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# Response of Tempranillo (*Vitis vinifera* L.) clones to climate change-related factors (elevated temperature, high CO<sub>2</sub>, and water deficit): plant performance and berry composition

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THÈSE EN COTUTELLE PRÉSENTÉE

POUR OBTENIR LE GRADE DE

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SPÉCIALITÉ BIOLOGIE VÉGÉTALE

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**Réponse de clones de Tempranillo (*Vitis vinifera* L.) à des  
facteurs de l'environnement liés au changement climatique  
(température élevée, haut niveau de CO<sub>2</sub> et déficit en eau) :  
réponse physiologique de la plante et composition de la baie**

Sous la direction de Ghislaine HILBERT  
et de Inmaculada PASCUAL

Soutenue le 18 de Décembre de 2019

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# Réponse de clones de Tempranillo (*Vitis vinifera* L.) à des facteurs de l'environnement liés au changement climatique (température élevée, haut niveau de CO<sub>2</sub> et déficit en eau) : réponse physiologique de la plante et composition de la baie

## RESUMÉ

Le changement climatique devrait modifier les conditions environnementales dans le future, affectant ainsi l'agriculture. Le Tempranillo, une variété de vigne rouge (*Vitis vinifera* L.) largement cultivée au niveau international, pourrait être affecté par l'augmentation des températures moyennes mondiales et des niveaux de CO<sub>2</sub> dans l'atmosphère, ainsi que par la diminution de la disponibilité en eau sur sa zone traditionnelle de culture. L'utilisation de la diversité intra-variétale a été proposée comme une stratégie pour essayer de conserver la typicité du vin et les variétés régionales dans les conditions de culture futures, en déplaçant la phase de maturation vers des périodes aux conditions environnementales plus favorables. L'objectif de cette thèse était donc de déterminer la réponse de différents clones de Tempranillo aux conditions environnementales simulées de 2100, en se concentrant sur la croissance et le développement des plantes, ainsi que sur la composition des baies. Des boutures fructifères de clones de Tempranillo, dont la longueur du cycle de reproduction était différente, ont été exposées à différents scénarios climatiques dans des serres à gradient de température (TGG) et des serres de chambre de croissance (GCG) depuis la fructification jusqu'à la maturité. Les impacts de la température élevée (+4 °C), du CO<sub>2</sub> élevé (700 ppm) et du déficit en eau, combinés ou non, ont été évalués. Les résultats montrent une augmentation de la croissance végétative et une réduction de la production dues aux températures élevées. La concentration élevée de CO<sub>2</sub> a également augmenté la croissance végétative et l'activité photosynthétique. Néanmoins, un processus d'acclimatation a été observé, celui-ci étant plus fort lorsqu'un haut niveau de CO<sub>2</sub> est combiné à une température élevée. Le déficit en eau a fortement réduit l'activité photosynthétique et la croissance végétative, occultant les effets de la température et du CO<sub>2</sub>. La température élevée, que ce soit individuellement ou associée à des niveaux élevés de CO<sub>2</sub>, a accéléré l'accumulation de sucres et la date de maturité a été avancée, mais ces effets ont été atténués par le déficit en eau. La dégradation de l'acide malique a également été favorisée par l'augmentation de la température, en particulier lorsque cette dernière est associée à une concentration élevée de CO<sub>2</sub> et à un déficit en eau. La concentration et le profil des acides aminés ont été influencés par les températures élevées, un niveau de CO<sub>2</sub> élevé et, en particulier, par un déficit en eau. L'augmentation de CO<sub>2</sub> a réduit l'effet de la température sur le découplage de l'accumulation des anthocyanes par rapport à celle des sucres ; cependant, la



combinaison d'une température élevée, d'un haut niveau de CO<sub>2</sub> et d'un déficit en eau a conduit à un déséquilibre entre ces deux composés du raisin. Le profil des anthocyanes a été modifié par le changement climatique, une température élevée augmentant la proportion des formes acylées tandis qu'un haut niveau de CO<sub>2</sub> et un déficit hydrique ont favorisé quant à eux l'abondance relative de la malvidine, et des formes acylées, méthylées et trihydroxylées. Les clones étudiés ont montré des différences dans leur développement phénologique, leur croissance végétative et reproductive, ainsi que dans la composition de leurs raisins. En outre, les résultats révèlent l'existence d'une réponse différentielle des clones de Tempranillo aux conditions environnementales prévues pour 2100 en termes de performance de la plante et de composition du raisin. De façon générale, parmi les clones étudiés, RJ43 fut le plus affecté par les conditions de croissance futures (températures élevées, haut niveau de CO<sub>2</sub> et déficit en eau) aussi bien en termes de développement phénologique qu'en termes de concentration en anthocyanes et de leur profil. A l'inverse, VN31 a maintenu la plus haute teneur en anthocyanes et le ratio anthocyanes sur TSS le plus élevé tandis que 1084 a montré les teneurs les plus faibles en sucres, acide malique et en anthocyanes et le ratio anthocyanes sur TSS le plus bas. Les différences observées dans la réponse des clones au changement climatique ne dépendent pas toujours de la longueur du cycle de reproduction.

