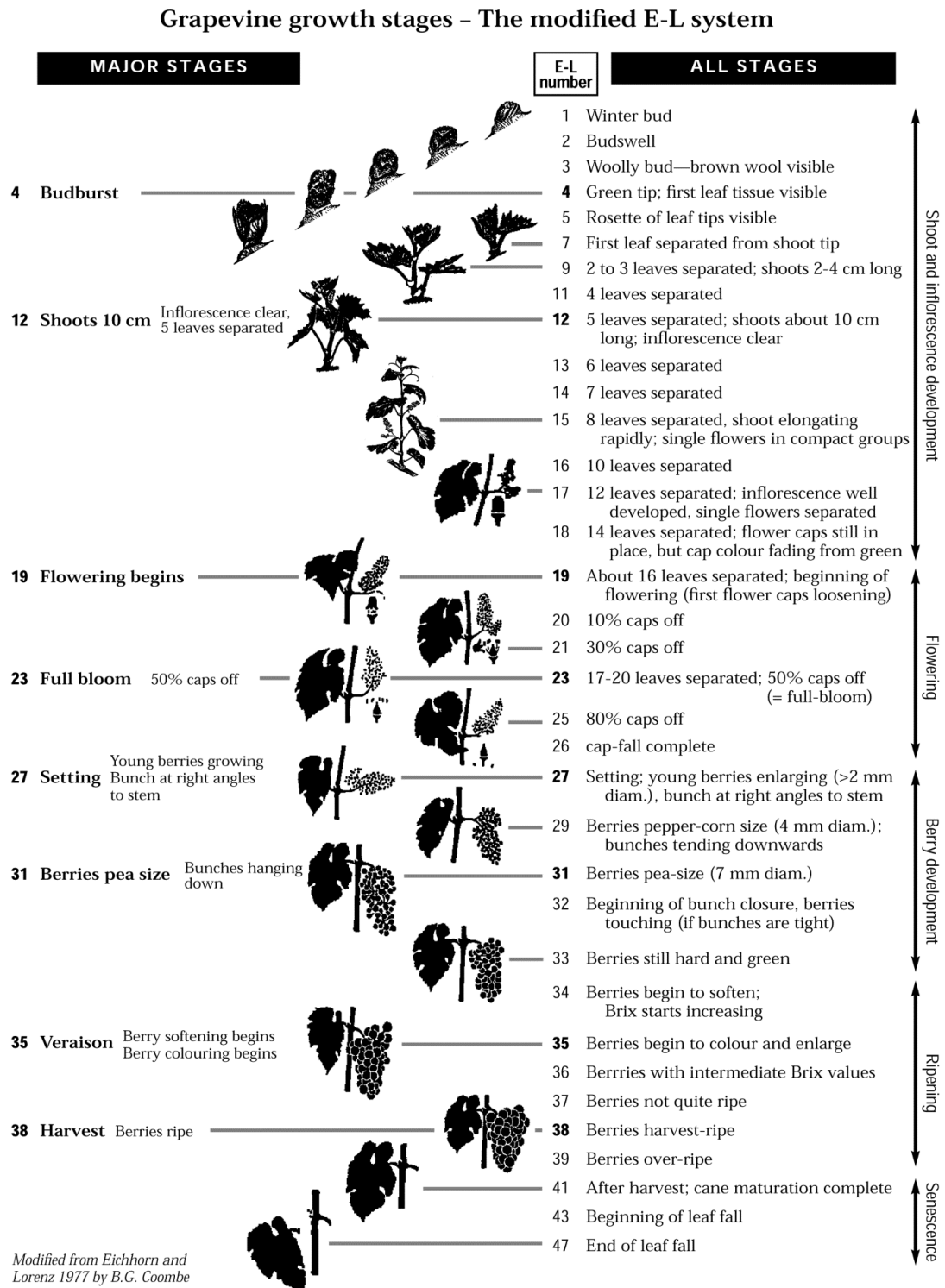


Figure 2. Grapevine growth stages (Coombe, 1995).

1.2.2. Grapevine vegetative cycle

Grapevine morphological changes occur within an annual cycle with distinct phenological events. The main phenological stages in the grapevine are budburst, flowering, fruit-set, veraison, maturity and leaf fall. The below diagram illustrates the annual cycle in the northern hemisphere (**Fig. 3**).

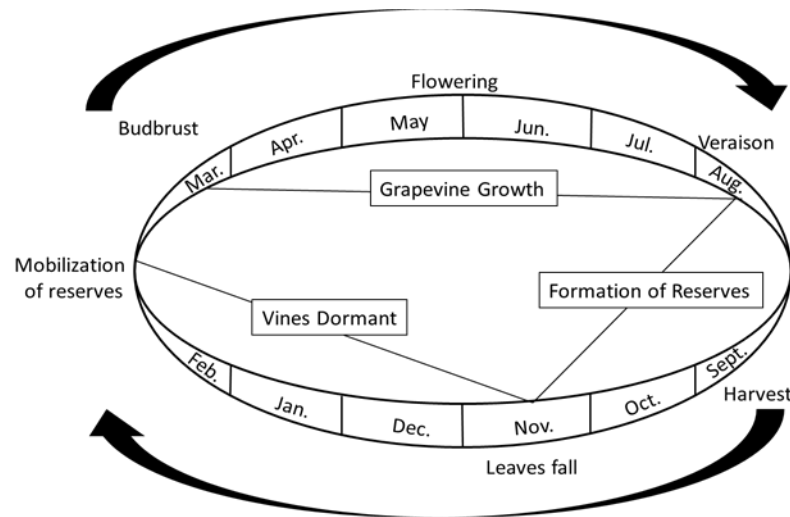


Figure 3. Vegetative and reproductive annual cycle of grapevine (adapted from Salazar and Melgarejo, 2005).

Seed propagation for grapevines requires several years to reach reproductive phase (Champagnol, 1984). However, grapevines in vineyard are generally propagated asexually, as cuttings grafted into rootstocks. Over winter, dormant grapevines are pruned to the required number of buds for the coming growing season (pruned dormant grapevines start to exude sap (“bleeding”) that may last from several days to few weeks; Martínez de Toda, 1991). When in spring temperature reaches approximately a mean of 10°C, dormant vines begin the sprout of new shoots (“Budburst”), announcing the beginning of the new growing season (Salazar and Melgarejo 2005; Reynier, 2012). At the end of spring or early summer, the mean temperature of 10-15°C provokes “flowering”, followed closely by the beginning of berry development called “fruit-set” (Martínez de Toda, 1991). At this stage, grapevine growth depends entirely on nutrient reserves stored in roots and stems formed the previous year, and the small berries begin to grow (Keller, 2010). These reserves are consumed both to build structures and to be respired, until the new leaves become photosynthetically active. Vegetative growth usually stops at mid or end summer. The following phenological stage is “veraison” that

indicates the beginning of ripening, with the grapevine focusing on moving sugar to the berries until reaching optimal ripeness for “harvest”. When berries reach the veraison phenological stage, berry has almost reached its final size (although its volume may, however, continue to augment until harvest). After harvest, shoots begin to form a periderm, turning from green to yellowish-brown color. Formation of storage nutrients for the next season may then commence (Reynier, 2012). Leaf senescence starts even before grapes are harvested, with re-absorption of part of the nutrients invested in leaves during growth. Leaves then fall from the base of the petioles at the abscission zone. Bud winter dormancy is related to abscisic acid levels change in buds and nodes (Rohde and Bhalerao, 2007). Shoots dehydrate, as part of the acclimation process to cold when unfavorable conditions for plant growth appear in winter.

1.2.3. Grapevine reproductive cycle

After fruit-set, a first phase of growth is the result of massive cell divisions and expansion causing rapid growth. This rapid berry growth stage is followed by a lag phase, in which little or no growth occurs. Thereafter, the second growth phase starts with the onset of ripening (veraison) (**Fig. 4**). Veraison produces berry softening and coloring. Overall, the berry size approximately doubles between the initial stage of veraison and harvest. At the end of the berry development, organic acid accumulation (including tartaric and malic acid concentration) is down-regulated, while sugar accumulation is increased. On the other hand, secondary metabolites, such as synthesis and accumulation of anthocyanins in berry skins, appear during veraison and continue until harvest (Panagiotis et al., 2012; **Fig. 4**).

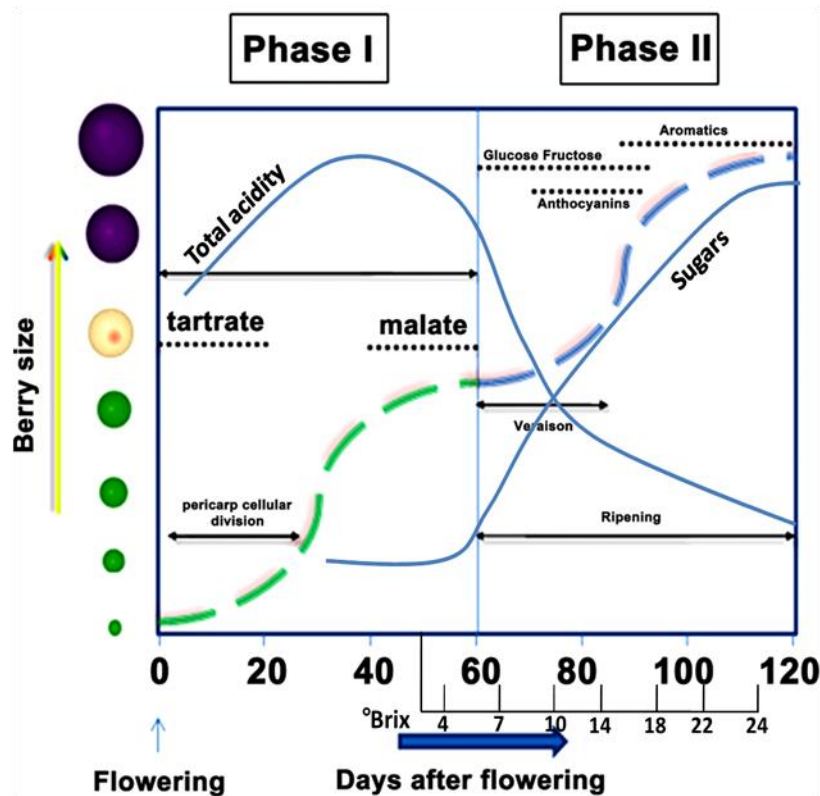


Figure 4. Double sigmoidal curve of grape berry growth (size, color) and development (main metabolites; sugars and organic acids) that are divided in two phases with the progression of berry size exhibiting a lag phase which is equally distributed between the two phases. Adapted from Coombe (2001), and Panagiotis and co-authors (2012).

1.3 Grape berry

1.3.1. Grape morphology

Grape berries are fleshy fruit constituted from a single ovary and carpel and its structure consists mainly of three tissues: skin, pulp (flesh) and seeds (**Fig. 5**). The outermost layer of cells of the exocarp (epidermis) also referred as “skin” is covered by a kind of wax which calls “cuticle” that protects berries from dissection and pathogen invasion (Rosenquist and Morrison, 1988). Depending on the variety, developmental stage and growing conditions, skin may vary to occupy from 6.5% up to 24% of the total fresh weight. The single cell layer of the mesocarp is formed the exocarp, where polyphenols and anthocyanins accumulate from veraison stage, responsible for both red pigmentation in red grapes and wine quality (Hardie et al., 1996). Exocarp cell walls are 20 times thicker than mesocarp, contributing to reduce water loss, but being also a limitation to berry enlargement.

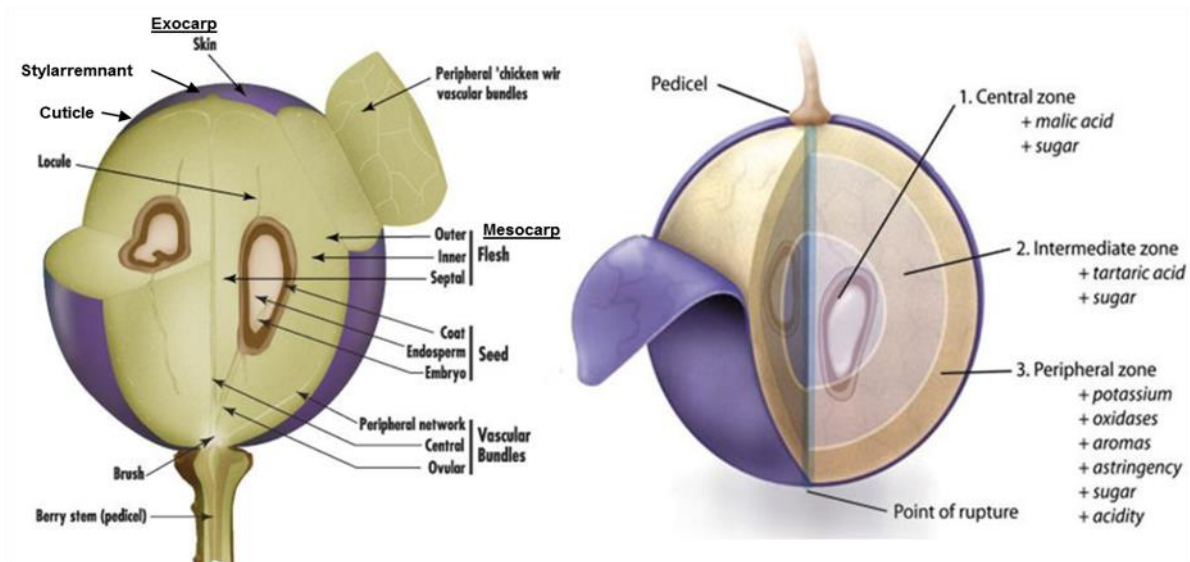


Figure 5. Structure of a ripe grape berry partially sectioned on the long and the central axis to show internal parts (Illustration adapted by [EnoViti](#)).

The flesh is constituted by the endocarp and mesocarp, which are specialized in the accumulation of sugars (glucose, fructose), organic acids (malic and tartaric acid) and water. Most of the berry enlargement is caused by the cells of the mesocarp, and their vacuoles represent from 65% up to 99% of the total cell volume (Hardie et al., 1996; Diakou and Carde, 2001). The innermost part of the endocarp composes several layers of cells surrounding the seeds; however, this is rather hard to differentiate from the mesocarp at the end of the maturity (Carmona et al., 2008). Seeded grapevine cultivars that contain embryos include usually one to four seeds that represent a 2-6% of the volume of the berry (Galet, 2000; **Fig. 5**).

1.3.2. Grape composition

Grape berry biosynthesize the primary metabolites essential for survival (such as carbohydrates, organic acids, etc.) but the berry has the ability to synthesize all other berry components, known as secondary metabolites such as phenolic and aroma compounds that define a particular wine.

Water is the most abundant constituent of grape berries (70 to 80%). Increases in berry volume are primarily associated with water until veraison, and consequently soil water availability is one of the determining factors for berry size (Coombe and McCarthy, 2000). During ripening, water influx pathway to berry shifts from the xylem to predominantly phloem supply (Etchebarne et al., 2010).

Carbohydrates are present in both simple forms as sugars (mainly glucose and fructose), and more complex ones as pectines and glucans at small quantities in the berry must (Conde et al., 2007). Glucose is the predominant sugar until ripening but later glucose and fructose co-exist usually in approximately equal quantities in the berry. The fructose / glucose ratio can however vary among grape varieties. Sugars are imported into the berry from the leaves through the phloem as photosynthetic product, and are predominantly stored in the berry vacuoles of flesh cells for providing carbon skeletons to other compounds such as organic acids and amino acids (Dokoozlian, 2000).

The main grape berry acids are tartaric and malic acids, as both represent approximately 90%. Grape juice also contains low levels of citric, ascorbic and acetic acids. At early berry formation, organic acids synthesize and accumulate in the berry at high concentration. These high concentrated organic acids decline with starting berry ripening due to dilution (Dokoozlian, 2000). The balance between the two major organic acids has huge influence on wine quality (Conde et al., 2007).

Phenolic compounds are also synthesized in the berry, and are important constituents of grape berry for determining the wine's color and flavor. They are primarily located in the seeds and skins of the berry, and the berry must contain a very small amount (3 to 5% of total phenols). Phenolic compounds are a group of substances can be divided into: non-flavonoids (with a simple C₆ backbone), such as hydroxybenzoic acids, hydroxycinnamic acids, volatile phenols and stilbenes, and flavonoid compounds, such as flavones, flavanones, flavonols, flavan-3-ols, proanthocyanidins and anthocyanins. Non-flavonoid phenolics, except hydroxycinnamic acids, are presented in grapes in small amounts (Kennedy et al., 2006; Conde et al., 2007). Flavonoids constitute a significant portion of the phenolic material in grapes (Conde et al., 2007). According to the oxidation state of the C ring in the ring structure of flavonoids, phenolic compounds are divided into structural classes that include flavonols, flavan-3-ols (that include simple flavan-3-ols and their polymeric forms proanthocyanidins), and anthocyanins (Castellarin et al., 2012).

Anthocyanins are perhaps the most important phenolic compounds present in the grape, which are synthesized via the flavonoid pathway and largely responsible for the red and purple color of grapes and wines (He et al., 2010). They exist in both colored and

colorless forms and generally are found only in the skin, except in “teinturier” or “sharks” varieties with skin and pulp colored.

In grape berries, many volatile aroma compounds can be found. Their concentrations vary by many factors, such as variety and plant manipulation during berry development (crucial to reach desired flavor level at harvest). The aroma compounds in berry are largely localized in the skin and the layers of cells immediately beneath it. Their concentration tends to increase during ripening, and it is important that the grape berries be harvested when the aroma is at its peak.

Tannins are very complex flavonoid compounds containing huge molecules, responsible for bitterness and astringency. They appear in white and red varieties and are located both in the skin and seeds (yellow, brown or red colored). The total content in the skin slightly increases with maturation. Increase in molecular size with maturation makes these compounds insoluble and, consequently, they precipitate, and the wine's astringency decreases.

Grapes contain various nitrogenous compounds that include inorganic nitrogen mostly as free ammonium (NH_4^+), and organic nitrogenous compounds such as amino acids, peptides, and proteins (Conde et al., 2007). Total nitrogen concentration of the berry increases during ripening, and varies with variety, climate, soil, fertilization and other cultural practices. Yeast activation in the berry juice needs nitrogen sources.

Approximately 0.2 to 0.6% of fresh weight of grape berry are minerals that are taken up by roots of the grapevine from the soil and can be directly accumulated in the berries. Potassium, calcium, sodium, phosphate and chloride are the principle mineral ions present in the berry and among them, potassium is the most important mineral because of its crucial role in enzyme activation, transmembrane potential control, and osmotic potential regulation (Conde et al., 2007). The potassium content in berry increases during ripening and its presence in the berry leads to the formation of potassium bitartrate, which decreases acidity and rises berry must pH.

2. Climate change related factors

Our climate is changing due to human activities, particularly fossil fuel burning that causes accumulation of atmospheric carbon dioxide (CO₂), methane (CH₄), mono-nitrogen oxides (NO_x) and CFC compounds since the beginning of industrial development. The most important gas within these gases (so-called heat-trapping gases or anthropogenic gases) is CO₂, because its atmospheric concentration has increased from 280 μmol mol⁻¹ air (ppm) in the pre-industrial period to 389-400 ppm nowadays, is expected to reach ca. 700 ppm at the end of this century, and it is a greenhouse-effect gas (IPCC, 2013).

Due to the continuous increase of CO₂ atmospheric concentration, current average air temperature is 0.6 °C higher than the average temperature recorded in the pre-industrialization period, because of its greenhouse-effect impact. Models anticipate an augmentation of temperature between 1.8 and 4.8 °C in the year 2100, depending on the future CO₂ emissions and mitigation measures adopted (IPCC, 2013).

Global evaporative demand has also been changed, altering cloud patterns due to the increase in atmospheric temperature. Non-uniform distributed precipitations caused by high evaporation result in water scarcity and increased aridity in other areas. For the Mediterranean region, long-term simulations (up to 2100) foresee a reduction in precipitations of ca. 30%, in part due to a decrease of cloud coverage (Trenberth and Fasullo, 2009; IPCC, 2013). Therefore, the Mediterranean area is particularly vulnerable to a temperature and aridity increase, as well as sustained severe weather (Tubiello et al., 2000).

2.1. Effect of elevated atmospheric CO₂ concentration

Any change in atmospheric CO₂ concentration has potential to alter directly plant photosynthetic efficiency and consequently plant growth, yield and physiology. These effects have been investigated in agricultural crops (Cure and Acock, 1986), trees (Ceulemans and Mousseau, 1984) and other plant species (Poorter, 1993; Idso and Idso, 1994).

For C3 plants, such as grapevine, this rise in atmospheric CO₂ concentration may increase photosynthesis because the enzyme ribulose-1,5 biphosphate

carboxylase/oxygenase (Rubisco) is unsaturated at the current CO₂ conditions (Drake et al., 1997). Up to 800 ppm, CO₂ concentration limits carbon fixation (Long et al., 2006). Any CO₂ increase therefore may enhance CO₂ fixation rates (Long and Drake, 1991). Nevertheless, plants may experience photosynthetic acclimation (down-regulation) in long-term elevated CO₂ exposure, a process that decreases photosynthesis capacity below its maximum potential (Jifon and Wolfe, 2002; Long et al., 2004; Erice et al., 2006).

Because grapevine photosynthesis is at the current CO₂ concentration CO₂-limited, any increment of atmospheric CO₂ concentration can directly enhance grapevine growth and yield (Bowes, 1993; Rogers et al., 1994) with higher accumulation of vegetative biomass and fruits (Bindi et al., 1996).

Not only yield can be affected. Elevated CO₂ may affect all phases of berry growth and the accumulation of grape constituents with potential influence on quality (Mira de Orduña, 2010). Under elevated CO₂ conditions, berry acids and sugars accumulation were stimulated in early stages of grape development, although effects disappeared at maturity (Bindi et al., 2001). Moreover, berry sugars, total anthocyanins and tannins (Gonçalves et al., 2009), and must color tonality and intensity (Salazar-Parra et al., 2010) were not influenced by elevated CO₂.

2.2. Effect of elevated atmospheric temperature

The impact of future increased global temperature on the distribution of wine producing areas was also modeled (White et al., 2006; Webb et al., 2007; Hannah et al., 2013). Model outputs include grapevine phenological changes and a reduction of the extension of the current premium wine regions.

Elevated temperature may accelerate grapevine phenology up to three weeks, leading to a reduction of the vegetative and reproductive period (Lebon, 2002; Seguin and Cortazar, 2005), and increasing the risk of berries sunburn.

Also, high temperature impacts berry quality because of the temperature sensitivity of the enzymes of several metabolic routes. It reduces anthocyanin accumulation because several enzymes of this metabolic pathway are temperature sensitive (Kliwer, 1970; Mori et al., 2007). Furthermore, temperature may reduce grape acidity and particularly

can modulate grape malic acid concentration (Spayd et al., 2002; Sweetman et al., 2014). As a consequence, cooler regions tend to produce wine with high malic acid levels (Blouin and Guimberteau, 2003). Other important effect of temperature is the induction of phenological changes, leading to changes in traits such as sugar accumulation, color, acidity and bitterness (Sadras and Moran, 2012; Bonada et al., 2013; Sadras et al., 2013).

2.3. Effect of water scarcity

Water stress is one of the environmental factors that regulate plant vegetative growth, photosynthesis and yield and it has been investigated due to the seasonal drought experienced in many viticultural regions. In the Mediterranean area, grapevines usually face water deficit during summer, as a consequence of the low soil water content and the elevated gradients of water vapor pressure between leaves and air. Grapevine, as a typical Mediterranean species, demonstrates good adaptation to summer drought due to a deep root system, efficient reduction of stomatal conductance and a high capacity of osmotic adjustment (Patakas and Noitsakis, 1999).

Soil water availability affects grapevine vegetative growth. Low soil water availability causes shoot growth stopping (Matthews and Anderson, 1989; Williams and Matthews, 1990), reduction in leaf size (Matthews et al., 1987) and high leaf senescence (Kliwer and Weaver, 1971). Reduction in carbon assimilation and leaf area may compromise yield and berry quality (Chaves et al., 2010).

Contradictory effects have been documented in the literature due to the wide range of situations encompassed by water deficit studies (Chaves et al., 2010). Some of these effects on berry quality, are (i) increases in malic acid concentration (López et al., 2007), sugar content (Matthews et al., 1990; Ojeda et al., 2002; Antolín et al., 2006; Koundouras et al., 2006) and pH values (Bahar et al., 2011) that influenced negatively wine quality, (ii) reduction in malic acid concentration (De Souza et al., 2005; Koundouras et al., 2006; Intrigliolo and Castel, 2009; Salazar-Parra et al., 2010), berry volume, total soluble solids, potassium and tartaric acid concentration (Bahar et al., 2011) or (iii) no change on malic acid concentration (Esteban et al., 1999). However, generally strong water stress reduces grapevine yield and quality (Myburgh, 2003). Water stress during flowering and veraison period reduces berry size (Matthews and

Anderson, 1989; McCarthy, 1997; Ojeda et al., 2002), resulting in changes in concentration of must phenolics including anthocyanins (Matthews et al., 1990; Petrie et al., 2004; Castellarin et al., 2007).

3. Interactions between elevated CO₂, high temperature and water deficit

Future conditions are expected to have impacts due to the combined effects of climate change-related factors (i.e., elevated CO₂, high temperature and water deficit). So far, influence of climate change-related factors on grapevine has been investigated individually but rarely their interactions. When the abovementioned stress factors were combined, impacts on berry weight were stronger (McCarthy, 1997) and changes in berry quality were more complicated (Schultz, 2000).

Thus, when elevated CO₂ was combined with high temperature, the individual effect of elevated CO₂ stimulating grapevine yield was decreased or completely cancelled (Bindi et al., 2001).