**Mots-clés** : Changement climatique; Vigne; Tempranillo; Clones; Diversité intra variétale; Développement végétatif; Composition de la baie; Profil en anthocyanes.

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## **Response of Tempranillo (*Vitis vinifera* L.) clones to climate change-related factors (elevated temperature, high CO<sub>2</sub>, and water deficit): plant performance and berry composition**

### **SUMMARY**

Climate change is expected to modify future environmental conditions, therefore affecting agriculture. Tempranillo, a largely cultivated worldwide grapevine (*Vitis vinifera* L.) red variety, will be affected by the increase of global mean temperature and atmospheric CO<sub>2</sub> levels and the decrease of water availability in its cultivation area. The use of the intra-varietal diversity has been proposed as a strategy for keeping wine typicity and regional varieties cultivation under future growing conditions by shifting the ripening phase to more favourable environmental conditions. The aim of the thesis was to determine the response of different clones of Tempranillo to simulated 2100 environmental conditions, focusing on plant growth and

development, as well as on berry composition. Fruit-bearing cuttings of Tempranillo clones, which differed in the length of their reproductive cycle, were exposed from fruit set to maturity to different scenarios of climate change in temperature gradient greenhouses (TGG) and growth chamber greenhouses (GCG). The impact of elevated temperature (+4 °C), elevated CO<sub>2</sub> (700 ppm) and water deficit, both in combination or independently, were evaluated. The results show an increment of vegetative growth and a reduction of yield due to high temperatures. Elevated CO<sub>2</sub> concentration also increased vegetative growth and photosynthetic activity, even though an acclimation process was observed, being stronger when combined with high temperature. Water deficit reduced severely the photosynthetic activity and vegetative growth, overshadowing the temperature and CO<sub>2</sub> effects. Elevated temperature, both individually and combined with high CO<sub>2</sub> levels, hastened sugar accumulation and advanced maturity, but these effects were mitigated by water deficit. Malic acid degradation was also enhanced by high temperature, especially when combined with elevated CO<sub>2</sub> and water deficit. Amino acid concentration and profile were affected by high temperature, elevated atmospheric CO<sub>2</sub> and, especially, water deficit. Elevated CO<sub>2</sub> reduced the effect of temperature decoupling the anthocyanin and TSS accumulation; however, the combination of elevated temperature, high CO<sub>2</sub> and water deficit led to the imbalance between these two grape components. Anthocyanin profile was modified by climate change, high temperature increasing the relative abundance of acylated forms and both elevated CO<sub>2</sub> and drought favouring the relative content of malvidin and acylated, methylated and tri-hydroxylated forms. The clones studied showed differences in their phenological development, vegetative and reproductive growth, as well as in their grape composition. In addition, the results reveal the existence of a differential response of Tempranillo clones to the environmental conditions projected for 2100 in relation to plant performance and grape composition. In general, RJ43 was the most affected by the future growing conditions (high temperature, elevated CO<sub>2</sub> and water deficit) among the clones studied in terms of phenology and anthocyanin concentration and profile. Conversely, VN31 maintained the highest anthocyanin and anthocyanin:TSS ratio, whereas 1084 had the lowest sugar, malic acid and anthocyanin levels. The differences observed in the response of the clones to climate change not always depended on their reproductive cycle length.

**Keywords:** Climate change; Grapevine; Tempranillo; Clones; Intra-varietal diversity; Vegetative development; Grape composition; Anthocyanin profile.

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# **Respuesta de clones de Tempranillo (*Vitis vinifera* L.) a factores relacionados con el cambio climático (temperatura elevada, CO<sub>2</sub> elevado y déficit hídrico): fisiología de la planta y composición de la baya**