With increases in the number of warm days during flowering and veraison period and with a reduced precipitation during maturation, Merlot and Cabernet Sauvignon produce berries with a higher sugar to total acid ratio and greater berry weights (Jones and Davis, 2000). In the case of Tempranillo, phenology was accelerated, berry malic acid and total anthocyanin concentrations were reduced and anthocyanin extractability was improved by climate change conditions (Salazar-Parra et al., 2010). The literature indicates that effects of single stress factors are sometimes inconsistent (see above the case of water stress) and multiple stress factors researches on grapevine are limited. However, during the last 30 years and due to climate change, a shorter grapevine phenology has been reported correlated with changes in wine composition (Mira de Orduña, 2010). Adaptive strategies have been successfully carried out to counteract or mitigate these effects (Martínez de Toda and Balda Manzanos, 2011, 2014; van Leeuwen et al., 2013). Therefore, understanding the environmental influence on grapevine may provide clues to design the best adaptive strategies.

4. Climate change research under controlled conditions

4.1. Fruit-bearing cuttings model

Grapevines are perennial plants and several years are needed for converting them in reproductive. Roots of grapevines in vineyards tend to grow both horizontally and especially vertically to penetrate into the soil. Obtaining a full-grown reproductive grapevine plant for experimental research may, therefore, be not convenient when space and time are limited.

The fruit-bearing grapevine hardwood cuttings model is based on developing reproductive grapevines starting with rooting cuttings (Mullins, 1966). This model provides a feasible system that allows the development of vegetative (roots, leaves and shoots) and reproductive (inflorescences and clusters) organs as for the vineyard grapevines, under fully controlled environmental conditions in a short time period. Grapevines obtained in this way respond like vineyard plants to different cultural and environmental conditions (Ollat et al., 1998; Lebon et al., 2005). Moreover, effects of diverse environmental factors on grapevine have been successfully examined using this model (Antolín et al., 2010; Salazar-Parra et al., 2010, 2012a, 2012b; Carbonell-Bejerano et al., 2013; Dai et al., 2013; Niculcea et al., 2013, 2014; Martínez-Lüscher et al., 2016; Torres et al., 2016). More details about the model are available in Morales et al. (2016).

4.2. Temperature Gradient Greenhouses (TGGs) to simulate climate change conditions

Studying the influence of climate change-related factors (elevated CO₂, high temperature and water deficit) individually and interacting on grapevine biology is difficult to accomplish. Within different facilities (growth chambers, greenhouses or FACE (Free Air CO₂ enrichment) facilities), the Temperature Gradient Greenhouses (TGGs) are a convenient way to investigate climate change-related factors both individually and interacting among them (Morales et al., 2014). A complete set of four TGGs allows working on effects of temperature (ambient, or ambient +4°C), CO₂ concentrations (400 ppm ambient, or 700 ppm elevated) and water availability (full irrigation, or water deficit) individually or in interaction (Morales et al., 2014). Furthermore, influence of climate change-related factors on grapevine physiology was successfully examined using these TGGs (Salazar Parra et al., 2015).

5. References

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OBJETIVOS

The general objective of this PhD Thesis was to assess the response of Red and White Tempranillo grapevine (*Vitis vinifera* L.) to different climate change scenarios (elevated CO₂, high temperature and water deficit).

The general objective was divided into the following objectives:

- **Objective 1.** Evaluate the changes in red and white Tempranillo grapevine vegetative and reproductive growth affected by the water deficit, elevated CO₂ and elevated temperature.
- **Objective 2.** Assess the changes in red and white Tempranillo grape berry composition affected by the water deficit, elevated CO₂ and elevated temperature.
- **Objective 3.** Characterize the photosynthetic acclimation to elevated CO₂ in red and white Tempranillo grown under different climate change scenarios (water deficit, elevated CO₂ and elevated temperature).

The above exposed objectives have been developed in the following chapters:

- **Chapter 1.** Effects of climate change including elevated CO₂ concentration, temperature and water deficit on growth, water status, and yield quality of grapevine (*Vitis vinifera* L.) cultivars. Published in *Agricultural Water Management* (2015) 159: 155-164.
- **Chapter 2.** Using fruit-bearing cuttings of grapevine and temperature gradient greenhouses to evaluate effects of climate change (elevated CO₂ and temperature, and water deficit) on the cv. red and white Tempranillo. I. Growth, and substrate and plant water status in three consecutive growing seasons (2013-2015). Submitted to *Agricultural Water Management* (it requires major revision).
- **Chapter 3.** Using fruit-bearing cuttings of grapevine and temperature gradient greenhouses to evaluate effects of climate change (elevated CO₂ and temperature, and water deficit) on the cv. red and white Tempranillo. II. Yield and must quality in three consecutive growing seasons (2013-2015). Submitted to *Agricultural Water Management* (it requires minor revision).

- **Chapter 4.** Photosynthetic acclimation to elevated CO₂ in red Tempranillo grapevine and its natural, spontaneous mutant white Tempranillo under different climate change scenarios. To be submitted to *Photosynthesis Research*.

***Anexo.** Sensitivity of grapevine phenology to water availability, temperature and CO₂ concentration. Published in *Frontiers in Environmental Science*, 4: 48.

CAPÍTULO 1

Effects of climate change including elevated CO₂ concentration, temperature and water deficit on growth, water status, and yield quality of grapevine (*Vitis vinifera* L.) cultivars.

(Agricultural Water Management (2015) 159: 155-164.)

Effects of climate change including elevated CO₂ concentration, temperature and water deficit on growth, water status, and yield quality of grapevine (*Vitis vinifera* L.) cultivars

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CAPÍTULO 2

Using fruit-bearing cuttings of grapevine and temperature gradient greenhouses to evaluate effects of climate change (elevated CO₂ and temperature, and water deficit) on the cv. red and white Tempranillo. I. Growth, and substrate and plant water status in three consecutive growing seasons (2013-2015).

Submitted to Agricultural Water Management

(it requires major revision)

Using fruit-bearing cuttings of grapevine and temperature gradient greenhouses to evaluate effects of climate change (elevated CO₂ and temperature, and water deficit) on the cv. red and white Tempranillo.

I. Growth, and substrate and plant water status in three consecutive growing seasons (2013-2015)

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CAPÍTULO 3

Using fruit-bearing cuttings of grapevine and temperature gradient greenhouses to evaluate effects of climate change (elevated CO₂ and temperature, and water deficit) on the cv. red and white Tempranillo. II. Yield and must quality in three consecutive growing seasons (2013-2015).

Submitted to Agricultural Water Management

(it requires minor revision)

Using fruit-bearing cuttings of grapevine and temperature gradient greenhouses to evaluate effects of climate change (elevated CO₂ and temperature, and water deficit) on the cv. red and white Tempranillo. II. Yield and must quality in three consecutive growing seasons (2013-2015)

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CAPÍTULO 4

Photosynthetic acclimation to elevated CO₂ in red Tempranillo grapevine and its natural, spontaneous mutant white Tempranillo under different climate change scenarios

(To be submitted to Photosynthesis Research)

Photosynthetic acclimation to elevated CO₂ in red Tempranillo grapevine and its natural, spontaneous mutant white Tempranillo under different climate change scenarios.

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Abstract

Atmospheric CO₂ concentration has increased from preindustrial level of 280 μmol CO₂ mol⁻¹ air (ppm) to currently more than 400 ppm. Intergovernmental Panel on Climate Change foresees that atmospheric CO₂ concentration will continue increasing to reach at the end of this century 700 ppm (ranging from 421 to 936 ppm, depending on mitigation measures adopted). Due to its greenhouse effect, elevated atmospheric CO₂ concentration is leading to higher atmospheric temperatures, a phenomenon that is being accompanied by episodes of less water availability or drought periods. All these three climate change-related factors affect photosynthesis in C3 plants. Grapevine (*Vitis vinifera* L.) is a plant species very sensitive to those environmental factors. Under elevated CO₂ concentration, grapevine photosynthesis increases, modulated by temperature and water availability. However, under prolonged exposure to elevated CO₂, grapevine down-regulates photosynthesis (also known as photosynthetic acclimation), decreasing photosynthetic capacity. The aim of the present study was to investigate the photosynthetic acclimation phenomenon in red Tempranillo grapevine plants and its natural, spontaneous mutant white Tempranillo growing under elevated CO₂, elevated temperature and water deficit. Experiments in temperature gradient greenhouses were used to recreate these climate change conditions. Grapevine plants underwent photosynthetic acclimation after a long exposure to elevated CO₂ (700 ppm), regardless of temperature and water availability. Evidence comes from photosynthetic capacity decreases (net photosynthetic rates measured at 700 ppm), leaf starch accumulation, and increases in leaf carbon/nitrogen ratio. Photosynthetic acclimation was well correlated to leaf starch, but not to soluble sugars, accumulation. The white Tempranillo mutant has altered its response to prolonged exposure to elevated CO₂. For any given sink size (bunch, total vegetative or total plant dry matter) or any given leaf starch accumulation, white Tempranillo always had higher levels of photosynthetic acclimation than the red one (quantified by the extent of the increases in leaf carbon/nitrogen ratio). Data suggest that mutation in white Tempranillo has affected loci other than grape color.

Keywords Climate change, Elevated CO₂ concentration, Grapevine, Photosynthetic acclimation, Red and white Tempranillo

Abbreviations A_N - net photosynthesis; BSA - bovine serum albumin; Chl - chlorophyll; C_i - sub-stomatal CO_2 concentration; DM - dry matter; DW - dry weight; FACE - free air CO_2 enrichment; FW - fresh weight; g_s - stomatal conductance; IPCC - Intergovernmental Panel on Climate Change; LED - light emitting diode; LSD - least significant difference; PPFD - photosynthetic photon flux density; PVPP - polyvinyl polypyrrolidone; RH - relative humidity; Rubisco - Ribulose-1,5-biphosphate carboxylase oxygenase; T - temperature; TGG - temperature gradient greenhouse; TSP - total soluble proteins; TSS - total soluble sugars

Introduction

Cumulative emissions of CO_2 largely have established global mean surface warming during the late 20th century and beyond. CO_2 , with an increased concentration from preindustrial level of $280 \mu\text{mol } CO_2 \text{ mol}^{-1}$ air (ppm) to currently more than 400 ppm, is the most relevant greenhouse-effect gas in volume and global effect (IPCC 2014). Thus, its concentration has increased by about 40% from 1800 to 2012 (National Academy of Sciences 2014), mainly as a result of fossil-fuel burning, deforestation and intensive animal husbandry (Irigoyen et al. 2014). It has been predicted to continue increasing, ranging from 421 to 936 ppm for the end of the century (Meinshausen et al. 2011). Multiple lines of evidence define a powerful, coherent, and almost linear relationship between cumulative CO_2 emissions and global warming (Collins et al. 2013). Projected average global temperature increases for the year 2100 range from stabilization at 1.5°C higher than the current reference period to a more than 4°C increase, depending on the mitigation measures adopted (IPCC 2014). Most climate models predict higher and more variable temperatures with a significant increase of summer air temperature, higher frequency of extreme events, and changes in precipitation patterns with lower and more erratic rainfall leading to water stress (Moutinho-Pereira et al. 2009). The main impacts of global warming predicted by most climate models in the Mediterranean area an increase in winter temperature combined with changes in rainfall amount and distribution (Ashour and Al-Najar 2012). Major wine growing regions must face all these changes in the climate (atmospheric CO_2 concentration increases, enhanced

temperatures and decreased rainfall) in order to maintain grapevine yield and grape, and in turn wine, quality (Tubiello et al. 2000).

Grapevine physiology and growth is sensitive to environmental factors, including atmospheric CO₂ concentration, air temperature and water availability (Salazar-Parra et al. 2010), thus making grapevine vulnerable to climate change (Fraga et al. 2014). In C₃ plants, including grapevine, any CO₂ short-term increase would lead to higher photosynthetic rates due to the no substrate-saturated requirement for CO₂ fixation of the enzyme Ribulose-1,5-biphosphate carboxylase oxygenase (Rubisco) (Long 1991; Drake et al. 1997). However, the initial CO₂ stimulation of photosynthesis frequently is not maintained for long periods of time, and under long-term elevated CO₂ exposures photosynthetic rates down-regulate in a process known as photosynthetic acclimation (Jifon and Wolfe 2002; Long et al. 2004; Erice et al. 2006). Photosynthetic acclimation to prolonged elevated CO₂ exposure is not a generalized process. Photosynthesis acclimation to elevated CO₂ can be prevented or at least reduced, allowing plant growth stimulation by providing additional carbon sinks to the plants such as occurs in nodulated leguminous plants (Erice et al. 2006; Bertrand et al. 2007). In these nodulated species, plants did not show signs of acclimation after several years of free air CO₂ enrichment systems (FACE) experiments (Zotz et al. 2005; Springer and Thomas 2007). On the contrary, grapevine pruning, reducing largely plant carbon sink, aggravates photosynthetic acclimation to elevated CO₂ symptoms (Salazar-Parra et al., submitted). Plant carbon sink size is therefore a factor key when plant responses to elevated CO₂ are investigated.

A second important factor when investigating plant response of foreseen climate change is obviously temperature. Photosynthesis is a temperature-dependent process. Beyond a temperature threshold where photosynthetic rates reach a maximum, photosynthesis declines. Grapevine photosynthesis is severely impeded at high temperatures (Greer and Weston 2010). Temperatures higher than 30°C caused a 30–50% reduction in the rates of leaf grapevine photosynthesis (Greer and Weedon 2013). In the literature, both stomatal and non-stomatal limitations of grapevine photosynthesis have been reported in response to elevated temperature. In grapevine, stomatal conductance declined during and after a high temperature event, accompanied by decreased photosynthesis (Greer et al. 2010). By contrast, other reports ascribed the reduction in grapevine photosynthesis during the heat treatment to the process of carbon

assimilation by Rubisco (Greer and Weedon 2013). Reports from several species (Law and Crafts-Brandner 1999; Haldimann and Feller 2004; Salvucci and Crafts-Brandner 2004) including grapevine (Greer and Weedon 2013) suggest that the high temperature inhibits Rubisco activation state. In addition, flowering is one of the phenological stages highly susceptible to be impacted by elevated temperature (that can cause a huge reduction in yield), while elevated temperature during late fruit set and beyond can have an impact on berry development and/or cause sunburn damage, reducing bunch yield and berry quality (Greer and Weston 2010). Grapevine yield reductions mediated by elevated temperatures may affect photosynthesis and the photosynthetic acclimation to elevated CO₂, because, as already mentioned, any carbon sink reduction may aggravate the process of photosynthetic acclimation to elevated CO₂.

Soil water availability is the third factor related to climate change that has a large influence on grapevine growth and therefore in the total grapevine carbon sink. Symptoms of leaf senescence (Kliever and Weaver 1971), reduction in leaf size (Matthews et al. 1987) and shoot growth stopping (Matthews and Anderson 1989; Williams and Matthews 1990; Kizildeniz et al. 2015) have been reported in grapevine in response to low water availability. Water deficit reduces the Rubisco CO₂ availability due to the decreased CO₂ concentration in the chloroplasts, as a consequence of both reduced stomatal (gS) and mesophyll conductance to CO₂ (Flexas et al. 2002, 2009). Therefore, low water availability reduces the grapevine photosynthetic capacity, which affects negatively grapevine vegetative and reproductive growth.

The acclimation process to elevated CO₂ has been scarcely investigated (and not in depth) in grapevine growing under foreseen climatic change conditions. Some reports (see above) have documented effects of elevated CO₂ concentration, water deficit and elevated temperature independently, but a few in combination because multi-factorial studies are complex, difficult and expensive to execute under field conditions (Bindi et al. 2001; Salazar-Parra et al. 2012b; Albert et al. 2011; Leibar et al. 2015). Today, advanced methodologies are available for using greenhouses to simulate climate change scenarios (Morales et al. 2014). In the present work, we used a new greenhouse concept for plant research, the temperature gradient greenhouse (TGG) (Morales et al. 2014). TGGs allow comparison of current and future conditions and to attribute to one specific factor a physiological response, enabling for more mechanistic researches on the causes

of the physiological responses to future, foreseen climate conditions (Morales et al. 2014).

Tempranillo is a local red grape variety widely cultivated in northern and central Spain, currently expanded to many other viticulture areas in the world. A new white berry somatic variant of Tempranillo (“white Tempranillo”) is the result of a spontaneous, natural mutation that comprised chromosomal deletions and reorganizations (with genetic material loss), which was discovered in an old red Tempranillo vineyard in 1988 in Murillo de Río Leza, La Rioja, Spain (García-Escudero et al. 2011) and vegetatively propagated from one single grapevine plant.

Therefore, the aim of the current study was to investigate the effects of elevated CO₂, elevated temperature and water deficit, either acting individually or interacting among them, on the photosynthetic acclimation process to elevated CO₂ in two grapevine (*Vitis vinifera* L.) cultivars, red Tempranillo and its natural, spontaneous mutant white Tempranillo. One of the strengths of the present work lies in the assessment of three-way interactions between CO₂, temperature and water availability under fully controlled conditions using the TGGs.

Materials and methods

Plant material and growth conditions

Dormant cuttings of *Vitis vinifera* L. cv. red (accession T43, clone RJ-43) and white (accession CI-101 in “La Grajera” germplasm bank from the Government of La Rioja, Spain) Tempranillo were obtained from an experimental vineyard of the Institute of Sciences of Vine and Wine (ICVV, Logroño, La Rioja, Spain).

Cuttings were chosen to obtain fruit-bearing cuttings according to Mullins (1966), as described in Morales et al. (2016). Rooting was induced using indole butyric acid (300 mg L⁻¹) in a rock-wool heat-bed (27°C) kept in a cool room (4°C). After one month, the rooted-cuttings were planted in 0.8 L plastic pots that contained a mixture of sand, perlite and vermiculite (1:1:1, in volume) and then transferred to the first greenhouse. Only a single flowering stem was allowed to develop on each plant, in order to get only one berry bunch per plant. Until fruit set, vegetation was controlled and only 4 leaves per plant was allowed to grow. At fruit-set, the cuttings were transplanted to 13 L

plastic pots containing a peat and perlite (2:1, v/v) mixture. During growing period, the greenhouse was set at 26/15°C and 60/80% relative humidity (RH) (day/night) and photoperiod of 15 h with natural daylight supplemented with high-pressure metal halide lamps (OSRAM[®], Augsburg, Germany), providing a photosynthetic photon flux density (PPFD) of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at inflorescence level that was triggered when natural daylight dropped below 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Nutrient solution was applied as proposed by Ollat et al. (1998): NH_4NO_3 (64.5 mg L⁻¹), $(\text{NH}_4)_2\text{HPO}_4$ (75 mg L⁻¹), KNO_3 (129 mg L⁻¹), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (125 mg L⁻¹), $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (248 mg L⁻¹), $(\text{NH}_4)_2\text{SO}_4$ (66 mg L⁻¹), Fe-EDDHA (280 mg L⁻¹), H_3BO_3 (2.86 mg L⁻¹), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.81 mg L⁻¹), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.22 mg L⁻¹), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.08 mg L⁻¹) and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (0.016 mg L⁻¹). These growing conditions were maintained until fruit set from March to May 2014.

Temperature gradient greenhouses and experimental design

Treatments were applied in the temperature gradient greenhouses (TGG), installed at the University of Navarra (42°48'9.3" N, 1°40'6.9" W) in Pamplona (Navarra, Spain), from June to August 2014 (from fruit set to maturity). TGGs are based on temperature gradient tunnels (Rawson et al. 1995), which allow exploring the effects on plants of environmental changes, such as elevated temperature, elevated CO₂ and drought, acting separately or in combination. They were established with a modular design with three temperature modules, which constitutes a temperature gradient ranging from near-ambient temperature in module 1 to ambient temperature +4°C in module 3. CO₂ can be flushed into the TGG to increase the air CO₂ concentration as desired (see details in Morales et al. 2014).

A week after fruit set, cuttings of red and white Tempranillo were subjected to a combination of two temperature regimes (ambient or ambient +4°C, no experimental plants were set in module 2 with intermediate temperature) and two CO₂ concentrations (current ca. 400 ppm and elevated ca. 700 ppm) until plants reached maturity (berries with a total soluble solids (mainly sugars) content of 21-23°Brix). Five phenological stages were chosen for sampling: (I) one week before veraison (around 60 days after flowering), (II) mid-veraison, (III) one week after mid-veraison, (IV) two weeks after mid-veraison and (V) maturity (21-23 °Brix). Plants were maintained without pruning.

CO₂ concentration, temperature, relative humidity and radiation were measured and/or controlled by an informatics monitoring system (Morales et al. 2014).

Soil moisture sensors (EC-5 Soil Moisture Sensors, Decagon Devices Inc., Pullman, Washington, USA) were placed into the pots and used to monitor substrate water content from fruit set to maturity. Plants with full irrigation were maintained at ca. 80% of the substrate capacity (sensor value between 30 to 40%, ($\text{m}^3 \text{H}_2\text{O m}^{-3}$ substrate) $\times 100$). Cyclic drought was induced withholding irrigation. When plants manifested visual signs of water deficit, such as tendrils and leaves downwards (sensor value between 10 and 0%, ($\text{m}^3 \text{H}_2\text{O m}^{-3}$ substrate) $\times 100$), they were irrigated with the same amount of nutrients that received plants under full irrigation in that cycle. Irrigations (full irrigation and cyclic drought) were based on our previous experience with grapevine fruit-bearing cuttings and TGG conditions for the red cultivar (Salazar-Parra 2011).

Measurements and analyses

With the aim of assessing sink size, vegetative and reproductive growth was measured. Complete bunch (berries and rachis), samples of 1 cm² leaf discs (taken from fully developed leaves with a calibrated cork borer), leaves, petioles, stems, and roots were harvested at maturity (defined at 21–23°Brix), weighed (fresh weight, FW) and then oven-dried (dry weight, DW) at 80°C for 48h (until constant weight).

Plant leaf area was estimated at the five sampling dates mentioned above using a model (Costanza et al. 2004), adapted for cv. Tempranillo. The model relates actual leaf area, which was measured with a leaf area meter (LI-300 model; Li-Cor Biosciences, Lincoln, USA) (y), and shoot length (x) ($y=15.5x + 24.8$, $R^2=0.93$). Regression model was built over 40 shoots sampled throughout the growing season from extra plants.

Gas exchange traits were measured on young, fully developed leaves at the five sampling dates, using a portable photosynthesis system (LCi-SD with the PLUS5 compact light unit, ADC BioScientific, England). For both plants grown at ambient and elevated CO₂, net photosynthesis (A_N), stomatal conductance (gS) and sub-stomatal CO₂ concentration (C_i) were measured at 700 $\mu\text{mol CO}_2 \text{mol}^{-1}$ air, 1400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white LED light, and air cuvette temperature between 25 and 30°C.

Leaves used for gas exchange measurements at maturity were harvested, and immediately frozen in liquid nitrogen and stored in a freezer at -80°C for further analysis. Leaf tissue (0.1 g) was extracted with a buffer containing 100 mM Bicine-NaOH (pH 7.8), 10 mM MgCl_2 , 10 mM β -mercaptoethanol, 2% PVPP (polyvinyl polypyrrolidone) (w/v), 1% bovine serum albumin (BSA) (w/v) and 1% Triton X-100 (v/v). Then, the supernatant was clarified, by centrifugation at 13,000g, and used to determine Rubisco in vitro enzyme activity by measuring the oxidation of NADH at 340 nm in a coupled enzyme assay (Sharkey et al. 1991).

Leaf water content (from 1 cm^2 leaf discs, see above) was determined at the five sampling dates as $(\text{FW} - \text{DW})/\text{area}$ ($\text{g H}_2\text{O cm}^{-2}$).

Another set of 1 cm^2 leaf discs (see above) were sampled at the five sampling dates for photosynthetic pigment analyses. Extraction was made in 5 mL of ethanol 95% in an 80°C bath, and then absorbance was measured at 665, 649 and 470 nm (Šesták 1971). Chlorophyll (Chl) *a*, Chl *b*, and carotenoid concentration were evaluated according to the following equations (Lichtenthaler 1987):

$$(i) \text{ Chl } a = (13.7 \times \Delta_{665}) - (5.76 \times \Delta_{649})$$

$$(ii) \text{ Chl } b = (25.8 \times \Delta_{649}) - (7.6 \times \Delta_{665})$$

$$(iii) \text{ Chl } a+b = (6.1 \times \Delta_{665}) - (20.04 \times \Delta_{649})$$

$$(iv) \text{ Carotenoids} = [(1000 \times \Delta_{470}) - (2.13 \times \text{Chlorophyll } a) - (97.64 \times \text{Chlorophyll } b)]$$

Total soluble proteins (TSP), total soluble sugars (TSS) and starch were also determined. At maturity, fresh leaf tissue (0.1 g) was extracted using 0.1 M K-phosphate (pH 7) buffer. Extracts were filtered and centrifuged at 6,000 rpm for 10 min at 4°C . Supernatants were used to quantify TSP and TSS, using the method of Bradford (1976) and Yemm and Wills (1954), respectively. Pellets were stored in a freezer at -80°C for starch analyses. Previously to the starch analyses, photosynthetic pigments were removed from the pellet using 5 mL ethanol 96% (v/v), avoiding interferences in the starch quantification. Then, the pellet was used for starch determination (Jarvis and Walker 1993).

As a measure of the extent of the photosynthetic acclimation (Irigoyen et al. 2014), leaf C/N ratio was determined at maturity. Oven-dried leaf samples (1 mg) were used. Total C and N concentrations were analyzed, using a Thermo-Finnigan 1,112 EA elemental analyzer (Thermo-Fisher Scientific).

Data analysis

A factorial ANOVA was carried out with XLStat 7.5.2 Pro[®] statistical software (Addinsoft, Paris, France). A Fisher's least significant difference (LSD) post-hoc test was applied for comparing means. Results (means \pm standard error) showing $P < 0.05$ were considered statistically significant. Linear regressions were performed between variables with XLstat-Pro (Addinsoft). Coefficients of determination (R^2) were calculated on the basis of Pearson's correlation coefficients, since all variables followed a normal distribution (Shapiro-Wilks test).

Results

Climate change and substrate water status from fruit set to maturity in grapevine cv. red and white Tempranillo

Upon irrigation, water either drains via gravitation or remains in soil pores by capillarity. Substrate water content is defined as the amount of water retained in the substrate after irrigation and drainage. Substrate water content was measured daily in all pots of the grapevine plants. Grapevine plants grown under full irrigation were watered at ca. 80% of the substrate capacity, showing the sensors values ranging from 30 to 40% ($(\text{m}^3 \text{H}_2\text{O m}^{-3} \text{substrate}) \times 100$) (Fig. 1). Substrate water content of full irrigation treatments combined with ambient temperature under ambient (Fig. 1a) or elevated (Fig. 1b) CO_2 showed similar values for both cultivars. In full irrigation treatments combined with elevated temperature under both CO_2 conditions, however, red Tempranillo pots had lower substrate water content than pots of the white cultivar (Fig. 1b and 1d). Cyclic drought plants were grown withholding irrigation until plants showed visual signs of water deficit and the sensors readings were between 10 to 0% ($(\text{m}^3 \text{H}_2\text{O m}^{-3} \text{substrate}) \times 100$) (Fig. 1). When cyclic drought plants had symptoms, they were

irrigated with nutrient solution. Thus, 7 cycles of drought were observed from fruit set to maturity (Fig. 1). In the first drought cycle, plants had visual signs of water deficit when they had ca. 20-10% of substrate water content, and spent more time than the rest of the cycles (Fig. 1). The substrate water content required for showing drought symptoms continuously decreased along the time in pots of red Tempranillo grown under ambient CO₂ and temperature (Fig. 1a). During the whole experiment (ca. 2.5 months), when both cyclic drought-treated red and white Tempranillo showed signs of water stress, pots of red Tempranillo had less substrate water content than those of the white ones, when ambient CO₂ and temperature were used for growing the plants (Fig. 1a). Therefore, under different experimental conditions, red Tempranillo consumed more water than the white cultivar. When grown under elevated temperature (irrespective of the growing CO₂ concentration; Fig. 1b and 1d) and under ambient temperature but elevated CO₂ (Fig. 1c), drought cycles were very similar in both cultivars. Sampling dates are indicated by arrows in Figure 1.

Climate change and plant growth at maturity in grapevine cv. red and white Tempranillo

Generally, the vegetative growth (g DW plant⁻¹) of the different organs (leaf, petiole, stem and root) from plants that grew under full irrigation was higher than that from plants that grew under cyclic drought (Table 1). Vegetative growth was less affected by elevated CO₂ or elevated temperature than by drought (Table 1). In most experimental conditions, root DW increased in red but not in white Tempranillo when plants grew in presence of elevated CO₂ (Table 1). Effects of elevated CO₂ on other vegetative organs were scarce (Table 1). Few effects of elevated temperature were observed. When such effects occurred, they were negative in leaf, petiole, stem and root DW in red but positive in white Tempranillo (Table 1). Under most experimental conditions, changes in leaf area (Fig. S1) were in line with those of leaf DW (Table 1).

Drought decreased and elevated CO₂ increased bunch DW under most experimental conditions (Table 2). These changes were mostly due to berry rather than rachis DW (Table 2). Elevated temperature induced few effects on bunch, berry and rachis DW (Table 2).

Climate change and gas exchange traits from fruit set to maturity, and extractable Rubisco activity at maturity, in grapevine cv. red and white Tempranillo

Under elevated CO₂, there is usually no decrease of photosynthetic activity, but rather a decrease in photosynthetic capacity. A photosynthetic capacity reduction in plants grown under elevated CO₂ suggests photosynthetic acclimation. This is generally evidenced in comparisons of plants grown at ambient and elevated CO₂ and measured at the same CO₂ concentration, either current or elevated. Therefore, gas exchange traits of all plants were measured at 700 ppm CO₂ irrespective of the CO₂ concentration they were growing.

Plants had lower stomatal conductance under elevated CO₂ concentration in most of the treatments during all sampling dates compared with plants grown at ambient CO₂ concentration (Fig. 2). This difference was more noticeable one week before veraison (sampling I), two weeks after mid-veraison (sampling IV) and at maturity (sampling V) (Fig. 2). As the experiment progressed, stomatal conductance values decreased, from ca. 0.1-0.5 mmol m⁻² s⁻¹ in sampling I to ca. 0.1-0.3 mmol m⁻² s⁻¹ in sampling V (Fig. 2). Few changes in stomatal conductance were observed in response to elevated temperature, increasing the values (when changes occurred) in both red and white Tempranillo (Fig. 2). Cyclic drought decreased stomatal conductance but these changes were not generalized (Fig. 2). It should be noted here that gas exchange measurements in the cyclic drought-treated plants were not always coincident with the lowest substrate water content in the different sampling dates (see Fig. 1).

Photosynthetic rates were measured in grapevine plants grown under different CO₂ concentrations, temperatures and water availabilities (Fig. 3). Growing red and white Tempranillo under elevated CO₂ resulted in lower photosynthesis in almost all the treatments, compared to plants grown at ambient CO₂ concentration (Fig. 3). This is the first evidence that red and white Tempranillo acclimated to elevated CO₂. Although there was always (from one week before veraison to maturity) a tendency to have lower photosynthetic rates in plants grown under elevated CO₂, the largest number of significant differences was found at the beginning (one week before veraison) and the end (maturity) of the treatment period in both red and white Tempranillo (Fig. 3). In the rest of the samplings, the response was quite similar; however, it should be noted here that there were more number of significant differences in white than in red Tempranillo (Fig. 3). Elevated temperature tended (sometimes, not always) to increase

photosynthesis in plants grown at ambient CO₂, in both full irrigated and cyclic droughted plants in red and white Tempranillo one week before veraison and at maturity (Fig. 3). Drought effects were not clear (Fig. 3), possibly because photosynthetic rates measurements were not always coincident with the lowest substrate water content in the different sampling dates.

Sub-stomatal CO₂ concentration (C_i) of the red and white Tempranillo leaves of all treatments at maturity were between 400 and 500 ppm (Fig. 4), indicating that the elevated CO₂ (700 ppm) imposed during the measurements has entered the leaves. Although there was a tendency to show lower C_i in elevated CO₂-grown plants when compared to the ambient-CO₂-grown ones, the only significant difference was observed in full-irrigated white Tempranillo grown under elevated temperature. Effects of elevated temperature and cyclic drought on C_i values were rather scarce (Fig. 4).

Extractable Rubisco in vitro activity was only measured at maturity. As a general rule, extractable Rubisco activity was not affected by any of the environmental factors related to climate change applied in this study (elevated CO₂, elevated temperature and cyclic drought) (Fig. 5). The only exceptions were white Tempranillo plants grown under elevated CO₂, elevated temperature and cyclic drought that had lower extractable Rubisco activity than both those grown under ambient temperature and cyclic drought, and those grown under elevated temperature and fully irrigated (in both cases in plants grown under elevated CO₂) (Fig. 5).

Climate change, leaf water content and photosynthetic pigments from fruit set to maturity in grapevine cv. red and white Tempranillo

Water status of red and white Tempranillo leaves was estimated by measuring the water content, expressed in leaf area basis. With very few exceptions, none of the treatments applied in this study (elevated CO₂, elevated temperature and cyclic drought), acting individually and/or in interaction, had impact on the leaf water content (Fig. S2). Values ranged from 0.008 to 0.014 g H₂O cm⁻² from one week before veraison until maturity, with no significant changes (Fig. S2).

Total Chl (Fig. S3) and carotenoid (Fig. S4) concentrations were unchanged (but with tendency to decrease) or decreased in both red and white Tempranillo from one week before veraison to maturity in plants grown under elevated CO₂ when compared to

those grown at ambient CO₂. Elevated CO₂ decreased both leaf Chl a and Chl b concentrations (not shown), resulting in an unchanged (with very few exceptions) Chl a/Chl b ratio (Fig. S5).

Climate change, leaf starch, total soluble sugars and total soluble proteins at maturity in grapevine cv. red and white Tempranillo

Leaf starch, total soluble sugars and total soluble protein were analyzed in leaves sampled at maturity. Leaf starch concentration was generally higher in plants grown under elevated CO₂ than in those grown under ambient CO₂ (Fig. 6). Also, elevated temperature (when compared to ambient temperature) decreased leaf starch, but only in plants grown under ambient CO₂ (Fig. 6). Leaf total soluble sugars showed a tendency to increase as leaf starch did (in response to both CO₂ and temperature), but, in general, effects were lower or no significant (except for few cases) (Fig. 6). Elevated CO₂ and elevated temperature had no effect on leaf total soluble proteins (Fig. 6). Cyclic drought did not impact remarkably leaf starch, total soluble sugars and total soluble protein (Fig. 6).

Correlations between photosynthetic rates and leaf starch and total soluble sugars

When all plants of this study were included in the regression analyses, photosynthetic rates measured at 700 ppm CO₂ were negatively correlated to leaf starch concentrations ($y=30.26-0.19x$; $R^2=0.55$) (Fig. 7). No correlation was found between photosynthetic rates measured at 700 ppm CO₂ and leaf total soluble sugars ($y=21.81-0.17x$; $R^2=0.02$) (Fig. 7).

Climate change and leaf carbon/nitrogen ratio at maturity in grapevine cv. red and white Tempranillo

Leaf C/N ratio increased significantly in response to elevated CO₂ in both red and white Tempranillo, with the unique exception of fully irrigated white Tempranillo grown under elevated temperature in which no changes were observed (Fig. 8). This is the second evidence for photosynthetic acclimation to elevated CO₂ in red and white Tempranillo plants. C/N ratio increases were due to decreases in the leaf N concentrations, because leaf C concentrations remained fairly constant in most of the treatments (Fig. 8).

Correlations between leaf carbon/nitrogen ratio and leaf starch

Leaf C/N ratio is considered a measure of the extent of the acclimation to elevated CO₂. When red and white Tempranillo plants were included in the regression analyses, leaf C/N ratios were positively correlated to leaf starch concentrations ($y=13.46+0.16x$; $R^2=0.65$ for white Tempranillo, and $y=5.87+0.34x$; $R^2=0.70$ for red Tempranillo) (Fig. 9). It should be noted here that for any given leaf starch white Tempranillo had higher values of C/N ratio than red Tempranillo, suggesting a more intense acclimation in white than in red Tempranillo for a given leaf starch accumulation.

Correlations between leaf carbon/nitrogen ratio and grapevine sink size

Plant sink size determines to what extent the plant acclimates to elevated CO₂. Obviously, plants grown under elevated CO₂ had higher growths (Tables 1 and 2) but also are the plants with higher chance to get acclimated to the elevated CO₂. In this work, three possible sinks for the extra carbon supplied to the plants were considered: (i) bunch dry matter (DM), (ii) total vegetative DM, and (iii) total plant DM. When red and white Tempranillo plants were included in the regression analyses, leaf C/N ratios were positively correlated to bunch DM ($y=16.16+0.11x$; $R^2=0.24$ for white Tempranillo, and $y=9.73+0.16x$; $R^2=0.76$ for red Tempranillo), to total vegetative DM ($y=20.62+0.02x$; $R^2=0.08$ for white Tempranillo, and $y=16.95+0.03x$; $R^2=0.28$ for red Tempranillo), and to total plant DM ($y=19.60+0.02x$; $R^2=0.12$ for white Tempranillo, and $y=14.85+0.03x$; $R^2=0.44$ for red Tempranillo) (Fig. 10). It should be noted here that for any given sink size (bunch, total vegetative or total plant DM) white Tempranillo had higher values of C/N ratio than red Tempranillo, suggesting again a more intense acclimation in white than in red Tempranillo for a given plant sink size.

Discussion

In this work, three factors related to climate change have been considered: an increased atmospheric CO₂ concentration, an increased temperature and water availability. Over the short term, increases in the atmospheric CO₂ concentration enhance photosynthetic rates, and decrease transpiration and stomatal conductance (Drake et al. 1997; Lee et al. 2001; Long et al. 2004; Oliveira et al. 2010). Lasting increases in C fixation in response to elevated atmospheric CO₂ levels have been reported for certain species (such as oak and soybean; Campbell et al. 1990; Maroco et al. 2002). However, during long-term

exposure (days to weeks, or longer), this response may, at least partly, be abolished by photosynthetic acclimation to elevated CO₂ (a down-regulation of photosynthesis) (Jifon and Wolfe 2002; Long et al. 2004; Aranjuelo et al. 2005b, 2009; Erice et al. 2006; Ainsworth and Rogers 2007; Irigoyen et al. 2014; Salazar-Parra et al. 2015). This is generally evidenced in comparisons of plants grown at ambient and elevated CO₂, measuring all plants at either current or elevated CO₂ concentration (Irigoyen et al. 2014). Here, plants were measured at 700 ppm CO₂ irrespective of the CO₂ concentration they were growing. Data showed decreases in photosynthetic rates in both red and white Tempranillo grown under elevated CO₂, evidencing photosynthetic acclimation to prolonged exposure to elevated concentration of atmospheric CO₂. Another line of evidence comes from C/N data. Both red and white Tempranillo had higher leaf C/N ratio values when grown under elevated CO₂ than when grown under ambient CO₂, mainly due to N reduction as was also indicated for red Tempranillo by Salazar-Parra et al. (2015). Low N availability can negatively affect plant growth, reducing therefore the plant capacity to develop new sinks (Ainsworth and Rogers 2007; Aranjuelo et al. 2007). An increased C/N ratio is a clear symptom of photosynthetic acclimation to elevated CO₂ (Jifon and Wolfe 2002; Irigoyen et al. 2014). Thus, data reported in this work for red and white Tempranillo confirm previous reports working with red Tempranillo (Leibar et al. 2015; Salazar-Parra et al. 2015).

Traditionally, causes for the photosynthetic acclimation to elevated CO₂ have been related to (i) stomatal limitations resulting from stomatal closure and the corresponding decreased sub-stomatal CO₂ concentration (C_i), (ii) reduced Rubisco carboxylation activity and/or (iii) reduced amount of Rubisco or leaf total soluble protein (Drake et al. 1997; Ainsworth et al. 2002; Urban 2003; Long et al. 2004; Sánchez-Díaz et al. 2004; Aranjuelo et al. 2005b, 2009; Erice et al. 2006; Ainsworth and Rogers 2007; Leakey et al. 2009). None of these causes seems to be the origin of the photosynthetic acclimation to elevated CO₂ in red and white Tempranillo. First, although growing under elevated CO₂ decreased the stomatal conductance to CO₂ as has been reported in herbaceous crop species (Bunce 2001; Goicoechea et al. 2014), red and white Tempranillo C_i values were not affected and were similar in plants grown under elevated or ambient CO₂. These unchanged C_i values might be related to an increased photorespiration/carboxylation ratio in elevated CO₂-grown grapevine (Salazar-Parra et al. 2012a). Second, extractable Rubisco activity remained fairly constant independent of the treatment applied, including elevated CO₂. Finally, although the amount of Rubisco

was not quantified, total soluble protein (in which Rubisco contributes largely) was not affected by growing red and white Tempranillo under elevated CO₂ as was previously reported for alfalfa (Sanz-Sáez et al. 2013). Leaf water content and photosynthetic pigments (Chls and carotenoids) did not vary much among treatments, or impacts were not enough to limit photosynthesis (elevated CO₂, indeed, increased leaf water content, as a result of partial stomatal closure and increased water use efficiency (Battipaglia et al. 2013)). Other reasons should therefore cause the photosynthetic acclimation. On the one hand, photosynthetic rates were negatively correlated to leaf starch accumulation (but not soluble sugars), and therefore a product feedback inhibition is likely. Carbohydrate accumulation has been previously reported in plants grown under elevated CO₂ (Geiger et al. 1999; Mishra et al. 2008). On the other hand, it is difficult to explain a decreased photosynthesis without diffusional and/or biochemical (Rubisco or other enzyme/s) limitations. Stomatal closure did not limit photosynthesis (see above), but diffusional limitations include in addition mesophyll conductance to CO₂ (i.e., the movement of the CO₂ from the sub-stomatal cavity of the leaves to the chloroplast). Although unfortunately mesophyll conductance to CO₂ was not estimated in this work, previous reports working with red Tempranillo have found that elevated CO₂ decreases the CO₂ conductance within the mesophyll, decreasing the CO₂ concentration in the chloroplast available for the Rubisco enzymatic reaction (Salazar-Parra et al. 2012a). Feedback inhibition of other anabolic enzyme/s mediated by starch (or starch-derived products) cannot be excluded.

The extent of the photosynthetic acclimation to elevated CO₂ may be related to the ability of plants to develop new C sinks. If plants are unable to store C in new sinks by either growing more the existing organs, enhancing their metabolism rates or expanding their storage capacity, they will tend to balance their C source/sink capacity by decreasing their photosynthetic rates (Urban 2003; Aranjuelo et al. 2005a, 2009, 2014). Grapevine is a species with possibility of storing extra C from elevated CO₂ in sinks (grape bunch, root reserves for early growth in the next growing season, etc.) but limited (Leibar et al. 2015; Salazar-Parra et al. 2015). Plants that develop strong C sinks avoid carbohydrate accumulation in the leaves (Jifon and Wolfe 2002), which was not the case of red and white Tempranillo (see above). Red and white Tempranillo plants underwent photosynthetic acclimation after a long exposure to elevated CO₂ (700 ppm), regardless of temperature and water availability. Direct impacts of temperature on

photosynthesis were not remarkable in this work, in contrast to what has been observed in several species (Law and Crafts-Brandner 1999; Haldimann and Feller 2004; Salvucci and Crafts-Brandner 2004; Greer and Weedon 2013).

White Tempranillo is a natural, spontaneous mutation from red Tempranillo that comprises deletions and chromosomal reorganizations, losing or affecting hundreds of genes. Photosynthetic acclimation was not so severe in red than in white Tempranillo. Thus, for any given sink size (berry, total vegetative or total plant DM) or any given leaf starch accumulation, white Tempranillo always had higher levels of photosynthetic acclimation than red Tempranillo (quantified by the extent of the increases in leaf C/N ratio). It is obvious that loci other than grape color, like those implicated in the photosynthetic response to prolonged exposure to elevated CO₂, have been affected by the mutation.

In conclusion, this research reveals that red and white Tempranillo grapevine underwent photosynthetic acclimation after a long exposure to elevated CO₂, regardless of temperature and water availability. Photosynthetic acclimation in grapevine plants is evidenced by photosynthesis capacity decrease, leaf starch accumulation, and increased leaf carbon/nitrogen (C/N) ratios. Causes of photosynthetic acclimation were not associated to stomatal closure, extractable Rubisco activity or Rubisco amount. Probably, it appears to be induced by CO₂ mesophyll conductance limitations and/or inhibition of other anabolic enzyme/s mediated by starch (or starch-derived products). For any given sink size (bunch, total vegetative or total plant dry matter) or any given leaf starch accumulation, white Tempranillo had higher symptoms of photosynthetic acclimation than the red cultivar. This indicates that the white Tempranillo mutant has altered its response to prolonged exposure to elevated CO₂. Loci other than grape color have been affected in the white Tempranillo mutant.

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Table 1. Leaf, petiole, stem, and root dry weight (DW) in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) (A) or elevated (700 ppm) (E), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test (n = 5).

| Treatments | | | Leaf | Petiole | Stem | Root | |
|----------------------|---------|-------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|---------|
| | | | (g DW plant ⁻¹) | (g DW plant ⁻¹) | (g DW plant ⁻¹) | (g DW plant ⁻¹) | |
| Red Tempranillo | FI | T | A CO ₂ | 59.3 bc | 7.9 ab | 51.5 bc | 28.9 bc |
| | | | E CO ₂ | 71.8 a | 8.0 ab | 63.5 a | 37.8 a |
| | T + 4°C | A CO ₂ | 54.2 cd | 6.8 bc | 43.2 cd | 26.1 c | |
| | | E CO ₂ | 54.2 cd | 6.4 cd | 46.8 bcd | 35.8 a | |
| | CD | T | A CO ₂ | 19.3 hi | 2.5 fg | 12.6 e | 17.9 d |
| | | | E CO ₂ | 24.1 gh | 2.2 fg | 10.4 e | 16.0 d |
| | | T + 4°C | A CO ₂ | 17.0 hi | 2.1 fg | 8.6 e | 6.0 e |
| | | | E CO ₂ | 19.3 hi | 2.5 fg | 12.0 e | 16.7 d |
| White Tempranillo | FI | T | A CO ₂ | 38.8 ef | 5.5 de | 40.7 d | 27.0 c |
| | | | E CO ₂ | 36.9 f | 4.6 e | 39.2 d | 28.8 bc |
| | T + 4°C | A CO ₂ | 68.8 ab | 8.5 a | 54.4 ab | 34.8 ab | |
| | | E CO ₂ | 47.6 de | 7.1 bc | 45.0 bcd | 29.0 bc | |
| | CD | T | A CO ₂ | 11.4 i | 1.6 g | 7.0 e | 11.3 de |
| | | | E CO ₂ | 29.9 fg | 3.2 f | 15.2 e | 17.7 d |
| | | T + 4°C | A CO ₂ | 16.1 hi | 2.1 fg | 9.8 e | 12.7 d |
| | | | E CO ₂ | 26.0 gh | 2.8 fg | 12.1 e | 17.4 d |

Table 2. Bunch, berry, and rachis dry weight (DW) in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) (A) or elevated (700 ppm) (E), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test (n = 5).

| Treatments | | | Bunch (g DW plant ⁻¹) | Berry (g DW plant ⁻¹) | Rachis (g DW plant ⁻¹) | | |
|--------------------|----------------------|---------|--------------------------------------|--------------------------------------|---------------------------------------|------------|-----------|
| Red Tempranillo | FI | T | A CO ₂ | 58.3 cdef | 55.4 def | 2.9 bcd | |
| | | | E CO ₂ | 97.7 a | 95.0 a | 2.6 bcde | |
| | T + 4°C | | A CO ₂ | 65.1 bcd | 62.2 cde | 2.9 bc | |
| | | | E CO ₂ | 78.3 b | 76.7 b | 2.9 b | |
| | CD | T | | A CO ₂ | 39.0 gh | 36.9 fgh | 2.1 efgh |
| | | | | E CO ₂ | 74.0 b | 70.5 bc | 3.5 a |
| | | T + 4°C | | A CO ₂ | 44.8 fgh | 42.7 fgh | 2.1 efgh |
| | | | | E CO ₂ | 69.4 bc | 66.8 bcd | 2.6 bcdef |
| | White Tempranillo | FI | T | A CO ₂ | 65.6 bcd | 63.3 cde | 2.3 defgh |
| | | | | E CO ₂ | 68.8 bc | 66.9 bcd | 2.0 fgh |
| T + 4°C | | | A CO ₂ | 58.8 cde | 56.5 def | 2.3 cdefgh | |
| | | | E CO ₂ | 73.9 b | 71.1 bc | 2.8 bcd | |
| CD | | T | | A CO ₂ | 46.9 efg | 45.2 fg | 1.7 h |
| | | | | E CO ₂ | 44.4 fgh | 42.4 fgh | 2.1 efgh |
| | | T + 4°C | | A CO ₂ | 31.7 h | 29.7 h | 2.0 fgh |
| | | | | E CO ₂ | 52.5 defg | 50.3 efg | 2.2 efgh |

Figure Legends

Fig 1 Pot substrate water content from red and white Tempranillo grapevine (*Vitis vinifera* L.) grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), at ambient temperature (T) or ambient temperature +4°C (T+4) and under full irrigation or cyclic drought measured every day until maturity (n = 5).

Fig 2 Stomatal conductance in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD)). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test (n = 5).

Fig 3 Photosynthesis in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD)). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test (n = 5).

Fig 4 Sub-stomatal CO₂ concentration in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD)). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test (n = 5).

Fig 5 Extractable Rubisco activity in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD)). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test (n = 3).

Fig 6 Starch, total soluble sugars, and total soluble proteins in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD)). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test (n = 5).

Fig 7 Correlations between photosynthesis and leaf starch, and between photosynthesis and total soluble sugars in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD) (n = 5).

Fig 8 Leaf carbon (C), nitrogen (N) and C/N ratio in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test (n = 5).

Fig 9 Correlations between leaf C/N ratio and leaf starch in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD) (n = 5).

Fig 10 Correlations between leaf C/N ratio and bunch dry matter (DM), between leaf C/N ratio and total vegetative DM, and between leaf C/N ratio and total plant DM in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD) (n = 5).

Figure 1. Kizildeniz et al. (2017c).