## **RESUMEN**

Se espera que el cambio climático modifique las condiciones ambientales futuras, afectando a la agricultura. Tempranillo, una variedad tinta de vid (*Vitis vinifera* L.) cultivada en todo el mundo, se verá afectada por el aumento de la temperatura media global y la concentración atmosférica de CO<sub>2</sub> así como por la disminución del agua disponible en el área de cultivo de esta variedad. Entre las estrategias para mitigar el impacto del cambio climático sobre las características del vino y mantener en el futuro el cultivo de variedades ligadas a una región determinada, se encuentra el uso de la diversidad intravarietal para trasladar el momento de maduración a épocas con condiciones ambientales más favorables. El objetivo de esta tesis fue determinar la respuesta de distintos clones de Tempranillo a las condiciones ambientales previstas para 2100, centrándonos en el crecimiento y desarrollo de las plantas, así como en la composición de las bayas. Para ello, se utilizaron esquejes fructíferos de clones de Tempranillo caracterizados con distinta duración del ciclo reproductivo, siendo expuestos a distintas condiciones ambientales en túneles de gradiente térmico (*Temperature gradient greenhouses*, TGG) e invernaderos cámara (*Growth chamber greenhouses*, GCG) desde cuajado a madurez. Se evaluó el impacto del aumento de la temperatura (+4 °C), del CO<sub>2</sub> elevado (700 ppm) y del déficit hídrico, tanto de forma individual como combinada. Los resultados muestran un aumento del crecimiento vegetativo y una reducción de la producción de fruto debido a las altas temperaturas. El CO<sub>2</sub> elevado también aumentó el crecimiento vegetativo y la actividad fotosintética, aunque se observó un proceso de aclimatación, siendo más intenso en combinación con altas temperaturas. La sequía redujo drásticamente la actividad fotosintética y el crecimiento vegetativo, anulando los efectos de la temperatura y del CO<sub>2</sub>. La temperatura elevada, tanto de forma individual como combinada con altos niveles de CO<sub>2</sub>, aceleró la acumulación de azúcares y adelantó la madurez, siendo estos efectos mitigados por el déficit hídrico. La degradación del ácido málico también se incrementó con el aumento de la temperatura, especialmente en combinación con alto CO<sub>2</sub> y sequía. La concentración y el perfil de aminoácidos se vieron afectados por el aumento de la temperatura, por el alto CO<sub>2</sub> y, especialmente, por el déficit hídrico. El CO<sub>2</sub> elevado disminuyó el desacoplamiento en la acumulación de antocianinas y azúcares inducido por la temperatura elevada; sin embargo, la

combinación de temperatura elevada, alto CO<sub>2</sub> y sequía provocó el desequilibrio entre estos compuestos. Las condiciones de cambio climático alteraron el perfil de antocianinas; así, la temperatura elevada aumentó la abundancia relativa de formas aciladas y, tanto el CO<sub>2</sub> elevado como la sequía, favorecieron la acumulación de malvidina y de formas aciladas, metiladas y trihidroxiladas. Los clones estudiados mostraron diferencias en su desarrollo fenológico, crecimiento vegetativo y reproductivo, así como en la composición de la baya. Además, los resultados revelan la existencia de una respuesta diferente de los clones de Tempranillo estudiados a las condiciones ambientales proyectadas para 2100 en términos de crecimiento y desarrollo de la planta, como de composición de la uva. En términos generales, el clon más afectado por las condiciones ambientales que se esperan para finales del presente siglo (alta temperatura, alto CO<sub>2</sub> y déficit hídrico) fue el RJ43 en cuanto a su desarrollo fenológico, la concentración de antocianinas y la relación antocianinas:azúcares. Además, bajo dichas condiciones, VN31 presentó la concentración de antocianinas y la relación antocianinas:azúcares más alta, mientras que las bayas de 1084 presentaron los niveles más bajos de azúcares, ácido málico y antocianinas. Las diferencias en la respuesta de los clones al cambio climático no siempre dependieron de la duración de su ciclo reproductivo.

**Palabras clave:** Vid; Cambio climático; Tempranillo; Clones; Diversidad intravarietal; Desarrollo vegetativo; Azúcares; Antocianinas; Ácido málico; Aminoácidos.

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# **Réponse de clones de Tempranillo (*Vitis vinifera* L.) à des facteurs de l'environnement liés au changement climatique (température élevée, haut niveau de CO<sub>2</sub> et déficit en eau) : réponse physiologique de la plante et composition de la baie**

## **RESUMÉ ÉTENDU**

Les conditions environnementales à l'avenir devraient être modifiées dans le contexte du changement climatique, notamment par la hausse de la température moyenne mondiale et de la concentration de CO<sub>2</sub> dans l'air ainsi que par les fluctuations de la périodicité et de l'abondance des précipitations. Ces variations des conditions environnementales devraient affecter dans une large mesure l'agriculture et, en particulier, la culture de la vigne (*Vitis vinifera* L.) qui est une culture importante en raison de sa croissance mondiale mais aussi de sa valeur économique et culturelle. Parmi les différentes variétés, le Tempranillo est une variété de vigne à raisins rouges utilisée pour la production de vin et cultivée dans le monde entier. Elle est particulièrement présente dans les régions espagnoles de La