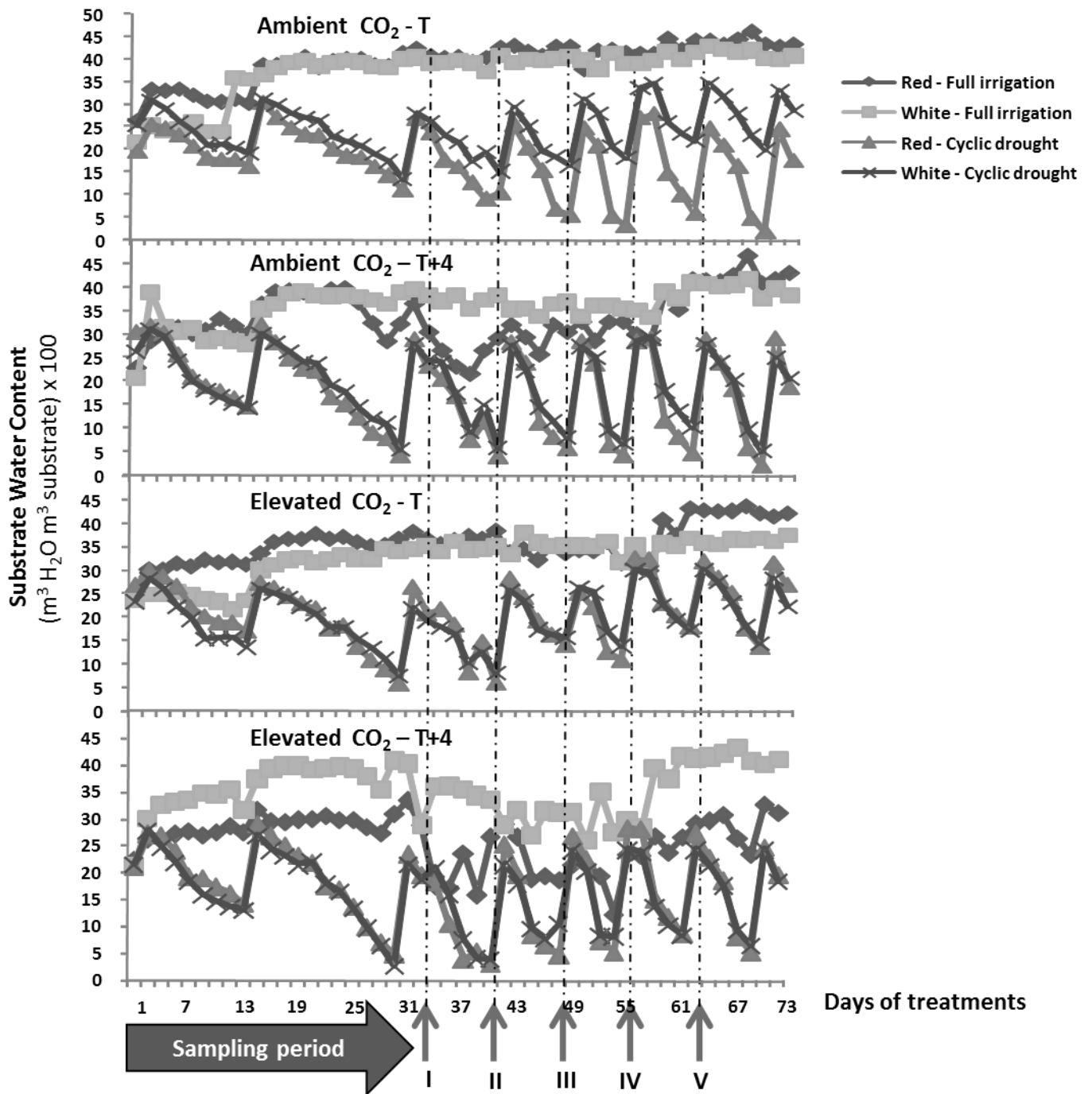


Figure 2. Kizildeniz et al. (2017c).

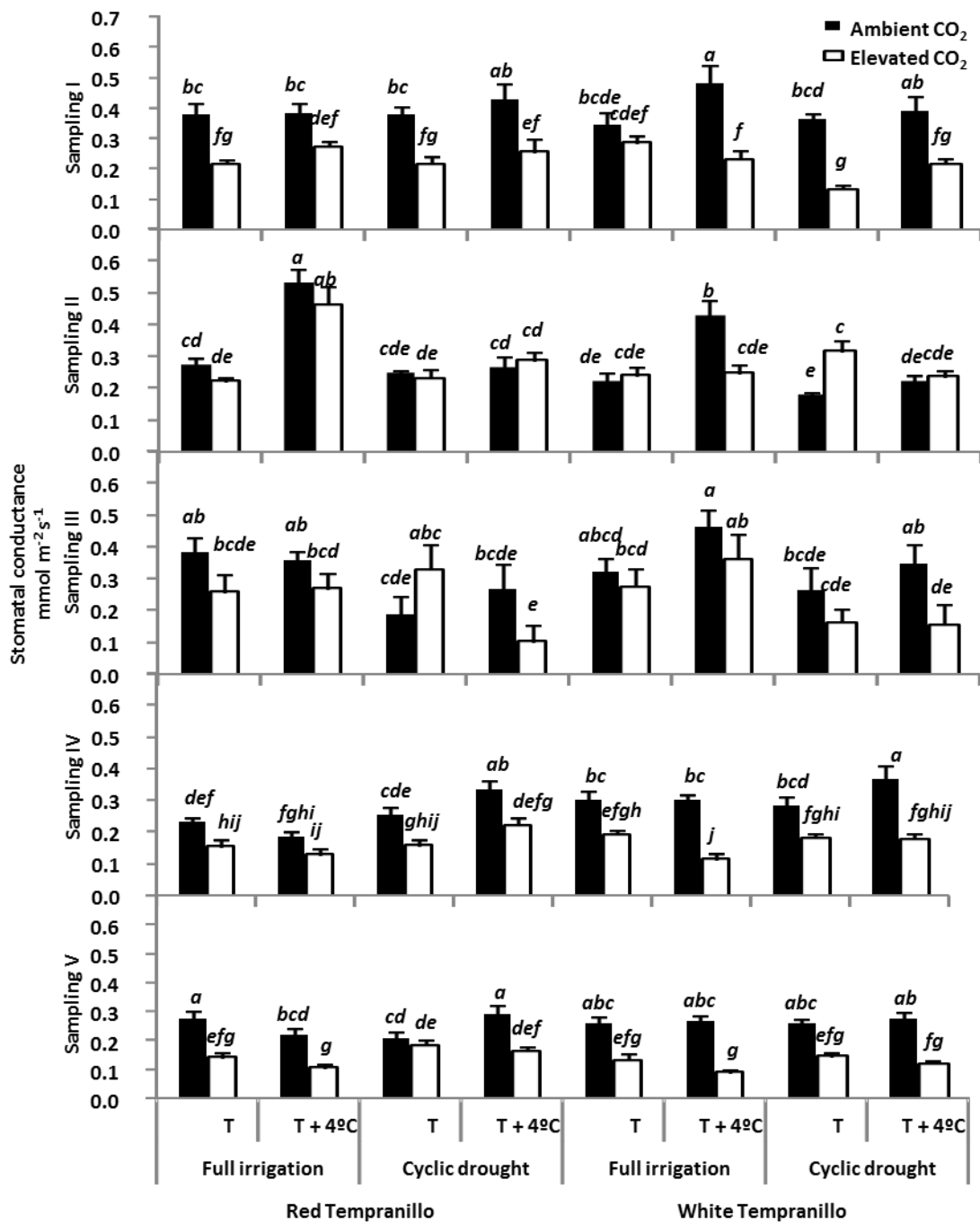
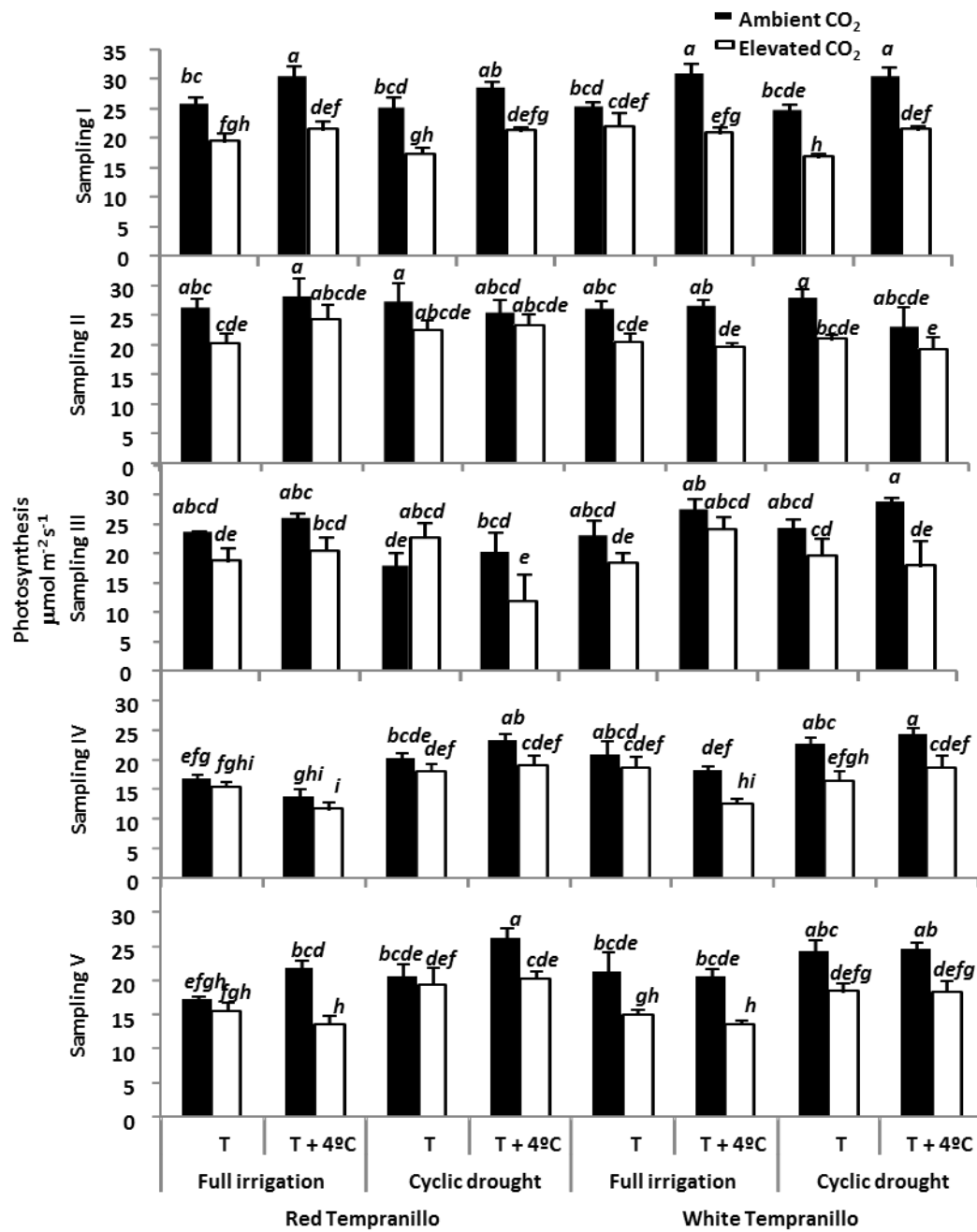


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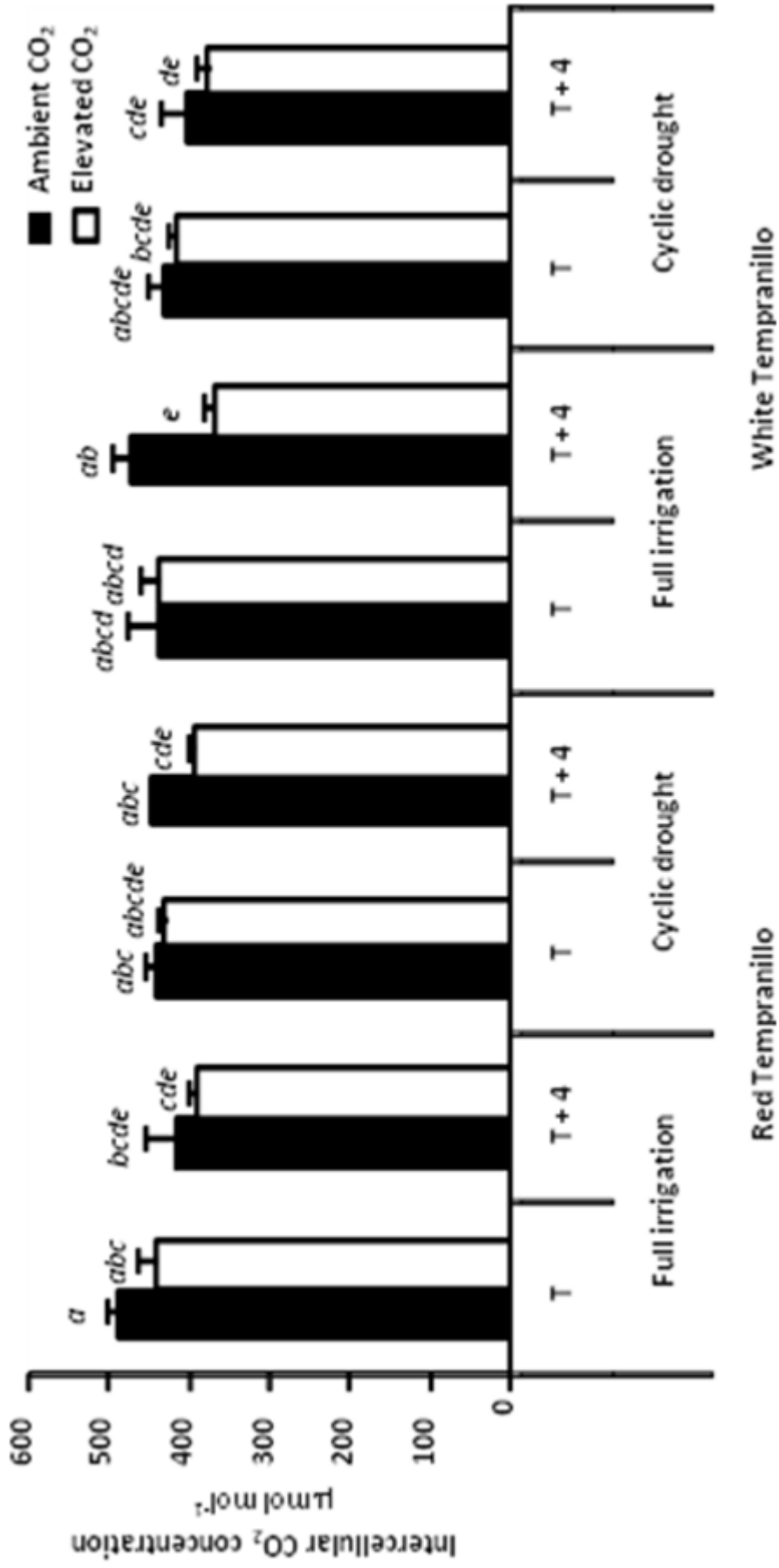


Figure 4. Kizildeniz et al. (2017c).

Figure 5. Kizildeniz et al. (2017c).

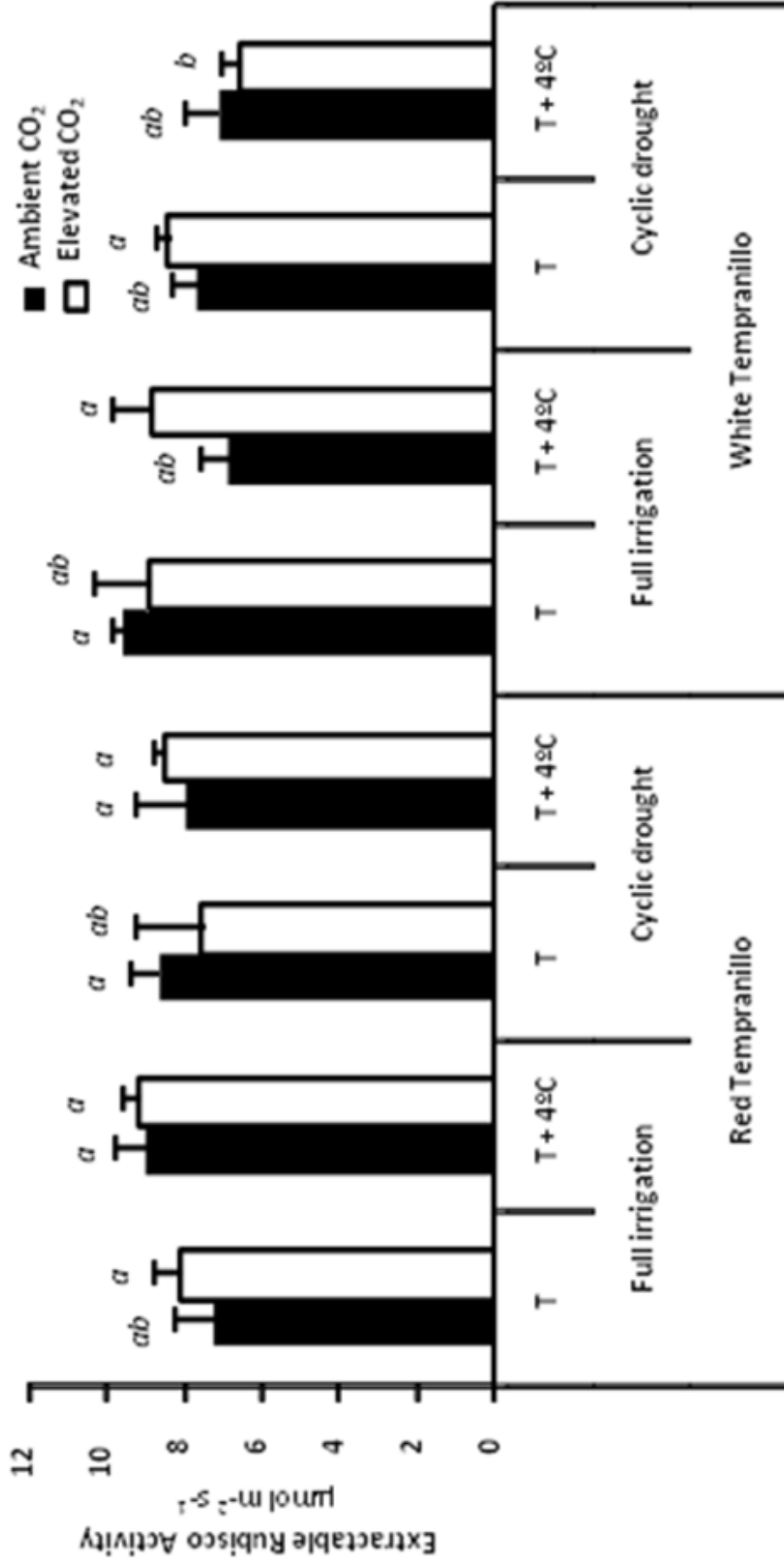


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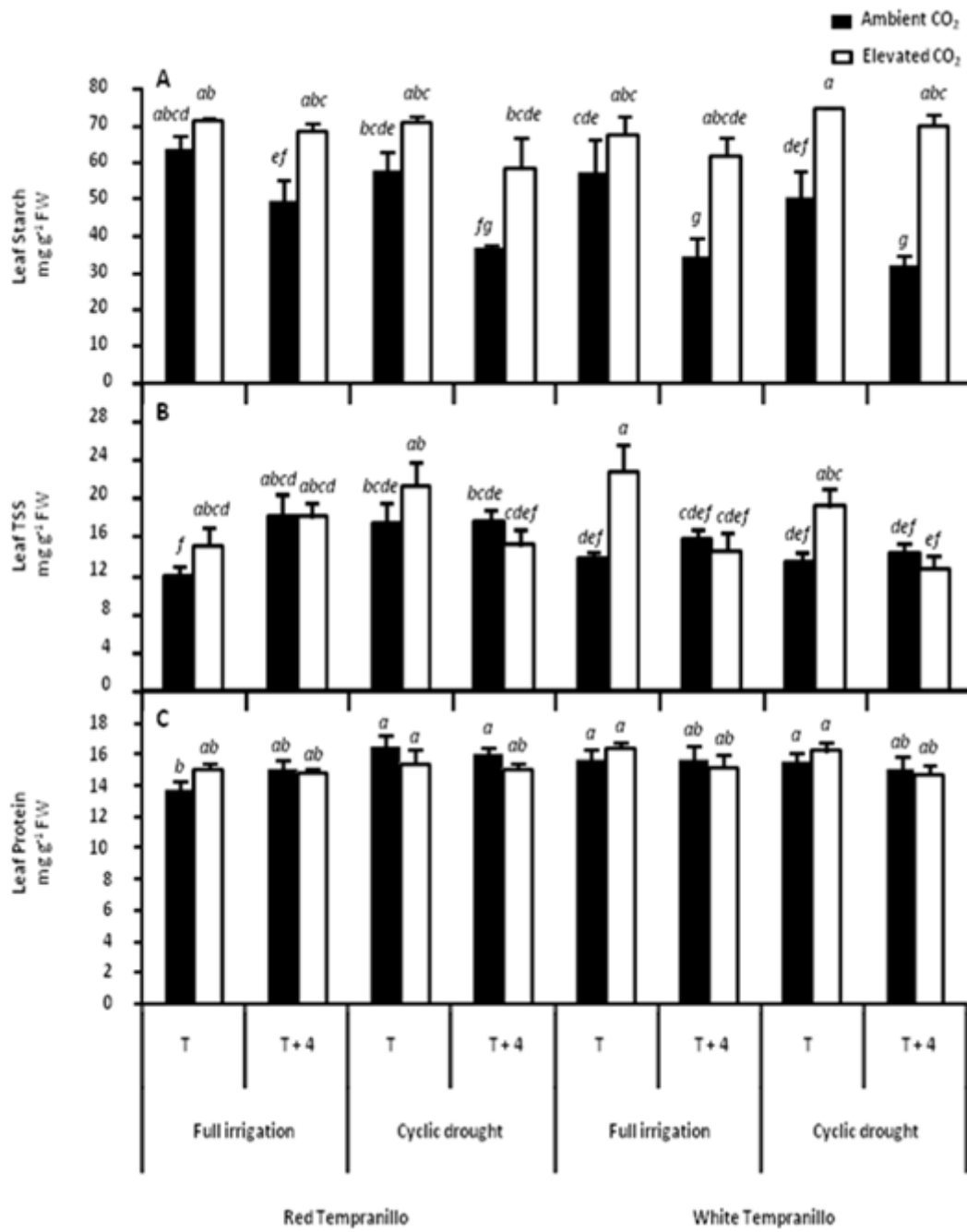


Figure 7. Kizildeniz et al. (2017c).

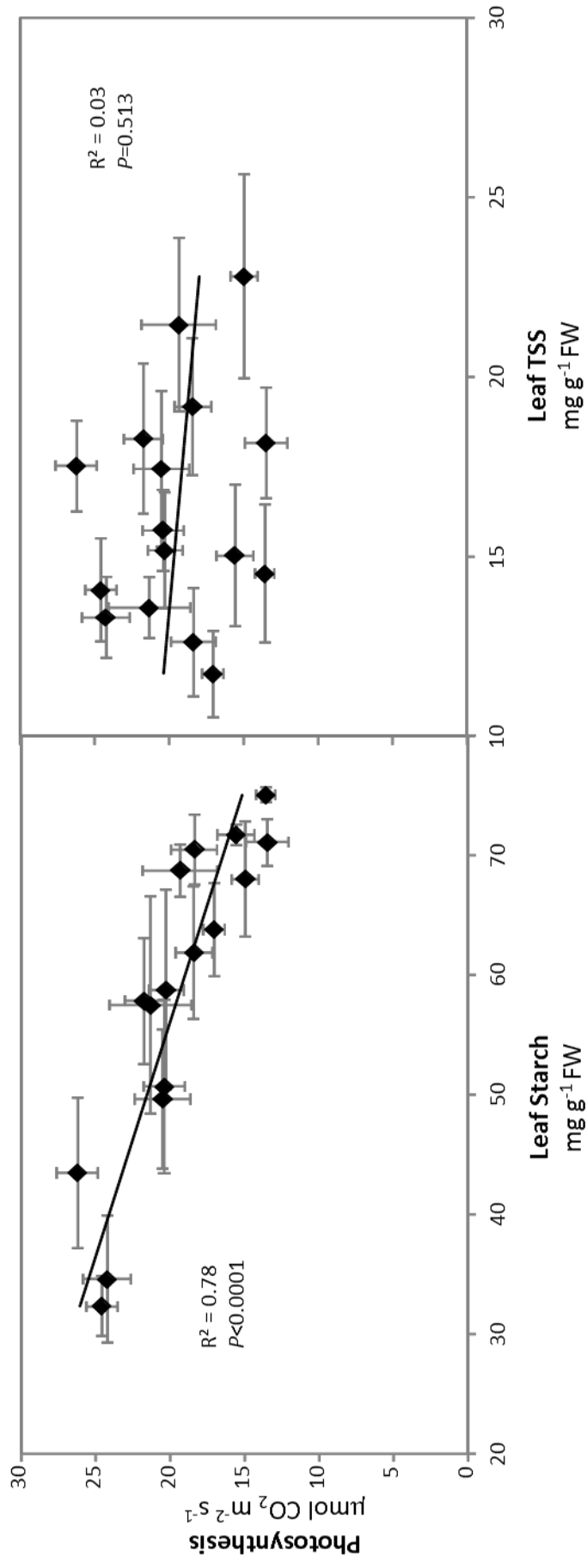


Figure 8. Kizildeniz et al. (2017c).

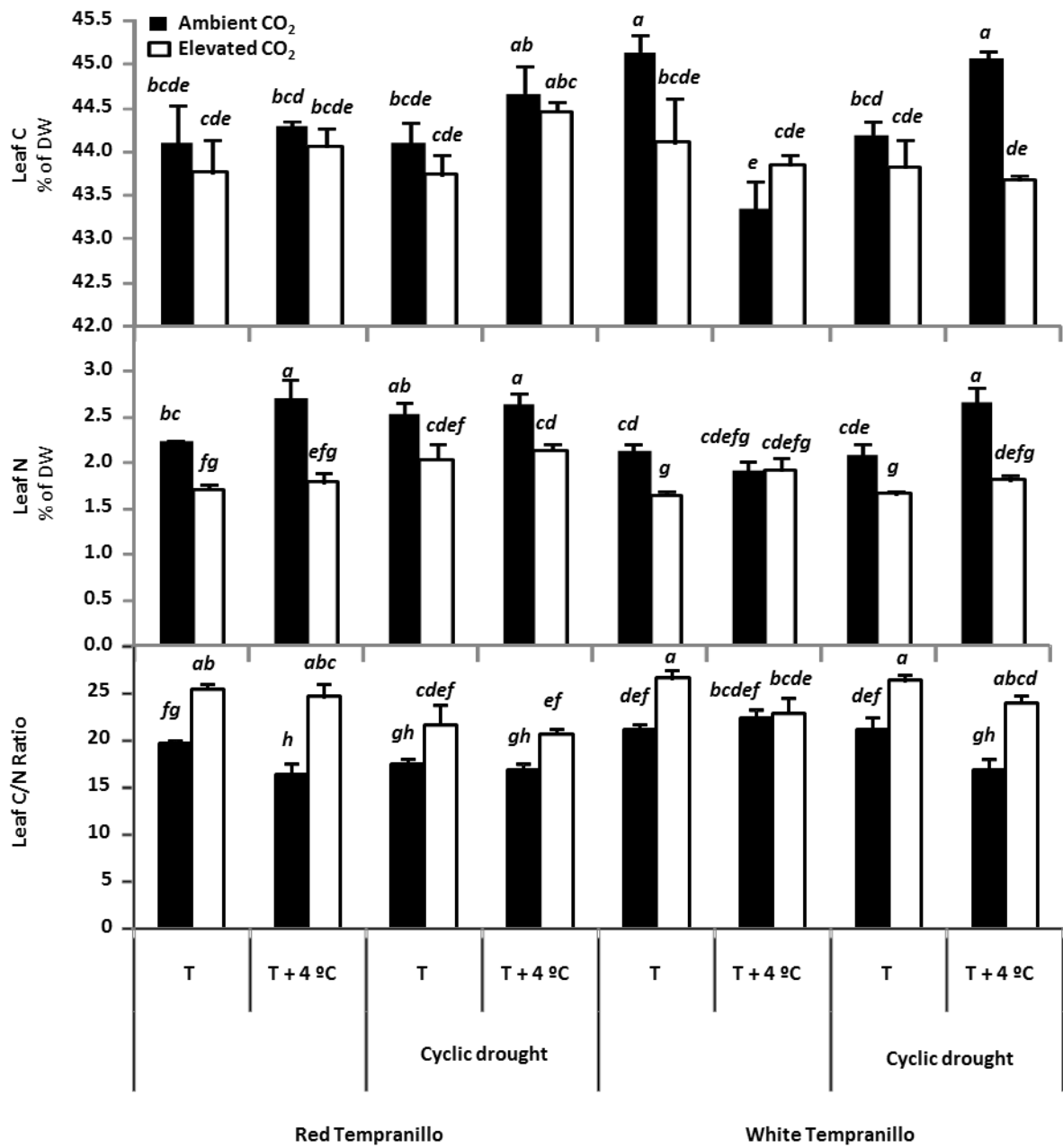


Figure 9. Kizildeniz et al. (2017c).

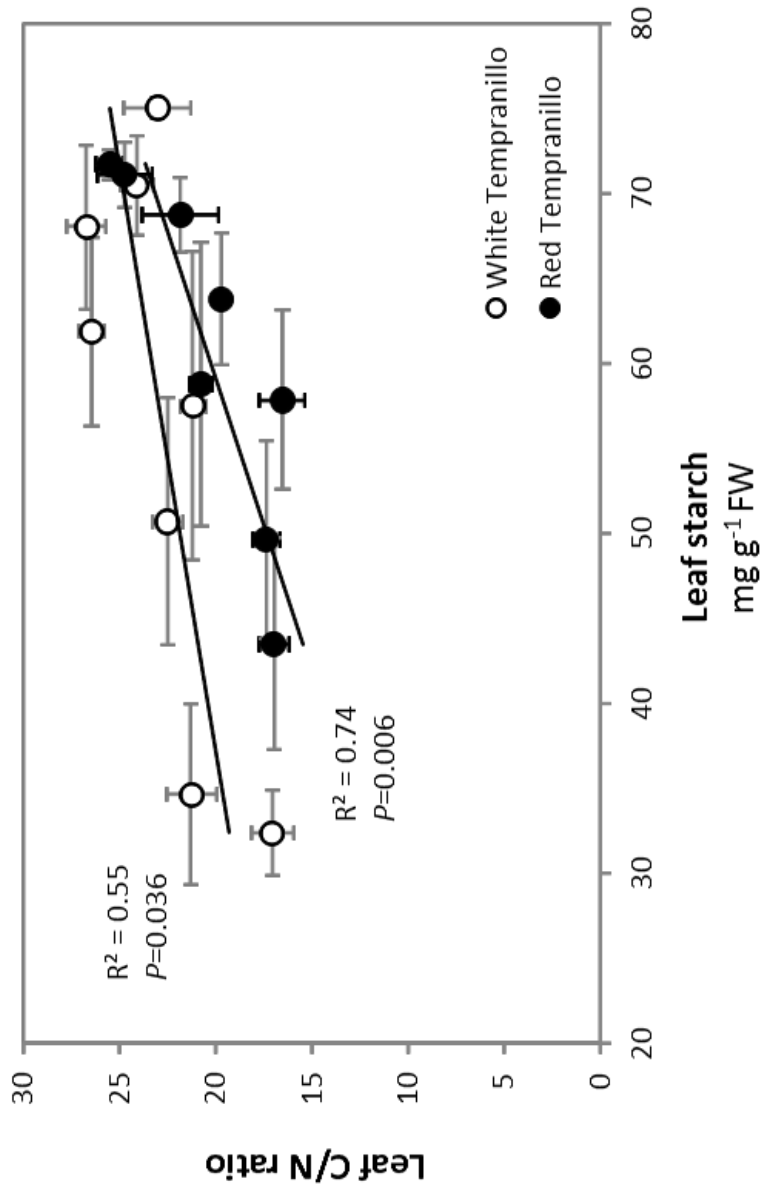
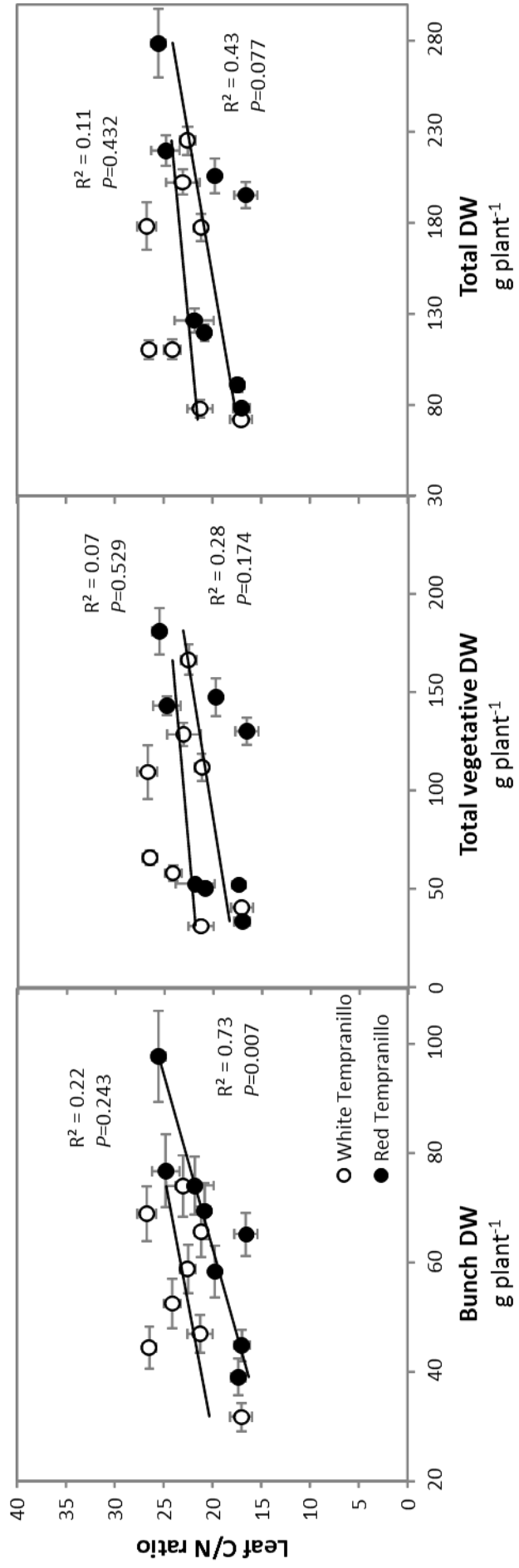


Figure 10. Kizildeniz et al. (2017c).



Supplementary Figure Legends

Fig. S1 Leaf area in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD)). Data were plotted as mean (n = 5). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test.

Fig. S2 Leaf water content in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD)). Data were plotted as mean (n = 5). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test.

Fig. S3 Chlorophyll a+b concentration in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD)). Data were plotted as mean (n = 5). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test.

Fig. S4 Carotenoid concentration in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD)). Data were plotted as mean (n = 5). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test.

Fig. S5 Chlorophyll a/b ratio in red and white Tempranillo grapevine (*Vitis vinifera* L.) plants grown under different CO₂ concentrations (ambient (400 ppm) or elevated (700 ppm)), temperature regimes (ambient (T) or ambient + 4 °C (T + 4 °C) and water availability (full irrigation (FI) or cyclic drought (CD)). Data were plotted as mean (n = 5). Different letters indicate significant differences among treatments (P < 0.05) based on LSD test.

Fig. S1. Kizildeniz et al. (2017c).

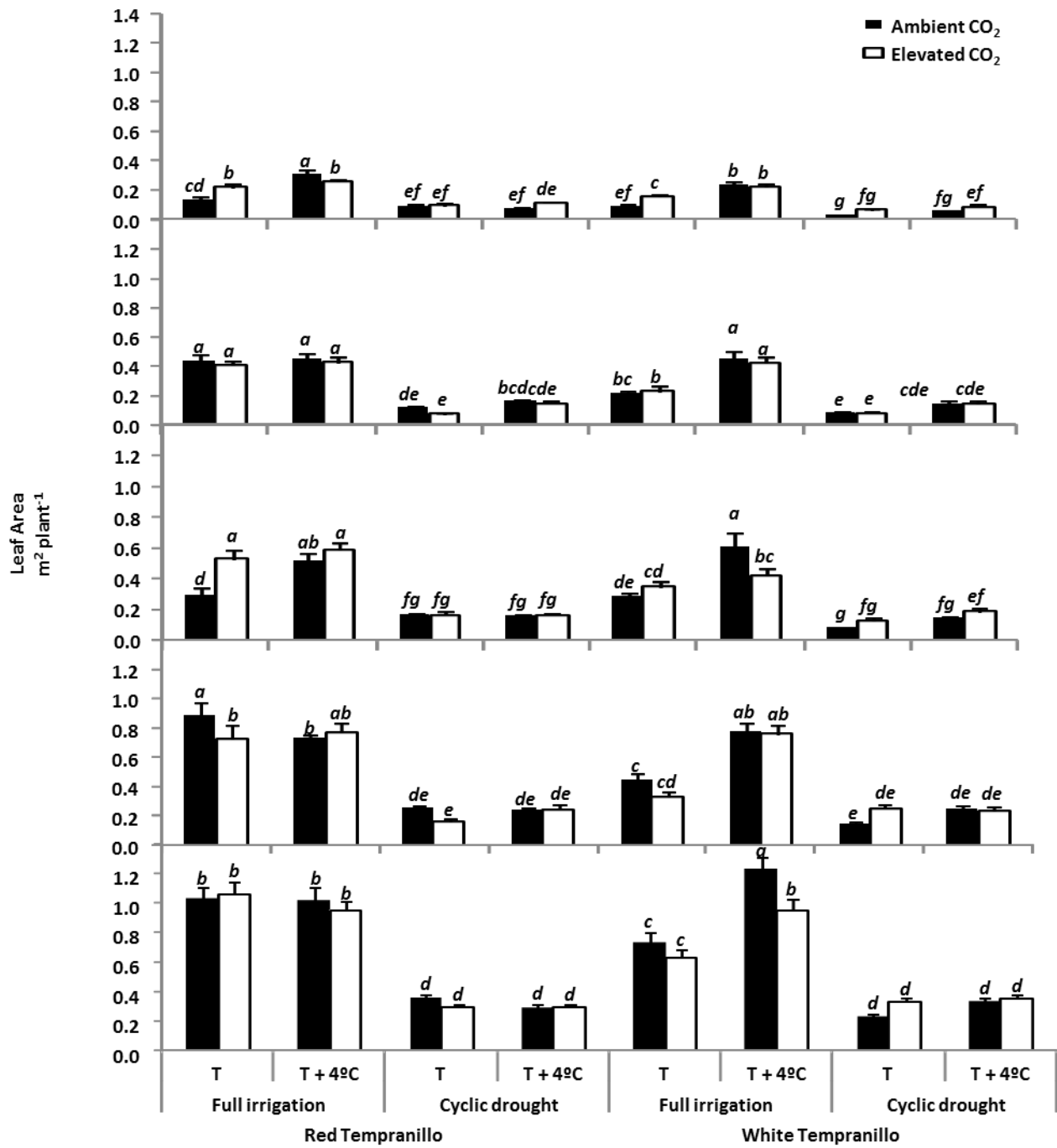


Fig. S2. . Kizildeniz et al. (2017c).

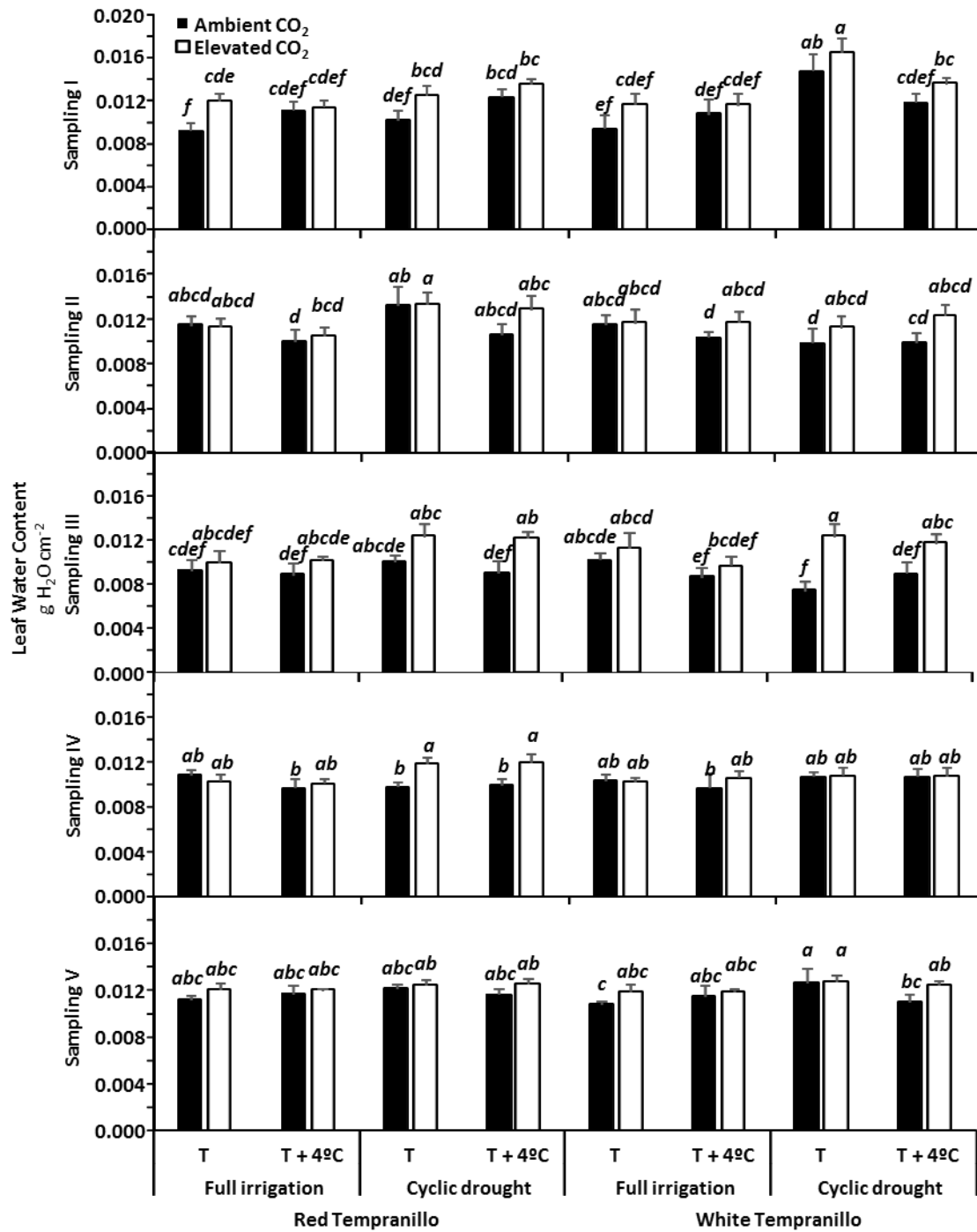


Fig. S3. Kizildeniz et al. (2017c).

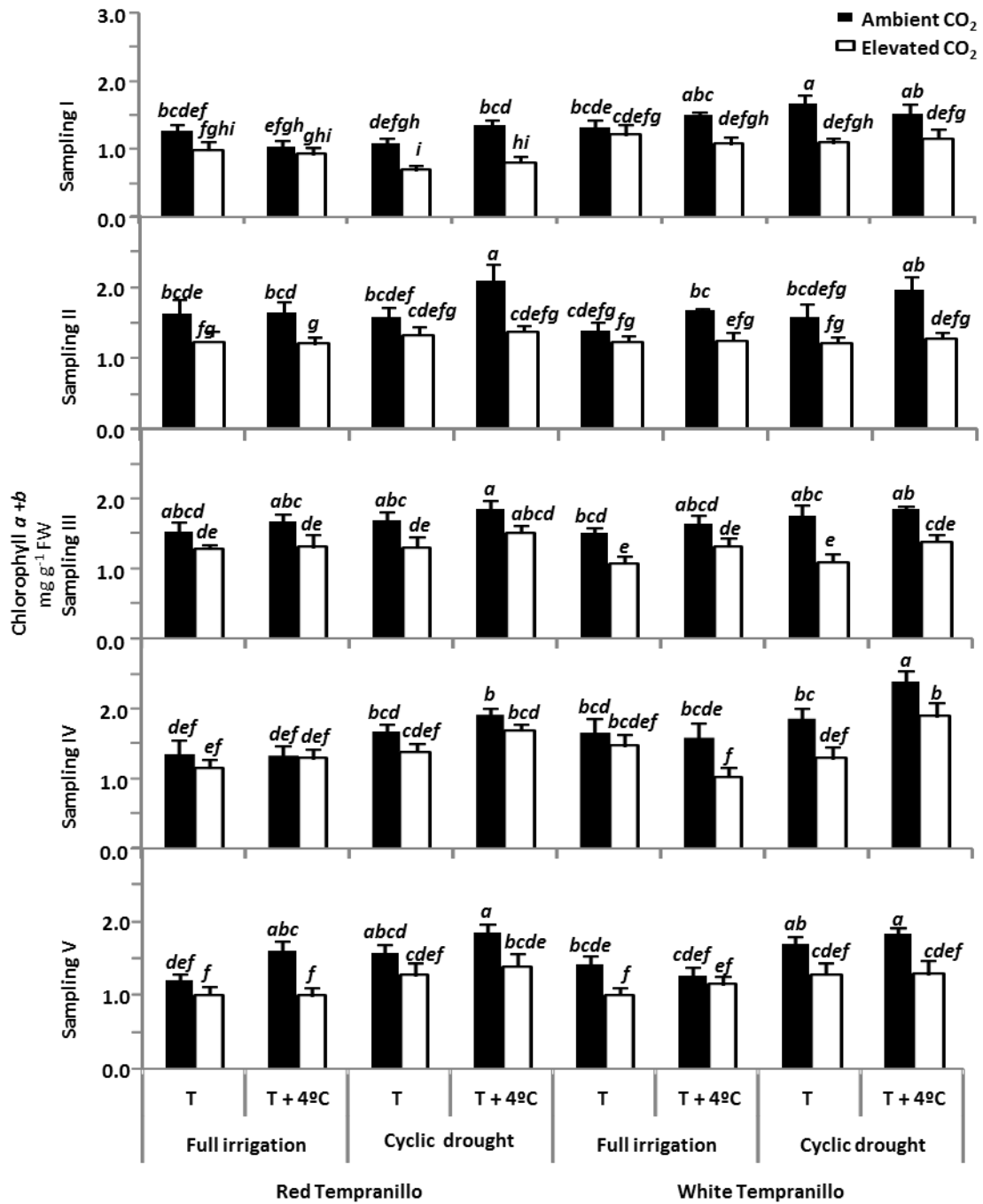


Fig. S4. Kizildeniz et al. (2017c).

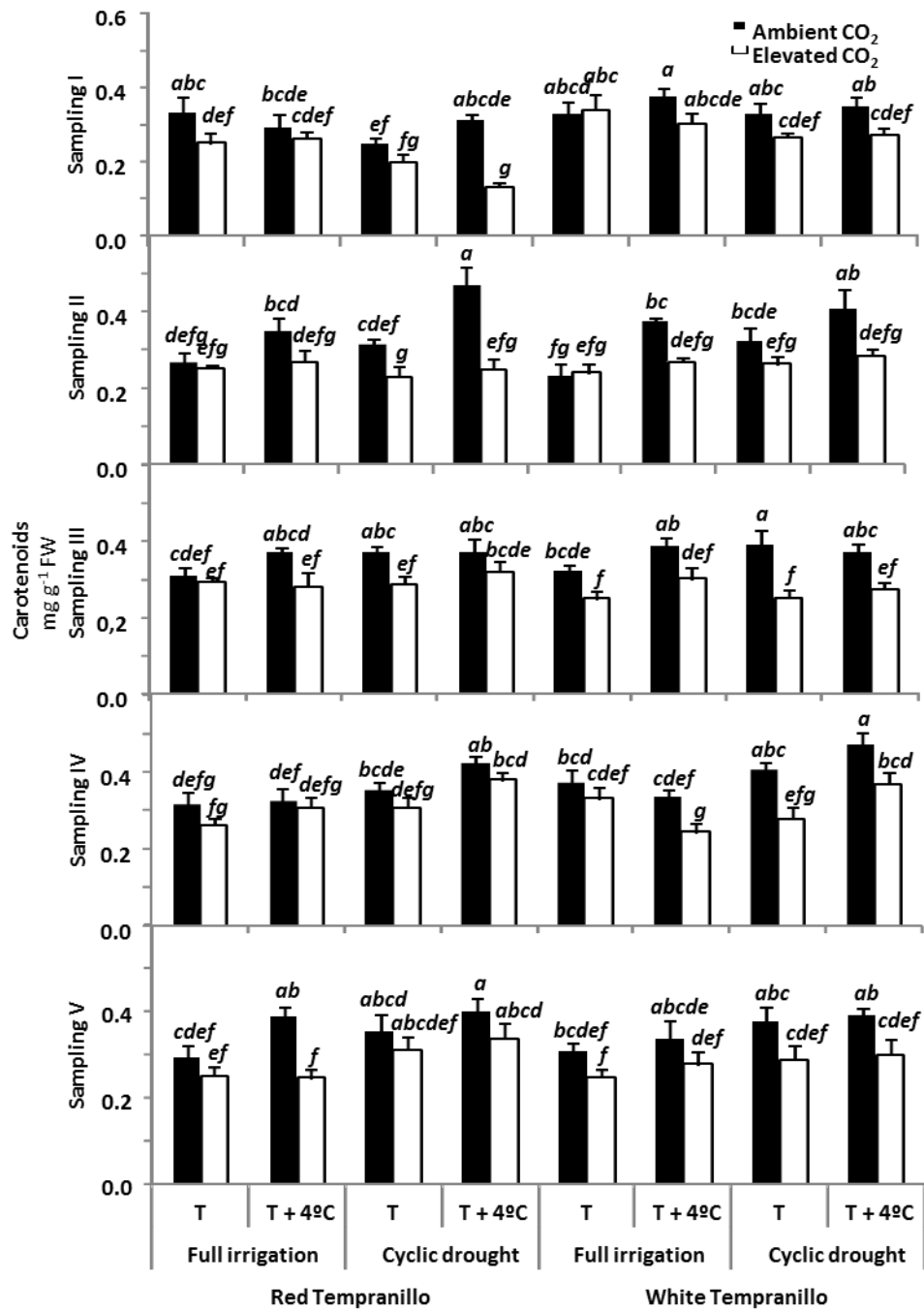
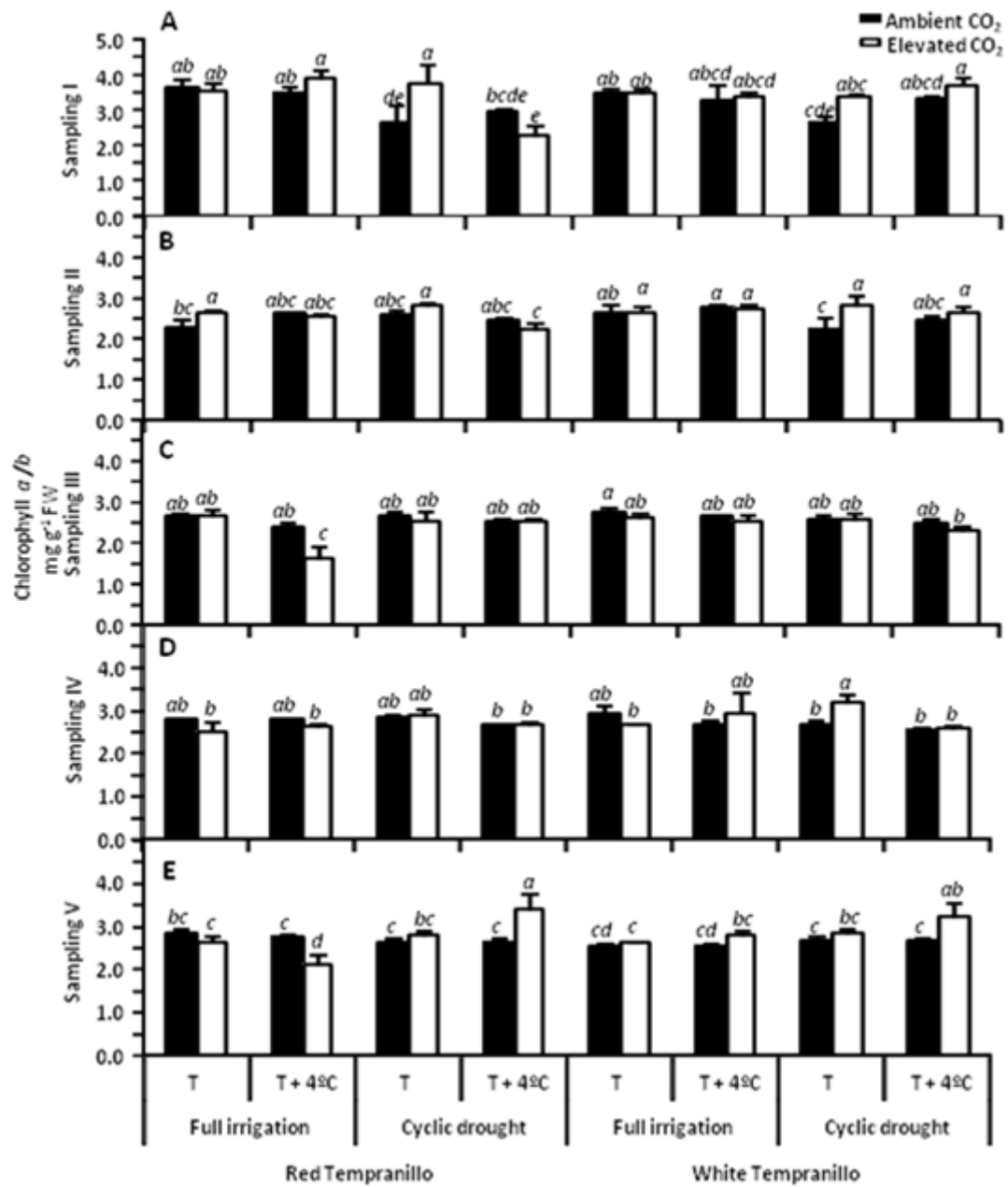


Fig. S5. Kizildeniz et al. (2017c).



DISCUSIÓN GENERAL

In recent years, effects of elevated CO₂ concentration, high temperature or water deficit on grapevine were investigated and reported as single stress factors in one particular or different grapevine cultivars. However, grapevine researches focused on multiple stress factors are scarce. In this PhD Thesis, a predicted scenario of climate change including elevated CO₂ concentration, elevated temperature and water deficit, acting individually and/or interacting, has been investigated. Effects on vegetative and reproductive growth, substrate and plant water status, photosynthetic acclimation and must quality in fruit-bearing cuttings of two grapevine (*Vitis vinifera* L.) cultivars (red and white Tempranillo) in the temperature gradient greenhouses (TGGs) during 2013, 2014 and 2015 have been studied. Results show that climate change triggers clear responses, modulated to a greater or lesser extent by other factors.

Grapevine photosynthesis increases in presence of elevated CO₂ (Salazar-Parra et al., 2012, 2015). Elevated CO₂ can stimulate plant growth if plants have enough nutrients and water (Mullins et al., 1992). The observed increases in leaf area and in the dry matter of different organs could be explained, at least in part, by an enhanced photosynthesis under elevated CO₂. Previous reports indicate that the beneficial effects of elevated CO₂ on growth are likely related to the higher rates of photosynthesis (Bindi et al., 1996, 2001; Da Silva et al., 2017; Salazar-Parra et al., 2012, 2015; Schultz, 2000).

With high temperature, a complex dilemma occurs between opening stomata with the aim of maintaining as low as possible leaf temperature and as high as possible photosynthesis, or closing them preventing unwanted water losses and excessive dehydration (Campitelli and Stinchcombe, 2013; Chaves et al., 2016; Jones, 2014; Nicotra et al., 2008; Peppe et al., 2011). Analyzing vegetative growth under elevated temperature, it seems that the extent of stomata aperture and its consequences on growth depend on the plant water availability (i.e., there was a clear interaction between temperature and water availability). In full-irrigated grapevines elevated temperature increased leaf, petiole, stem and root dry weight (range 4-26%), whereas decreases (from 7 to 19%) were observed in plants exposed to drought.

In the two Tempranillo cultivars water deficit inhibit both shoot and root growths, in line with previous reports (Schultz and Matthew, 1988). Furthermore, root growth was less sensitive to water deficit than shoot growth, as also indicated by Williams and Matthews (1990).

Besides, elevated CO₂ compensates partially for the negative effects of water stress (Chaves and Pereira, 1992), which could explain the increased growth of grapevine plants due to elevated CO₂ in water-stressed red Tempranillo.

A varietal difference could be observed. Grapevine above- and under-ground organs were higher in red than in white Tempranillo, and plant leaf area was lower in the white cultivar (especially, under full irrigation and current temperature), which may be related to a lower water consumption in the white Tempranillo possibly caused by a lower stomatal density in this variety (according to our preliminary and unpublished results).

Elevated CO₂ stimulated more vegetative (total vegetative mass) than reproductive (berry and rachis FW) growth. For instance, berry diameter, a parameter that is easily measured and highly variable in grapevine (Friedel et al., 2016), increased only 8% in response to elevated CO₂, showing a CO₂ positive, although small, effect in line with a previous report (Bindi et al., 2001).

Exposure to elevated temperature per se (+ 4°C) had no impact on grapevine yield. However, the high temperatures of the heat waves (with maxima temperatures above 35°C for at least five consecutive days or 40°C for three days) resulted in berry burn and browning, and finally loss of 50% of the bunch grapes.

Reproductive growth of grapevine is generally less sensitive to water stress than its vegetative growth (Korkutal et al., 2011; Williams and Matthews, 1990). At the beginning of the berry developmental stage (flowering and fruit set), water scarcity has high impact on berry growth and its damage cannot be reversed by supplemental irrigation during the following stages. After fruit set, however, Mekni (2014) reported that deficit irrigation does not result in changes on berry size. According to our data, there is not alteration in berry

diameter and fresh weight, relative skin mass, and number of seeds per berry, and small decreases in number of berries per bunch and rachis fresh weight in response to drought.

Also, a varietal difference could be observed regarding reproductive growth. Red Tempranillo produced bunches of higher size than the white cultivar, possibly related to higher water consumption and soil water depletion in the red one (see above).

Climate change-related factors impact on berry quality was year-dependent with high variability. Even though, some general conclusions can be obtained from the three years of experimentation in the TGGs. Thus, the simulated climate change scenarios affected to a greater extent the technological maturity parameters (primary metabolism) than the phenolic maturity (secondary metabolism).

Changes in quality observed were not due, or modulated by, changes in berry size. Water stress effects due to berry size reduction may have indirect influence on berry quality through concentration of phenolic compounds, especially when water stress occurs from flowering until veraison (Antolín et al., 2003; Hardie and Considine, 1976; Korkutal et al., 2011; Koundouras et al., 2006; Matthews et al., 1987; McCarthy, 1997; Niculcea et al., 2014; Ojeda et al., 2002). However, as already mentioned, deficit irrigation after fruit set in red Tempranillo does not result in changes on berry size (Mekni, 2014).

Tartaric and malic acids are the major organic acids in grape berries, with citric acid being present in lower amount. Grape acidity depends on their concentration, the ratio among them and the concentration of potassium (Conde et al., 2007). Organic acids start to accumulate during the first grape berry development period, peaking before the onset of ripening (veraison). Then, their concentration in berries decrease coincident with grape sugar accumulation, and this dynamic is highly affected by environmental variables (typically decreases in hot climates). Here, in our particular experiments, high temperature and drought significantly and consistently raise pH and diminish acidity. According to metabolic pathway of acids to sugar balance in grape berries, the loss of malic acid (Lakso and Kliewer, 1975; Shellie, 2006) and consequently reduction in acidity (Sadras and Moran, 2012) due to high temperatures has been attributed in controlled experiments to an increased degradation to glucose during ripening (Blouin and Guimberteau, 2003;

Matthews and Anderson, 1989; Ruffner et al., 1976). Also, it is well known that drought usually decreases malate concentration in grapevine due to malate breakdown (Matthews and Anderson, 1989). A single-year previously reported experiment for the same red Tempranillo variety (Salazar Parra et al., 2010) is confirmed by these multi-year results. In the literature, however, no clear results were reported; malic acid increases (López et al., 2007), decreases (Kondouras et al., 2006; Matthews and Anderson, 1989; Salon et al., 2005) or even no grape malic acid changes (Esteban et al., 1999) have been reported under water stress. On the contrary, it seems to be a specific effect of the elevated CO₂ on the tartaric acid concentration, increasing its biosynthesis and/or decreasing its degradation. As previously reported (Salazar-Parra et al., 2010), water stress had no influence on grape tartaric acid of red Tempranillo. Potassium has an important role as trait for quality and longevity of wines, because it impacts must and wine pH and wine stability (Kliewer, 1965b, 1966; Ribéreau-Gayon et al., 2006). Potassium binds to tartaric acid to form tartrate, reducing acidity (Kliewer, 1965a) and precipitating during fermentation and ageing of wine (Conde et al., 2007), which results in low quality wines. On the other hand, high potassium levels in grapevine reduce the rate of malate degradation (Etienne et al., 2013). According to our experiments, water stress increases and elevated CO₂ decreases significantly potassium in grapevine must. Overall, all these changes in the acidic properties of the berry must have direct impact on its quality and in the potentially resulting wine through changes in the wine stability and taste (Conde et al., 2007).

Phenolic compounds are present in grapes, and are very important constituents for wine quality (Downey et al., 2006; He et al., 2010; Kuhn et al., 2014). Environmental factors, such as light and temperature, influence these grape constituents (Blouin and Guimberteau, 2003; Kliewer, 1970; Lakso and Kliewer, 1975; Mori et al., 2007). Total polyphenol index (TPI) estimates the total content of phenolic compounds. In our experiments, high temperature significantly decreased grape TPI but with no changes in anthocyanin concentration, which demonstrates that phenolic compounds other than anthocyanins caused these decreases. Besides, elevated temperature also lowers flavonol concentrations (Pastore et al., 2017). No interaction between water availability and temperature on the TPI was found, in line with a previous report of Bonada and co-workers (2015). Water deficit combined with elevated CO₂ in the white cultivar raises TPI, in line

with Petrie et al. (2004). Nevertheless, water deficit combined with high temperature and elevated CO₂ had no influence on TPI in the white Tempranillo. This demonstrates the cancelation of the influence of some climate change-related environmental factors by others, in this case temperature, and highlights the importance of investigating multiple environmental factors, and their interactions, in grapevine studies. Furthermore, TPI in white Tempranillo was significantly lower than TPI in the red cultivar. White varieties may have other polyphenols (Santesteban, 2008).

Anthocyanins, synthesized via the flavonoid pathway in the red varieties, have an essential role in the grape and wine color. Grape total anthocyanins decreased by the impact of drought, which agrees with previous reports showing lower anthocyanin concentrations in non-irrigated than in those subjected to conventional (or regulated deficit) irrigation in red Tempranillo grapevines (Kuhn et al., 2014; Zarrouk et al., 2012). The increased tonality (hue) observed in these experiments in red Tempranillo grapes from water stressed plants agrees with previous reports (Martínez-Lüscher et al., 2014) and suggests a change in anthocyanin composition. Downey et al. (2006) related the blueness and redness tonality with the pattern of anthocyanin hydroxylation and methylation, respectively. More in detail, the changes in tonality of droughted red Tempranillo grapes were ascribed to an increased proportion of the 3'4'5' substituted forms (delphinidine, petunidine and malvidin) to the detriment of the substituted 3'4' (cyanidin and peonidine) ones (Martínez-Lüscher et al., 2014). There are also differences in their extractability (Downey et al., 2006) and their sensitivity to oxidation (He et al., 2010) with major effects on aged wine characteristics (Kontoudakis et al., 2011), because the more hydroxylated and methoxylated forms (of both anthocyanins and flavonols, the latter acting as co-pigments of the former) are those that have greater resistance to degradation, and therefore prevail during the vinification and wine aging process protecting it from oxidation (Arroyo-Currás et al., 2016; Cortell and Kennedy, 2006).

Grape veraison is characterized by sugar accumulation in the berry pulp, followed then by anthocyanin accumulation mainly in the skin for red varieties (Conde et al., 2007; Coombe and McCarthy, 2000; Lecourieux et al., 2014). Sugar level and anthocyanin accumulation appear to be highly related (Dai et al., 2014). Besides, these compounds are

decoupled by climate change in red varieties (Martínez-Lüscher et al., 2016). In our experiments, during three years, decreases in the anthocyanins to sugars ratio were observed when grapevine plants were grown under water stress.

White Tempranillo used in this work is a new white berry somatic variant of red Tempranillo that came up with a spontaneous, natural mutation, which comprised chromosomal deletions and reorganizations. Due to a multi-allelic mutation on white Tempranillo, the lack of at least two similar and adjacent regulatory genes (Walker et al., 2007) resulted in the loss of genes and/or changes in their activity or regulation related to primary and secondary metabolisms. In our experiments, differences regarding grape quality between red and white Tempranillo were as follows: (i) grape total soluble solids increased, and acidity decreased and pH increased, due to decreased malic and tartaric acids concentrations in white, when compared to the red Tempranillo, (ii) total polyphenol index was lower in the white variety when compared to the red one, which in part at least can be due to the absence of anthocyanins. All these findings may give clues to further research on the genetic differences between red and white Tempranillo.

Under elevated CO₂ concentrations, the photosynthetic rates of C₃ plants, such as grapevine, increases (see above). Also, stomatal conductance and transpiration rates (Flexas et al., 2014; Leakey et al., 2009; Salazar-Parra et al., 2012, 2015) as well as stomatal density (Moutinho-Pereira et al., 2009; Rogiers et al., 2011) were reported to diminish when plants are grown in presence of elevated CO₂, improving water use efficiency (Da Silva et al., 2017; Wullschleger et al., 2002). These clear effects have been observed with short-term elevated CO₂ exposure in grapevine plants (Bindi et al., 1996; Schultz, 2000). Under long-term exposure, however, effects of elevated CO₂ become attenuated not only in grapevine but also in other species (Aranjuelo et al., 2005; Bindi et al., 1996; Erice et al., 2006; Jifon and Wolfe, 2002; Long et al., 2004). Previous reports from our research group demonstrated this photosynthetic down-regulation after long-term exposure to elevated CO₂ in red Tempranillo (Leibar et al., 2015; Salazar-Parra et al., 2015). Red and white Tempranillo plants grown under elevated CO₂ concentration had lower net photosynthetic rates than those grown under ambient CO₂ concentration, a first symptom of photosynthetic

acclimation to elevated CO₂. A similar pattern was observed in diverse herbaceous C3 species (Bunce, 2000) and in alfalfa (Goicoechea et al., 2014).

One hypothesis under debate of the reasons of photosynthesis acclimation to elevated CO₂ is that it results from a decrease in the photosynthetic capacity caused by a reduction in Rubisco amount and/or activity (Ainsworth and Rogers, 2007; Aranjuelo et al., 2009; Erice et al., 2006). The results of our study are not in line with this hypothesis neither with inactivation of the CO₂ fixing enzyme Rubisco by high temperature as mentioned by other researchers (Greer and Weedon, 2013; Haldimann and Feller, 2004; Law and Crafts-Brandner, 1999; Salvucci and Crafts-Brandner, 2004).

Another line of evidence of photosynthetic acclimation to elevated CO₂ in red and white Tempranillo comes from C/N data. Both red and white Tempranillo had higher leaf C/N ratio values when grown under elevated CO₂ than when grown under ambient CO₂, mainly due to N reduction as was also indicated for red Tempranillo by Salazar-Parra et al. (2015). Low N availability can negatively affect plant growth, reducing therefore the plant capacity to develop new sinks (Ainsworth and Rogers, 2007; Aranjuelo et al., 2007; Sanz-Sánchez et al., 2013). In the photosynthetically acclimated plants, photosynthetic rates are reduced to balance C source activity and its sink capacity (Aranjuelo et al., 2009, 2013). An increased C/N ratio is a clear symptom of photosynthetic acclimation to elevated CO₂ (Jifon and Wolfe, 2002; Irigoyen et al., 2014). Thus, data reported in this work for red and white Tempranillo confirm previous reports working with red Tempranillo (Leibar et al., 2015; Salazar-Parra et al., 2015).

Plants grown under elevated CO₂ concentration had higher leaf starch contents, and more important the decreased photosynthesis correlated with the increased starch, suggesting a feedback down-regulation by starch. No correlations were observed with total soluble sugars. Previous reports have shown that plants acclimated to elevated CO₂ accumulate carbohydrates (Geiger et al., 1999; Mishra et al., 2008).

A second hypothesis proposes a leaf stomatal conductance reduction in plants grown under elevated CO₂ as the origin of the photosynthetic acclimation (Sánchez-Díaz et al., 2004). Decreases in stomatal conductance under elevated CO₂ concentration may be caused

by stomatal changes in density or aperture, driven by physiological changes during development (Ainsworth and Rogers, 2007). Elevated CO₂ concentration decreased stomatal conductance, however, the differences in photosynthetic rates of plants grown under ambient and under elevated CO₂ concentrations were not associated with CO₂ limitation at the leaf sub-stomatal cavities, as the values were similar in both groups of plants. Taking into account the lack of changes in Rubisco activity and the similar values in sub-stomatal CO₂ concentration, it can be concluded that probably the CO₂ mesophyll conductance is the limiting factor for the supply of CO₂ to Rubisco and, therefore, it is what leads to a reduction in the photosynthetic rates of the long-term grown grapevine plants under elevated CO₂ concentration. Although unfortunately mesophyll conductance to CO₂ was not estimated in this work, previous reports working with red Tempranillo have found that elevated CO₂ decreases the CO₂ conductance within the mesophyll, decreasing the CO₂ concentration in the chloroplast available for the Rubisco enzymatic reaction (Salazar-Parra et al., 2012). Feedback inhibition of other anabolic enzyme/s mediated by starch (or starch-derived products) cannot be excluded.

White Tempranillo is a natural, spontaneous mutation from red Tempranillo that comprises, as already mentioned, deletions and chromosomal reorganizations, losing or affecting hundreds of genes. Photosynthetic acclimation was no so severe in red than in white Tempranillo. Thus, for any given sink size (berry, total vegetative or total plant DM) or any given leaf starch accumulation, white Tempranillo always had higher levels of photosynthetic acclimation than red Tempranillo (quantified by the extent of the increases in leaf C/N ratio). It is obvious that loci other than grape color, like those implicated in the photosynthetic response to prolonged exposure to elevated CO₂, have been affected by the mutation.

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CONCLUSIONES GENERALES

1. Drought and heat shocks (but not elevated temperature per se) decreased grapevine yield in red and white Tempranillo.
2. Red Tempranillo was more productive than the white cultivar as a consequence of higher water consumption and soil water depletion.
3. Grape yield and quality effects of climate change conditions (elevated CO₂, elevated temperature and water deficit) were cultivar dependent, affecting different traits.
4. The climate change-related factors investigated (elevated CO₂ concentration, elevated temperature and drought) affected to a greater extent the technological maturity parameters (primary metabolism) than the phenolic maturity (secondary metabolism). The response of grape quality (technological and phenolic maturity) to the mentioned factors was variable and depended on the year, probably due to the climate variability and the interactions among factors defining the climate (temperature, humidity, and sunlight).
5. Elevated temperature and drought consistently increased must pH, due to malic acid decreases. On the contrary, elevated CO₂ decreased pH associated with consistent tartaric acid concentration increases. The changes in the must acidic properties affect its quality and potentially that of the resulting wine.
6. Grapevine reproductive growth was less impacted by environmental factors than vegetative growth. Both vegetative and reproductive growth responses to the climatic related factors (elevated CO₂, elevated temperature and water deficit) were variable between years, probably due to the year-to-year characteristics and the interactions among factors defining the climate (seasonal changes and diurnal cycles of temperature, humidity, sunlight, etc.).
7. Plant vegetative growth decreased especially with water deficit treatments, showing a similar pattern the three growing seasons (2013, 2014 and 2015).
8. The inhibitory effect of water deficit was more related with substrate water status than with leaf water content. The stimulatory effect of the elevated CO₂ was not associated with a better leaf water status, rather the contrary.
9. Elevated CO₂ stimulated more vegetative than reproductive growth, in particular total vegetative mass (leaf area was not affected). In white and red Tempranillo, the

largest increases when plants were grown under elevated CO₂ were observed in leaf and root growth, respectively. A clear interaction between temperature and water availability was evidenced. In full-irrigated grapevines, elevated temperature increased leaf, petiole, stem and root dry weight, whereas decreases were observed in plants exposed to drought.

10. Photosynthetic acclimation in grapevine plants was evidenced by photosynthesis capacity decrease, leaf starch accumulation, and leaf carbon/nitrogen ratio (C/N). The cause of photosynthesis acclimation was not associated with Rubisco in vitro extractable activity decrease (specific activity and/or amount) or CO₂ stomatal limitation (leaf sub-stomatal CO₂ concentration), and probably appears to be induced by mesophyll CO₂ conductance limitations and/or by feedback inhibition of other anabolic enzyme/s mediated by starch (or starch-derived products).
11. Photosynthetic acclimation may explain the low rates of growth stimulation observed in grapevine plants under elevated CO₂, as plants had no significant leaf area and dry weight differences between CO₂ treatments.
12. Photosynthetic acclimation and drought effects were not associated with changes in leaf water status. Drought-induced growth inhibition (leaf area and dry weight accumulation decline) was associated more with soil water status than with leaf water status.
13. Other factors, such as elevated temperature or drought did not influence significantly the photosynthetic acclimation process.
14. White Tempranillo showed higher level of photosynthetic acclimation than the red cultivar, for any given level of leaf starch accumulation or any given sink size.
15. Conditions expected for the end of the 21st century may strongly advance bud burst, flowering, and berry designated maturity. Some uncertainties still exist, such as whether the sensitivities to temperature and CO₂ or the delaying effect of water deficit, which have been observed in fruit-bearing cuttings, will be generally observable in vineyards in the future.

ANEXO

Sensitivity of grapevine phenology to water availability,
temperature and CO₂ concentration

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Sensitivity of grapevine phenology to water availability, temperature and CO₂ concentration

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Abstract

In recent decades, mean global temperatures have increased in parallel with a sharp rise in atmospheric carbon dioxide (CO₂) levels, with apparent implications for precipitation patterns. The aim of the present work is to assess the sensitivity of different phenological stages of grapevine to temperature and to study the influence of other factors related to climate change (water availability and CO₂ concentration) on this relationship. Grapevine phenological records from 9 plantings between 42.75°N and 46.03°N consisting of dates for budburst, flowering and fruit maturity were used. In addition, we used phenological data collected from two years of experiments with grapevine fruit-bearing cuttings with two grapevine varieties under two levels of water availability, two temperature regimes and two levels of CO₂. Dormancy breaking and flowering were strongly dependent on spring temperature, while neither variation in temperature during the chilling period nor precipitation significantly affected budburst date. The time needed to reach fruit maturity diminished with increasing temperature and decreasing precipitation. Experiments under semi-controlled conditions revealed great sensitivity of berry development to both temperature and CO₂. Water availability had significant interactions with both temperature and CO₂; however, in general, water deficit delayed maturity when combined with other factors. Sensitivities to temperature and CO₂ varied widely, but higher sensitivities appeared in the coolest year, particularly for the late ripening variety, 'White Tempranillo'. The knowledge gained in whole plant physiology and multi stress approaches is crucial to predict the effects of climate change and to design mitigation and adaptation strategies allowing viticulture to cope with climate change.

Key words: Climate change, viticulture, fruit development, ripening, chilling, dormancy, Partial Least Squares regression

1. Introduction

1.1 Evolution of environmental factors linked to climate change

Analyses of historic climatic changes indicate an increase in mean land surface temperature by 1.06°C over a period of more than a hundred years, with the lion's share of this amount – 0.85°C – occurring over the past two decades (IPCC, 2014b). Climate projections for the end of the 21st century forecast increases in temperature within a rather wide range, from stabilization at 1.5°C higher than the current reference period to a more than 4°C increase in average global temperature, depending on the mitigation measures adopted (IPCC, 2014a). The main driver of the temperature increase has been human emission of greenhouse gases. Among these, CO₂ is the most relevant in volume and global effect (IPCC, 2014a), with its concentrations increasing from a preindustrial level of 280 µL L⁻¹ to currently more than 400 µL L⁻¹ in 2016, with predictions for the end of the century ranging from 421 to a 936 µL L⁻¹ (Meinshausen et al., 2011). Rainfall in many major wine growing regions of the world has decreased and is expected to decrease further in the future (IPCC, 2014b).

1.2 Dependence of grapevine on temperature

Important effects of temperature on grapevine (*Vitis vinifera* L.) have long been recognized as influencing plant physiology, berry composition and ultimately wine characteristics (Jones et al., 2005; Bonada and Sadras, 2015). Thermal time integrals are even used as one of the main criteria to assess the suitability of a given cultivar to a given location (Gladstones, 1992). Premium commercial vineyards have been traditionally distributed across a relatively wide range of latitudes, ranging from the subtropics to temperate climates like the south of England (at 53° N). Across this range, temperature during dormancy and growing season varies widely (Jones, 2006; Jones, 2007). However, the distribution of grape growing regions is not necessarily aligned with the acclimation or adaptation thresholds of the species, and in the case of quality wine production, the upper temperature limits are hard to assess even at the variety level (van Leeuwen et al., 2013). Evidence of the plasticity of this species and the apparent lack of thermal restrictions for growing grapes is the recent increase in production of both table and wine grapes in subtropical and tropical areas (Demir, 2014).

1.3 Temperature thresholds for wine typicity

Although basic climatic conditions for grape growing are easily satisfied, rising temperatures may make it difficult to consistently fulfil specific criteria required for grape quality in many places without adjusting variety, clone or accession within a given variety, or changing management practices. This is especially relevant for wine grapes, as most wine appellations in the world aim to deliver a very specific product, resulting from the wine-making, grapevine genetic material (cultivar and clone), cultural practices, edaphic factors and climatic conditions (van Leeuwen et al., 2004). Final grape composition results from numerous processes leading to accumulation and/or decay of metabolites, which are affected to a considerable degree by climatic conditions (Kuhn et al., 2014). Consequently, aromas, aroma precursors, phenolic compounds, organic acids and sugars have very different accumulation patterns throughout grape development and, what is most relevant, their responses to increasing temperatures may differ in magnitude. The best example is that temperature increases can enhance both sugar accumulation and organic acid decay, but acidity is more affected than sugar levels. This results in lower acidity for the same sugar level in grapes grown under warmer temperature (Lakso and Kliewer, 1975; Sweetman et al., 2009; Etienne et al., 2013). This decoupling has been reported for other relevant metabolites, such as anthocyanins (Sadras and Moran, 2012; Martínez-Lüscher et al., 2016), proanthocyanidins (Cohen et al., 2012) and aromas (Bonada et al., 2015).

1.4 Relationship between wine composition and altered phenology

Despite the scarcity of data, some attempts have been undertaken to relate grape composition to records of environmental conditions (Bonada and Sadras, 2015). Grapevine phenology records, however, are relatively abundant and can be a good proxy for altered grape composition in response to environmental factors (Sadras and Moran, 2013; Bonada and Sadras, 2015). The duration of the phenophases can affect metabolite dynamics (Kuhn et al., 2014; Martínez-Lüscher et al., 2016), but it is also likely that advancing phenology shifts the ripening period towards the warmest part of the year (Webb et al., 2007; Duchene et al., 2010), which is not compatible with the production of high quality table wines (van Leeuwen and Seguin, 2006).

1.5 Annual cycle of grapevine development

Most temperate fruit crops need a period of cool temperatures before they can produce flowers (Campoy et al., 2011; Considine and Considine, 2016). Satisfaction of the chilling requirement influences the timing of budburst, flowering and subsequent phenological stages (Luedeling, 2012). However, grapevine flowering and veraison can be modelled quite successfully using spring temperatures as the only predictor variable (Parker et al., 2011), suggesting a weak effect of temperatures during bud dormancy (Garcia de Cortazar-Atauri et al., 2009). While the period from the breaking of dormancy to flowering is strongly determined by temperature, flowering to veraison is often influenced by other abiotic factors such as water deficit (Davies et al., 1994; Antolin et al., 2003; Martínez-Lüscher et al., 2015a), and the correlation is usually weaker (Duchene et al., 2010). This becomes even more evident for the period from flowering or veraison to fruit maturity, which is influenced by an even larger number of factors (Petrie and Sadras, 2008; Webb et al., 2012). Even though ripeness is defined by subjective criteria, and therefore is not a phenological event strictly, it can be reliably measured in relation to metabolite concentrations, sugars, anthocyanins and organic acids (Bonada and Sadras, 2015). For instance, regarding the implications of sugar content for the potential alcohol content of resulting wine, the concentration of total soluble solid (TSS) concentration is a straightforward and reliable marker for the progress of ripening (Bonada and Sadras, 2015).

1.6 Aim of the study

In recent years, phenology responses of perennial crops to projected future climates have been assessed. These projections have often focused on response to temperature, with a few cases including water availability, but they have not convincingly considered CO₂. The present study aims to give an overview of the effect of climate change-related phenomena (water deficit, increasing temperature and elevated CO₂) on the phenology of grapevine, a temperate perennial woody crop. For this purpose, we evaluated historical data of phenological records and conducted some experiments under controlled conditions. The combination of these two data sources – historical records and fruit-bearing cuttings under controlled conditions – allows immediate extrapolation to the field when analyzing historical data, but it also allows studying the direct effects

of these environmental factors at reasonable cost, when performing experiments under semi-controlled conditions.

2. Materials and Methods

2.1 Field phenology records

Grapevine phenological records were obtained from the pan European phenology project (PEP725 Pan European Phenology Data; dataset accessed on 2015-09-23 at <http://www.pep725.eu>). The records were used to calculate the time elapsed between March 1st and the beginning of budburst (BBCH 7), budburst to beginning of flowering (BBCH 60) and from flowering to fruit maturity (BBCH 87), to test the influence of temperature on grapevine development (Lorenz et al., 1995). The database consisted of 306 seasonal records collected between 1961 and 2013 from 9 different plantings in 6 locations in Croatia (Daruvar, Cepic, Hvar, Kricevzi, Mandicevac and Trsteno). These sites, which are distributed between latitudes 42.75°N and 46.03°N and between longitudes 14.13°E and 19.23°E, are representative of the average latitude for the distribution of northern hemisphere vineyards.

2.2 Temperature and rainfall records

Field temperature and rainfall records were extracted from the E-OBS European gridded data set (Haylock et al., 2008). As this database contains daily minimal and maximal temperatures, hourly records were constructed with procedures contained in the chillR package (Luedeling, 2016), for R programming language (R Development Core Team, 2015). These procedures follow the recommendations of Linvill (1990). Sunrise, sunset and day length data for this method were modelled using each site latitude (Spencer, 1971; Almorox et al., 2005).

2.3 Identification of chilling and warming periods

Partial Least Squares (PLS) regression was used at one of the locations (Mandicevac) to correlate variation in daily chill and heat accumulation to grapevine budburst dates. Daily chill accumulation (in Chill Portions) was calculated according to the so-called Dynamic Model (Fishman et al., 1987), which is regarded as the most accurate under a

wide range of circumstances (Campoy et al., 2011). Daily heat accumulation (in Growing Degree Hours) was calculated according to Anderson et al. (1986), with a curvilinear model using a base temperature of 4°C and an optimum temperature of 26°C, which are representative of grapevine response to temperature (Parker et al., 2011). Formulas for each model are given in Luedeling and Brown (2011) and Luedeling et al. (2009), respectively. Eleven-day running means were constructed to facilitate interpretation of the results (Luedeling and Gassner, 2012). 41 datasets were created consisting of 694 independent variables (i.e. daily heat and chill accumulation from June 1st (of the previous year of each budburst data) to May 14th, which was the latest budburst date recorded in the site; data for May 14th were omitted in leap years), and one bud break date each. PLS regression outputs – variable importance in the projection (VIP) and model coefficients – were used to delineate the periods where an increase in daily chill accumulation indicated a bloom-advancing effect (negative correlation between chill accumulation and budburst date; this was interpreted as the chilling phase) and where an increase in daily heat accumulation implied a bloom-advancing effect (negative correlation; warming phase). The VIP threshold for signification used was 0.8, which is commonly adopted by other studies (Wold, 1995). Further details of the procedures are described in Luedeling et al. (2013). Once these periods were identified, three-dimensional interpolation (Kriging) was used to illustrate the differential effects of temperature during the chilling and warming periods on budburst dates. This aims to facilitate the interpretation of the effect of two predictor variables that are highly correlated. The angle of the contour lines that are generated indicates, which one of the two factors is dominant in determining budburst dates (Guo et al., 2015).

2.4 Plant material and growth conditions

Dormant cuttings of *Vitis vinifera* L. cv. Red Tempranillo (accession T43, Clone RJ-43) and White Tempranillo (accession CI-101 in the “La Grajera” germplasm bank, Government of Rioja, Spain) were collected in January of 2014 and 2015 from an experimental vineyard of the Institute of Sciences of Vine and Wine (ICVV) in Logroño (La Rioja, Spain).

Three node cuttings of *V. vinifera* L. cv. Red and White Tempranillo were selected to produce fruit-bearing cuttings according to Mullins (1966), as described in Kizildeniz et

al. (2015). Rooting was induced using indole butyric acid (300 ppm) in a heated moist-bed (25-27°C) kept in a cool chamber (5°C). After one month, the rooted cuttings were planted in 0.8 L plastic pots containing a mixture of sand, perlite and vermiculite (1:1:1, v/v) and transferred to the greenhouse. At fruit set, plants were planted in 13 L plastic pots containing a mixture of peat and perlite (2:1, v/v). Only a single flowering stem was allowed to develop on each plant, resulting in only one grape bunch per plant. Pruning was used to control vegetative growth until fruit set, thus allowing only 4 leaves per plant to grow. Growth conditions in the greenhouse were 26/15°C and 60/80% relative humidity (RH) (day/night), with a photoperiod of 15 h with natural daylight supplemented with high-pressure metal halide lamps (OSRAM®, Augsburg, Germany). The supplemental system was triggered when photosynthetically active radiation (PAR) dropped below a photosynthetic flux density (PPFD) of 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$, providing a PPFD of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at inflorescence level. Plants were irrigated with the nutrient solution described by Ollat et al. (1998): NH_4NO_3 (64.5 mg L^{-1}), $(\text{NH}_4)_2\text{HPO}_4$ (75 mg L^{-1}), KNO_3 (129 mg L^{-1}), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (125 mg L^{-1}), $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (248 mg L^{-1}), $(\text{NH}_4)_2\text{SO}_4$ (66 mg L^{-1}), Fe (EDDHA) (280 mg L^{-1}), H_3BO_3 (2.86 mg L^{-1}), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.81 mg L^{-1}), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.22 mg L^{-1}), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.08 mg L^{-1}) and $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (0.016 mg L^{-1}). Plants grew under these conditions until fruit set (from March to May, both in 2014 and 2015).

2.5 Temperature gradient greenhouse experiment design and analyses

Treatments were applied in the temperature gradient greenhouses (TGGs), located at the University of Navarra (42.80°N, 1.67°W) in Pamplona (Navarra, Spain), from June to August (i.e., from fruit set to maturity) in 2014 and 2015. TGGs are designed as temperature gradient tunnels (Rawson, 1995), which allows investigating the effects on plants of environmental changes, such as elevated temperature, elevated CO_2 and drought, acting separately or in combination. They were constructed with a modular design with three temperature modules, which creates a temperature gradient ranging from near-ambient temperature in module 1 to ambient temperature +4°C in module 3. CO_2 can be injected into the greenhouse to increase the air CO_2 concentration as desired (more details in Morales et al., 2014). When fruit set was complete for all plants, fruit-bearing cuttings of Red and White Tempranillo cultivars were subjected to a combination of two temperature regimes (ambient and ambient +4°C, no experimental plants were set in module 2 with intermediate temperature) and two CO_2 concentrations

(current ca. 400 $\mu\text{L L}^{-1}$ and elevated ca. 700 $\mu\text{L L}^{-1}$) until maturity (defined as TSS=21-23°Brix). Each treatment consisted of 10 plants, which were selected on the basis of similar grape bunch sizes. Plants were maintained with free vegetative growth. CO₂ concentration, temperature, relative humidity and radiation were measured and/or controlled by an automated monitoring system. Data was analyzed with XLStat (Addinsoft, Paris, France) by a factorial ANOVA (2x2x2x2x2). Only main factors and two-level interaction P values were presented.

3. Results

3.1 Effect of temperature on grapevine dormancy breaking

PLS regression results for budburst dates (Fig. 1) showed several periods with significant variable importance in the projection (VIP) scores (>0.8) and negative coefficients between December 8th and February 28th, suggesting that during this period, increases in chilling were correlated to advanced budburst. Although few periods of negative correlations were observed before this period – between August 27th and October 23rd – due to the large period of significant scores and negative coefficients from October 24th to November 26th, it seemed reasonable to interpret the period between December 8th and February 28th as the chilling phase. Days with significant VIP scores coupled with negative correlation coefficients for daily heat accumulation were also discontinuous, but they were concentrated in three major periods: December 9th to December 25th, January 3rd to January 16th and January 22nd to May 1st, with only brief interruptions, during which model coefficients remained negative almost every day. Still, mean heat accumulation during the two first periods – December 9th to December 25th and January 3rd to January 16th – was very low and most likely did not have a strong effect in most years. It must be noted that this species may fulfill its critical chilling requirement much earlier than most perennial crops, and therefore, important and negative scores for heat accumulation could be expected at any time of the chilling period when adequate temperature levels are reached. We delineated the warming phase as the period between January 22nd and May 1st.

The delineation of chilling and warming phases allowed calculation of mean temperature during these phases. Results show a weak effect of changes in temperature during the chilling phase on budburst date (Fig. 2). Taking into account the angle and

the separation of the contour lines in figure 2, the sensitivity of budburst date to changes in temperature during the chilling period was small ($-0.01 \text{ d } ^\circ\text{C}^{-1}$), compared to $-4.47 \text{ d } ^\circ\text{C}^{-1}$ for temperatures during the forcing phase. The almost horizontal contour lines of the interpolating surface suggests that temperature during the warming period strongly affected budburst date compared to the effect of temperatures during the chilling period. In addition, variation in mean temperature during the delineated chilling phase is much lower than during the warming phase (ranges of -4.2 - 3.9°C and 3.0 - 9.2°C , respectively).

3.2 Decoupling of the effects of water availability and temperature on grapevine phenophases

The linear regression trend determined for all sites (Fig. 3) shows that the sensitivity is -4.49 days change in budburst date for each degree of temperature increase ($\text{d } ^\circ\text{C}^{-1}$) from March 1st to budburst. This sensitivity is higher than for any other phenophase displayed, as the stage from budbreak to flowering had a sensitivity of $-3.29 \text{ d } ^\circ\text{C}^{-1}$ and the stage from flowering to harvest had a sensitivity of $-2.57 \text{ d } ^\circ\text{C}^{-1}$. When the effect of temperature on field phenological data was decoupled from rainfall (Fig. 4), precipitation showed a differential effect on phenological periods. The interpolation surface of the period from March 1st to budburst (Fig. 4A) shows some delaying effects in rainy years and advancing effects in dry years, but this was not a general trend and it resulted in a high degree of patchiness. Contrarily, the period from budburst to flowering and flowering to ripe fruit resulted in smoother interpolating surfaces (Fig. 4B and Fig. 4C, respectively). In the case of budburst to flowering (Fig. 4B), only temperature and not rainfall showed an advancing effect. This is suggested by the decreasing the value of contour lines and interpolating surface with increasing temperature, but not with rainfall. However, in the case of the period from flowering to ripe fruit (Fig. 4C), this effect was shared by increases in temperatures and decreases in precipitation.

3.3 Effects of water availability, temperature, CO₂ concentration and their interactions under semi-controlled conditions

The two varieties (Red and White Tempranillo) showed different sensitivities to increasing temperatures (Fig. 5). The figures obtained were $-1.52 \text{ d } ^\circ\text{C}^{-1}$ in 2014 and $0.15 \text{ d } ^\circ\text{C}^{-1}$ in 2015 for Red Tempranillo and $-2.03 \text{ d } ^\circ\text{C}^{-1}$ in 2014 and $-0.90 \text{ d } ^\circ\text{C}^{-1}$ in

2015 for White Tempranillo. The lower sensitivity to temperature observed in 2015 for both varieties was most likely associated with higher temperatures recorded in that year and a higher number of days with temperatures above 35°C (Table 1). Atmospheric CO₂ did not change the sensitivity to temperature in any case, and this is supported by the lack of interactions in the two-way ANOVA analysis. The overall effect of CO₂ and temperature was very significant ($p(\text{CO}_2) < 0.001$ and $p(\text{Temp}) = 0.001$), elevated CO₂ had an advancing effect of 4.37 d and 3.54 d in T and T+4°C treatments, respectively. However, looking at the varieties separately, the effect was not significant for the Red Tempranillo variety ($p(\text{CO}_2)$ and $p(\text{Temp}) > 0.05$), whereas the effect for White Tempranillo was strong ($p(\text{CO}_2) < 0.001$ and $p(\text{Temp}) = 0.005$). In the overall (all years and varieties), high CO₂ advanced phenology by 6.75 d and 4.06 d in the T and T+4°C treatments, respectively.

It must be noted that several two-level interactions were significant for time from fruit set to veraison, veraison to harvest and fruit set to harvest (Table 2). Therefore, the significance of the main effects must be interpreted with caution. For instance, 5 out of 11 interactions involved the Year main effect, which reflects the inconsistency of some factor effects from year to year. Other important interactions were found between the Cultivar and CO₂ for time from veraison to maturity and fruit set to maturity. These findings point out the higher susceptibility of the white cultivar under all the combinations of conditions. Still, it must be noted that for most combinations of treatments, elevated CO₂ plants completed fruit development and ripening earlier than their ambient CO₂ homologue. Interactions between environmental factors were also found, including between water deficit and temperature and between water deficit and CO₂. In the main effect comparisons, water deficit extended the ripening period by an average of 3 days. However, looking at the effect of water deficit combined with other factors, cyclic drought (CD) had an advancing effect (i.e., Red Tempranillo under T – E CO₂ in 2015 and White Tempranillo under T – E CO₂ in 2014 and 2015), which explains the significant interactions between water availability and temperature and CO₂.

4. Discussion

4.1 Effect of temperature

Many articles have reported the accelerating effects of rising temperatures on phenology based on events typically occurring in spring (i.e. budburst, leaf unfolding and flowering). While most species show a clear advance in phenology, an exception to this general trend may be those species that are starting to experience difficulties in meeting their chilling requirements (Guo et al., 2015). In this respect, grapevine (*Vitis vinifera* L.) is presented in the literature as a species with a low chill requirement, despite its tendency to burst and flower rather late (Mullins et al., 1992). Although they are believed to need a very small exposure to chilling temperatures to resume growth and flower normally, incremental exposure to chilling temperatures reduces the time to respond to warm temperatures and increases the percentage of budburst, which suggests some sensitivity to chilling (Dokoozlian, 1999). In the present study, grapevines do not show a major change in budburst date in response to changes in temperature during the chilling period. Instead, temperature during the warming period was the overriding factor influencing this phenophase (Fig. 2). The high variable importance in the projection (VIP) values and negative coefficients for heat accumulation observed at the beginning of March highlight the importance of taking into account this period for the prediction of budburst and flowering. These results support previous studies that find best model performance for models using the March 1st as a start date for the accumulation of thermal time, instead of the classical approach of taking into account temperatures from January 1st (Garcia de Cortazar-Atauri et al., 2009; Duchene et al., 2010; Parker et al., 2011).

For later phenological events, such as onset of ripening, thermal time models have proven to be valuable tools. However, as the growing season goes on, the level of complexity increases and factors such as yield, cultural practices and water availability may also influence the timing of phenophases (Petrie and Sadras, 2008; Sadras and Petrie, 2011; Martínez de Toda et al., 2013). Simply, the ripening of non-climacteric fruits relies to a great extent on photoassimilation in the leaves, translocation and storage of photoassimilates, which are reactions greatly enhanced by temperature (Greer and Weedon, 2013). Historical data also support the relationship between temperature and commercial ripeness in grapes (Chuine et al., 2004; Daux et al., 2012). In the

historical data used in this study, the effect of temperature on the time between flowering and harvest was visible, but – as expected – the correlation was weaker than for the completion of previous stages, such as budburst or flowering. This advancement of the ripening period shifting dates from September to August in the northern hemisphere may have an additional effect by shifting this period to what is usually the warmest part of the year (Webb et al., 2007; Duchene et al., 2010). In this study, this effect resulted in an increase of the temperature during the ripening period by 1.27 °C for each °C increased in the average temperature (Figure S1).

4.2 Effect of water availability in relation to temperature

Our studies show differences in the effects of rainfall depending on the phenological period. Whereas events occurring before berry development do not show a clear dependence on rainfall, berry development shows a higher dependence both on temperature and water availability (Fig 4). Mild water deficit has proven to enhance ripening through several processes, such as altering plant abscisic acid signaling, reduction in berry size or concentrating berry contents (i.e. anthocyanins and sugars) (Deluc et al., 2009; van Leeuwen et al., 2009; Chaves et al., 2010). In fact, environmental cues such as water deficit, as well as solar radiation, and even heat within the suboptimal range of temperature of a variety, may promote signaling mechanisms, such as abscisic acid biosynthesis, and enhance ripening (Kuhn et al., 2014). Thus, despite hastening fruit ripening, mild water deficit normally has a desirable effect, increasing the concentration of some phenolic compounds in the grapes (Chaves et al., 2010). However, as climate change continues, places experiencing a risk of severe water deficit may encounter contrasting effects. Severe water deficit can induce stomatal closure, greatly reduce carbon fixation, and subsequently, impair berry ripening (Martínez-Lüscher et al., 2015a). This may explain the results obtained with fruit-bearing cuttings, where a water deficit appeared to generally delay maturity. In addition, water availability had significant interactions with both temperature and CO₂ concentration. For conditions such as ambient temperature and elevated CO₂, where plants were presumably less stressed, the general tendency of cyclic drought to delay ripening was reverted. Contrasting effects of water deficit have also been reported by Cook and Wolkovich (2016) in large scale field data analyses, where precipitation correlates positively with harvest date anomalies in France, while they correlate negatively in the dryer vineyards of Spain. Cook and Wolkovich (2016) highlight that

the relationship between water deficit and early harvest has weakened in recent decades in central Europe due to the decoupling of the incidence of high temperatures and drought.

4.3 Effect of CO₂ concentration in relation to temperature

Grapevine fruit-bearing cuttings grown under semi-controlled conditions showed similar sensitivities to increasing temperatures compared to field grown vines, although sensitivities were lower in the warmer year, 2015, and for the early cultivar, Red Tempranillo. The change induced by elevated CO₂ followed similar variation, but this effect was greater than the effect of the 4°C increase. In recent studies with grapevine fruit-bearing cuttings, a correlation between carbon fixation rates and grape development rates has been reported (Martínez-Lüscher et al., 2015b). This behavior has been described under field conditions as well, where leaf removal treatments, which presumably reduce overall grapevine carbon fixation, resulted in a delay in grape maturity (Martínez de Toda et al., 2013; Parker et al., 2014; 2015). Plants exposed to elevated CO₂ often show photosynthetic acclimation, which is characterized by an initial increase in carbon fixation rates and reduction to initial levels or even lower after a mid-term exposure (Leakey et al., 2009). However, this is not a generalized response and, even if this were the case, it would not mean that plant performance and fruit yield would be strongly affected (Idso and Kimball, 1991; Leakey et al., 2009). One of the greatest efforts to study the effect of elevated CO₂ on fruiting woody perennials is a 17-year experiment on sour orange trees (*Citrus aurantium* L.) with open-top-chambers, which showed a constant increase in yield despite photosynthetic acclimation (Kimball et al., 2007). To the best of our knowledge, a paper by Bindi et al. (2001) is the only FACE experiment study in the literature, where an increase in sugar accumulation in the grapes was reported. Although this effect was diluted in the latest stages of ripening, this is evidence for accelerated ripening in grapes grown under elevated CO₂. In previous reports, Salazar Parra et al. (2010) and Martínez-Lüscher et al. (2016) showed how grapes grown under both elevated CO₂ and increased temperature met the sugar criteria for harvest much earlier, and this led to a decrease in anthocyanin concentration. In contrast, Kizildeniz et al. (2015), who studies the interaction between elevated CO₂ and increasing temperature, reported that elevated CO₂ showed mitigating effects, generally increasing anthocyanin concentration and increasing either grape organic acid concentration or decreasing grape pH, probably related to the precocity of these

treatments. Thus, due to its complexity, the relationship between different environmental factors and grape composition should be assessed with caution, as phenology-mediated effects are likely.

Martínez de Toda and Balda Manzanos (2013) and Martínez de Toda et al. (2013) showed that cultural practices, such as canopy density reduction, can have an opposite effect to that resulting from high temperature, reducing TSS concentration for a fixed date. However, in that case, this contributed to restore grape pH, but reduced anthocyanin concentration. Interestingly, Martínez de Toda et al. (2014), in a similar experiment but harvesting grapes at a designated TSS concentration, found that delayed phenological development contributed to increasing anthocyanin concentration. These findings suggest that carbon translocation into the grapes can be controlled, thus altering the timing of phenological stages, which can contribute to mitigating the effects of climate change.

Conclusions

The results of the present study suggest that temperature, water deficit and CO₂ levels representative of the conditions expected for the end of the 21st century may strongly advance budburst, flowering, and berry designated maturity. Some uncertainties still exist, such as whether the delaying effect of severe water deficit or the decreasing sensitivity under extreme temperature, which have been observed both in fruit-bearing cuttings in the present study and in other field studies, will be generally observable in vineyards in the future. Future efforts should be directed to investigating how grapevine whole-plant physiology may be altered in response to climate change-related factors, and to uncoupling the effects of environmental factors such as temperature, water deficit and CO₂, from their potential effects on berry phenology. These are crucial topics to establish successful mitigation and adaptive strategies for viticulture in a changing environment.

Author's contribution

J.M.L. performed the analysis and elaborated the draft of the manuscript. Z.D. and E.L. designed the analysis of the field data. I.P., J.J.I. and F.M. designed the TGG experiments, T.K. performed the TGG experiments. J.M.L., T.K., Z.D., E.L., C.v.L.,

E.G., I.P., J.J.I., F.M. and S.D. contributed the interpretation of the results and elaboration of the final manuscript.

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Table 1. Temperature recorded in the temperature gradient greenhouse experiments with Red and White Tempranillo grapevine. T, ambient temperature and T+4°C, ambient temperature +4°C.

| Year | 2014 | | 2015 | |
|-------------------------|------|-------|------|-------|
| | T | T+4°C | T | T+4°C |
| Mean daily minimum (°C) | 14.8 | 18.8 | 15.4 | 19.65 |
| Daily mean (°C) | 22.1 | 26.5 | 22.9 | 27.1 |
| Mean daily maximum (°C) | 28.7 | 33.5 | 29.7 | 35.11 |
| Days above 30°C | 46 | 85 | 44 | 77 |
| Days above 35°C | 8 | 42 | 19 | 50 |

Table 2. Days to complete phenological periods: fruit set to veraison, veraison to designated maturity and fruit set to designated maturity in Red and White Tempranillo grapevine grown under two water availability levels (FI, full irrigation or CD, cyclic drought), two temperature regimes (ambient temperature, T or ambient temperature +4°C, T+4°C) and two CO₂ concentrations (A CO₂, ambient CO₂, or E CO₂, 700 µL L⁻¹ CO₂). Values are elapsed time (days) mean ± SE, n= 8-10. Cult, Cultivar; Temp, Temperature; WA, water availability and n.s., not significant.

| Cultivar | Water availability | Temperature | CO ₂ level | Years | Fruit set to veraison | Veraison to maturity | Fruit set to maturity | | | | | | |
|-----------------------|--------------------|-------------|-----------------------|------------|--------------------------|----------------------|-----------------------|------------|--|---------|--------|--------|--------|
| Red | FI | T | A CO ₂ | 2014 | 64.1 ± 1.5 | 29.8 ± 3.4 | 93.9 ± 3.0 | | | | | | |
| | | | | 2015 | 53.0 ± 1.1 | 25.6 ± 2.3 | 78.6 ± 2.5 | | | | | | |
| | | | E CO ₂ | 2014 | 57.9 ± 1.3 | 31.5 ± 1.9 | 89.4 ± 2.5 | | | | | | |
| | | 2015 | | 51.6 ± 1.1 | 27.0 ± 1.8 | 79.1 ± 1.8 | | | | | | | |
| | | T+4 | A CO ₂ | 2014 | 56.2 ± 1.1 | 25.3 ± 1.3 | 81.5 ± 1.8 | | | | | | |
| | | | | 2015 | 54.2 ± 1.5 | 20.8 ± 2.3 | 75.0 ± 1.1 | | | | | | |
| | E CO ₂ | | 2014 | 54.9 ± 1.5 | 27.1 ± 2.2 | 82.0 ± 3.6 | | | | | | | |
| | | 2015 | 51.1 ± 1.0 | 24.5 ± 1.4 | 75.6 ± 1.7 | | | | | | | | |
| | CD | T | A CO ₂ | 2014 | 68.7 ± 2.3 | 27.8 ± 2.2 | 95.4 ± 2.0 | | | | | | |
| | | | | 2015 | 48.7 ± 1.5 | 26.7 ± 1.3 | 75.4 ± 1.6 | | | | | | |
| | | | E CO ₂ | 2014 | 57.5 ± 1.7 | 37.3 ± 2.3 | 94.8 ± 1.7 | | | | | | |
| | | 2015 | | 51.4 ± 0.9 | 23.0 ± 1.4 | 74.3 ± 1.1 | | | | | | | |
| | | T+4 | A CO ₂ | 2014 | 66.5 ± 2.2 | 28.5 ± 1.8 | 95.0 ± 3.3 | | | | | | |
| | | | | 2015 | 54.8 ± 1.7 | 25.7 ± 2.1 | 80.5 ± 1.5 | | | | | | |
| | E CO ₂ | | 2014 | 57.0 ± 1.4 | 31.5 ± 2.4 | 88.5 ± 2.8 | | | | | | | |
| | | 2015 | 50.2 ± 1.4 | 27.8 ± 1.7 | 78.0 ± 0.9 | | | | | | | | |
| White | FI | T | A CO ₂ | 2014 | 66.5 ± 2.4 | 40.1 ± 2.0 | 106.6 ± 3.0 | | | | | | |
| | | | | 2015 | 50.4 ± 0.7 | 39.4 ± 3.5 | 90.0 ± 3.7 | | | | | | |
| | | | E CO ₂ | 2014 | 56.9 ± 0.6 | 47.5 ± 3.0 | 104.4 ± 3.4 | | | | | | |
| | | 2015 | | 49.5 ± 0.9 | 41.2 ± 3.6 | 90.7 ± 3.7 | | | | | | | |
| | | T+4 | A CO ₂ | 2014 | 58.9 ± 1.3 | 31.8 ± 1.6 | 90.7 ± 0.9 | | | | | | |
| | | | | 2015 | 51.5 ± 1.6 | 40.7 ± 4.3 | 92.2 ± 3.3 | | | | | | |
| | E CO ₂ | | 2014 | 55.0 ± 0.5 | 30.3 ± 1.0 | 85.3 ± 1.1 | | | | | | | |
| | | 2015 | 51.5 ± 1.4 | 33.1 ± 2.1 | 84.6 ± 2.8 | | | | | | | | |
| | CD | T | A CO ₂ | 2014 | 62.9 ± 2.1 | 50.4 ± 3.2 | 111.1 ± 3.3 | | | | | | |
| | | | | 2015 | 49.6 ± 0.8 | 49.2 ± 2.6 | 98.9 ± 2.6 | | | | | | |
| | | | E CO ₂ | 2014 | 59.6 ± 1.1 | 37.0 ± 4.2 | 93.3 ± 5.1 | | | | | | |
| | | 2015 | | 48.3 ± 0.5 | 33.6 ± 3.0 | 81.9 ± 2.9 | | | | | | | |
| | | T+4 | A CO ₂ | 2014 | 60.9 ± 0.9 | 48.1 ± 2.5 | 109.0 ± 2.5 | | | | | | |
| | | | | 2015 | 48.8 ± 0.6 | 38.3 ± 3.2 | 87.1 ± 2.8 | | | | | | |
| | E CO ₂ | | 2014 | 58.3 ± 1.2 | 40.4 ± 3.4 | 98.7 ± 3.6 | | | | | | | |
| | | 2015 | 48.1 ± 0.5 | 37.4 ± 3.7 | 85.5 ± 3.5 | | | | | | | | |
| Means of main factors | | | | | Red Tempranillo | 56.1 ± 0.6 | 27.5 ± 0.6 | 83.6 ± 0.8 | | | | | |
| | | | | | White Tempranillo | 54.8 ± 0.5 | 39.9 ± 0.9 | 94.4 ± 1.0 | | | | | |
| | | | | | Full irrigation | 55.2 ± 0.5 | 32.2 ± 0.8 | 87.5 ± 0.9 | | | | | |
| | | | | | Water deficit | 55.7 ± 0.6 | 35.2 ± 0.9 | 90.5 ± 1.1 | | | | | |
| | | | | | Ambient temperature | 56.0 ± 0.6 | 35.4 ± 0.9 | 91.1 ± 1.1 | | | | | |
| | | | | | Elevated temperature | 54.9 ± 0.5 | 32.0 ± 0.8 | 86.8 ± 0.9 | | | | | |
| | | | | | Ambient CO ₂ | 57.2 ± 0.6 | 34.3 ± 0.9 | 91.3 ± 1.1 | | | | | |
| | | | | | Elevated CO ₂ | 53.7 ± 0.4 | 33.1 ± 0.8 | 86.6 ± 0.9 | | | | | |
| | | | | | 2014 | 60.1 ± 0.5 | 35.3 ± 0.8 | 95.0 ± 1.0 | | | | | |
| | | | | | 2015 | 50.8 ± 0.3 | 32.1 ± 0.9 | 83.0 ± 0.8 | | | | | |
| | | | | | ANOVA P of main factors | | | | | P(Cult) | <0.001 | <0.001 | <0.001 |
| | | | | | | | | | | P(WA) | n.s. | 0.005 | 0.01 |
| | | | | | P(Temp) | 0.014 | <0.001 | <0.001 | | | | | |

| | | | | |
|-----------------------------------|------------------------------------|--------|--------|---------|
| | P(CO₂) | <0.001 | n.s. | <0.001 |
| | P(Year) | <0.001 | 0.001 | <0.001 |
| ANOVA 2 level interactions | P(Cult) x P(WA) | n.s. | n.s. | n.s. |
| | P(Cult) x P(Temp) | n.s. | n.s. | n.s. |
| | P(Cult) x P(CO₂) | n.s. | <0.001 | 0.004 |
| | P(Cult) x P(Year) | n.s. | n.s. | n.s. |
| | P(WA) x P(Temp) | n.s. | 0.007 | < 0.001 |
| | P(WA) x P(CO₂) | n.s. | 0.028 | 0.014 |
| | P(WA) x P(Year) | <0.001 | n.s. | < 0.001 |
| | P(Temp) x P(CO₂) | n.s. | n.s. | n.s. |
| | P(Temp) x P(Year) | <0.001 | n.s. | 0.002 |
| | P(CO₂) x P(Year) | <0.001 | n.s. | n.s. |

Figure legends

Figure 1. Results of Partial Least Squares (PLS) regression analysis for grapevine budburst dates in Mandicevac (Croatia), using the Dynamic Model and the GDH Model for quantifying chill and heat accumulation, respectively. Color bars in the figures indicate VIP above 0.8, the threshold for considering variables important. Green and red bars represent, besides importance, a positive and negative relationship, respectively, between budburst and daily chilling and heat accumulation. In the lower graphs (chill accumulation and heat accumulation), bars represent the standard deviation of daily chill and heat accumulation, with colors following the same pattern as for the variable importance in the projection (VIP) and model coefficients. Blue, red and grey shaded areas and dashed lines represent the designated chilling and warming period, range in budburst dates and average budburst date, respectively. GDH, Growing Degree Hours.

Figure 2. Response of the budburst date of grapevine to average temperatures during the chilling and warming periods (December 5th to February 26th and January 22nd to May 1st, respectively). Colours and contour lines represent predicted flowering dates expressed in Julian dates (days of the year) using observed data points of grapevine budburst dates in Mandicevac (Croatia; black dots).

Figure 3. Time from the March 1st to budburst (**A**), budburst to flowering (**B**) and flowering to ripe fruit stage (**C**) versus mean temperature between that period for each year and place. Data from 9 plantings in Daruvar, Cepic, Hvar, Kricevzi, Mandicevac and Trsteno (Croatia).

Figure 4. Response of the length of the periods March 1st to budburst (**A**), budburst to flowering (**B**) and flowering to ripe fruit (**C**) to mean temperature and mean rainfall for each planting and year. Colours and contour lines represent predicted deviation from the average length of each period expressed in days using observed data points of the length of each phenophase (black dots).

Figure 5. Days to complete fruit development (from fruit set to designated maturity) versus mean temperature between the phenophases in Red and White Tempranillo grapevine grown under two CO₂ concentrations (ambient CO₂ or 700 μL L⁻¹ CO₂; white and black points and bars, respectively) and two temperature regimes (ambient temperature, T or ambient temperature +4°C, T+4°C). Points and bars represent means ±

standard error (n = 16-20). P values based on four-way ANOVA taking as main factors: cultivar, temperature, CO₂ concentration and water availability.

Figure 1. Martínez-Lüscher et al. (2016)

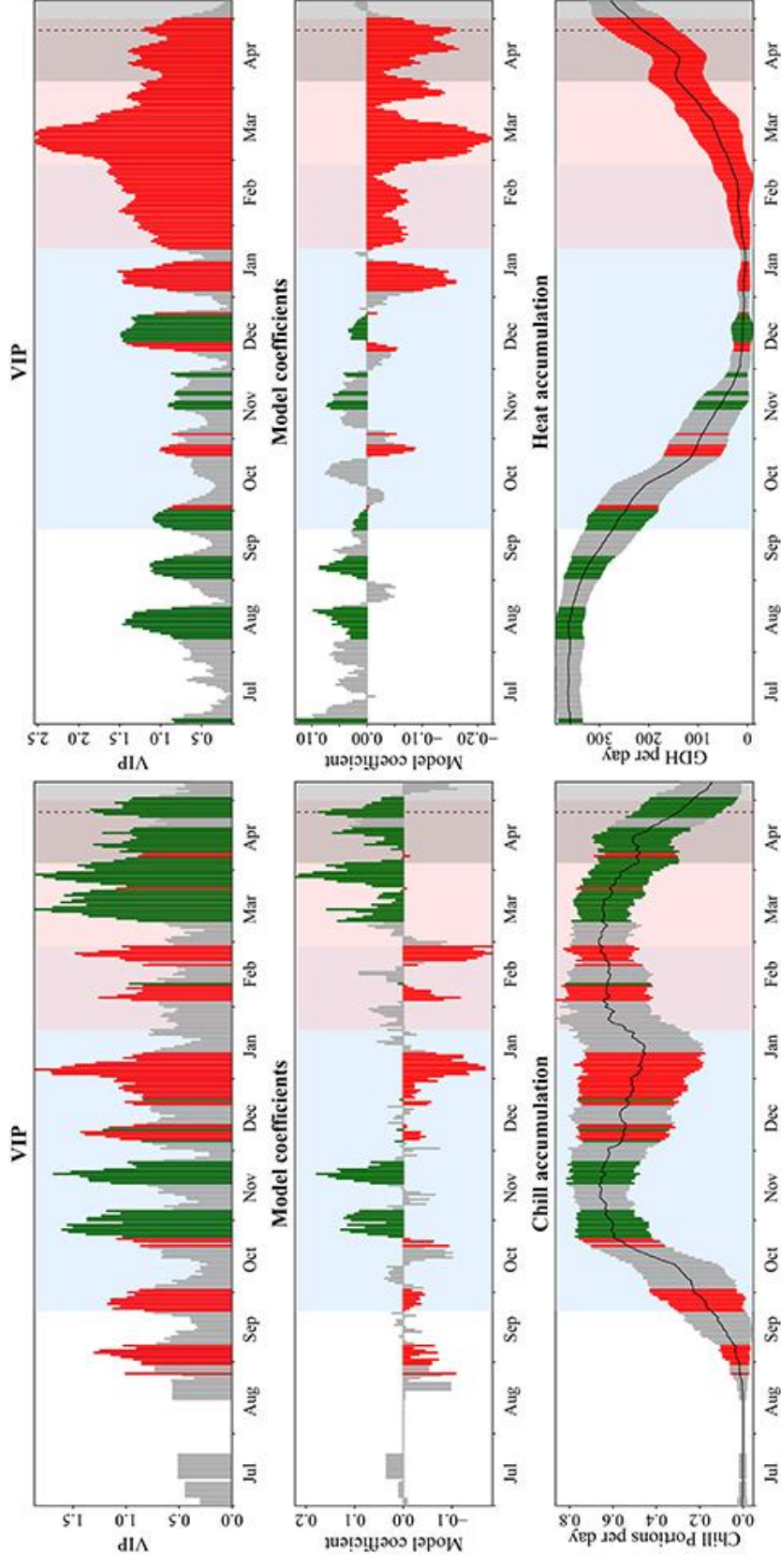


Figure 2. Martínez-Lüscher et al. (2016)

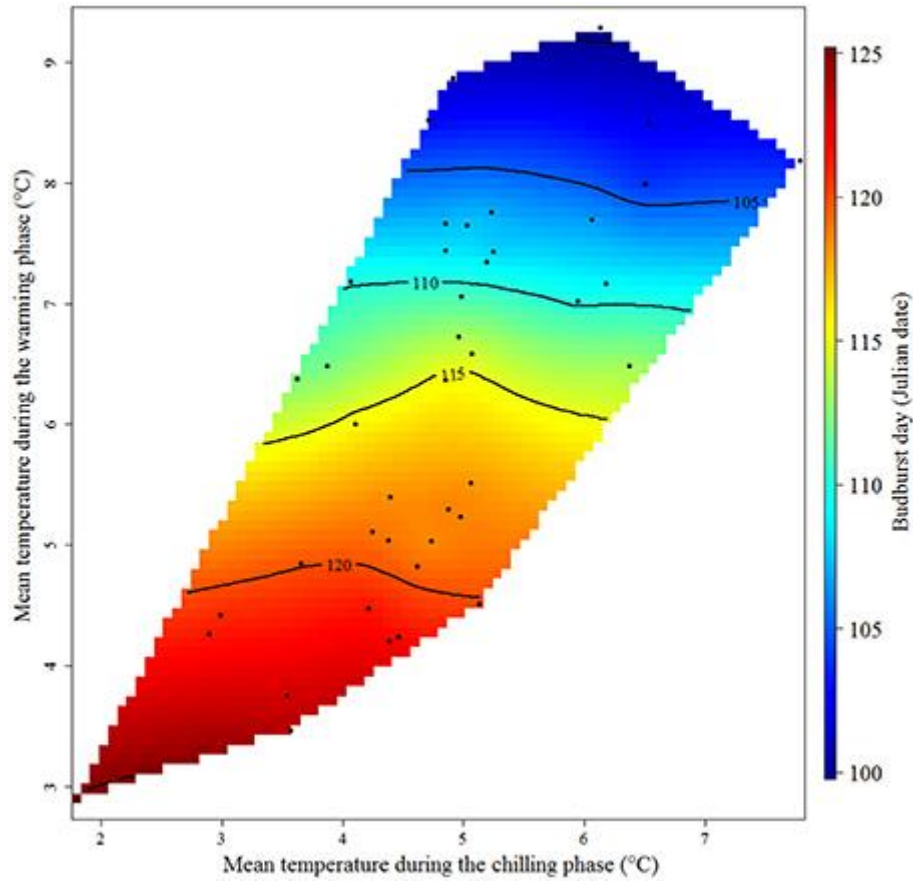


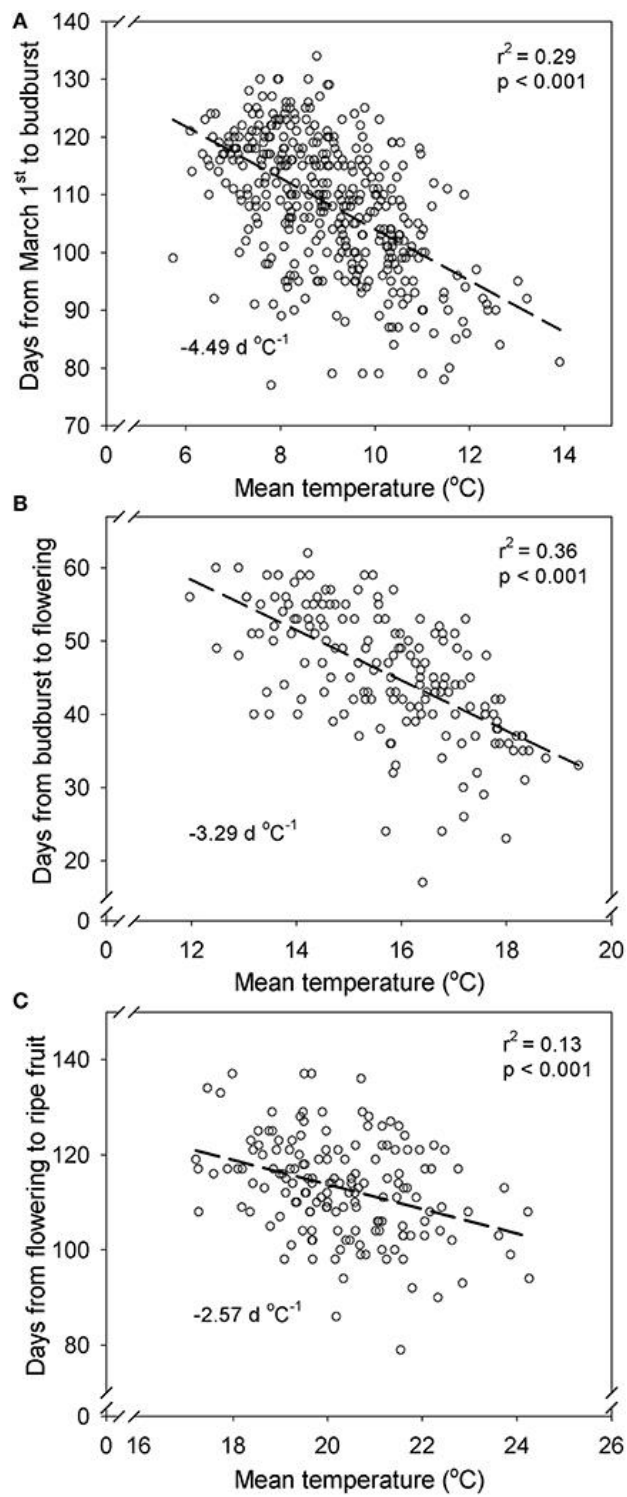
Figure 3. Martínez-Lüscher et al. (2016)

Figure 4. Martínez-Lüscher et al. (2016)

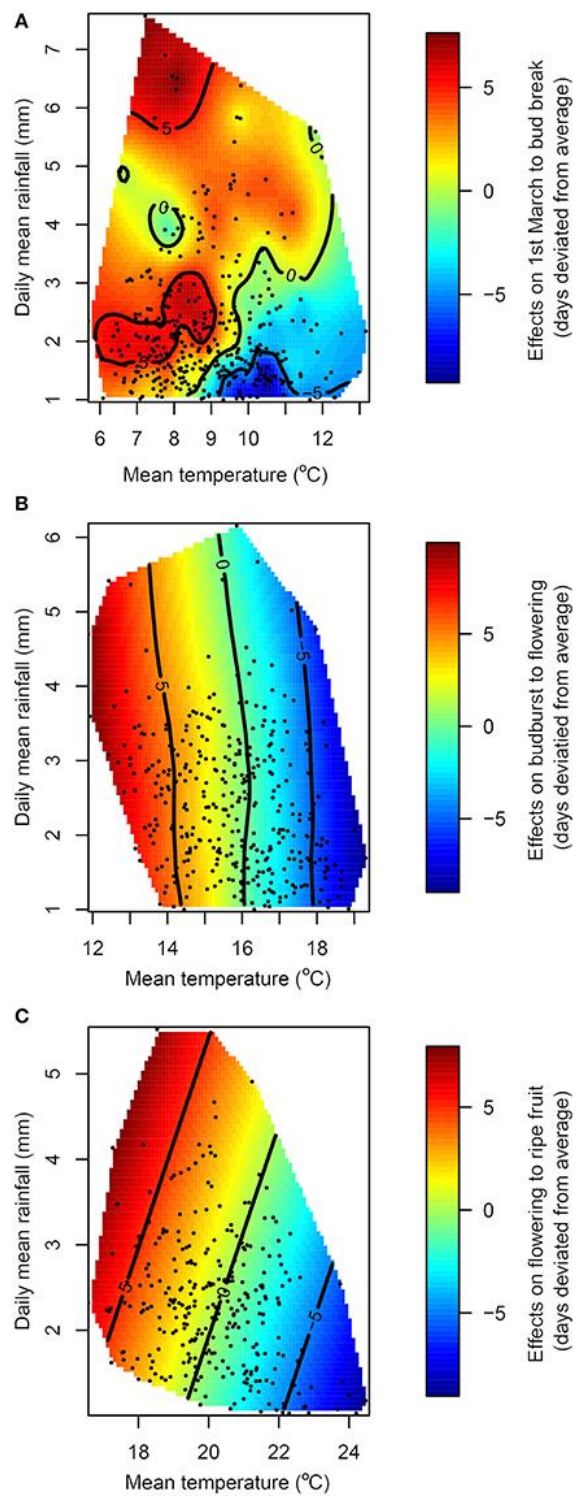
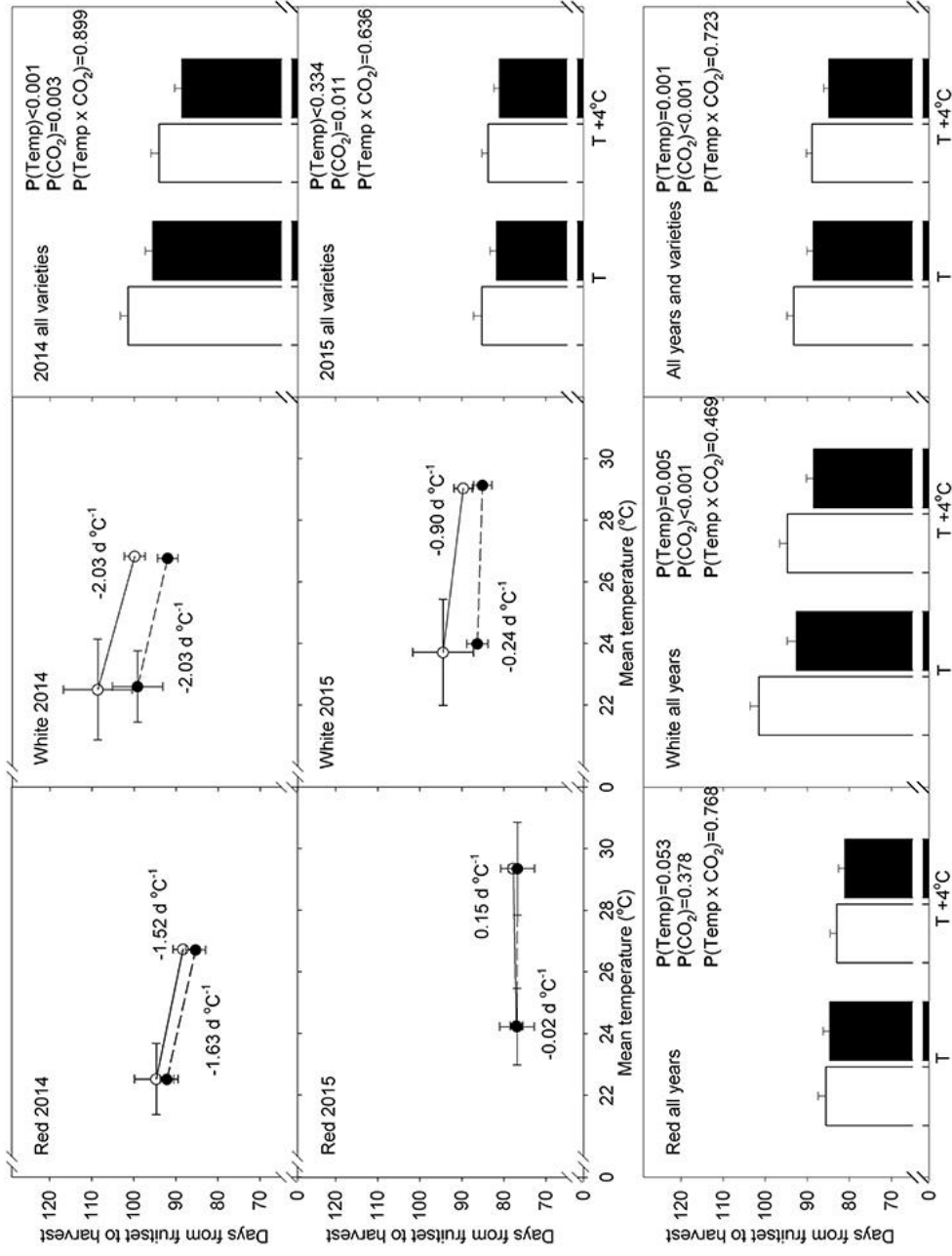


Figure 5. Martínez-Lüscher et al. (2016)



Supplementary material

Figure S1: Season mean temperature from (November 1st November to October 31st October) against versus mean temperature from veraison to harvest. Data obtained from PEP725 database from 7 plantings in Croatia. Dash line is linear regression with the data ($y = 1.27 * x + 3.26$) and continuous line is an adjusted line $y = a * x + b$ where $a=1$ and $b=5.75$, only for comparison purposes.

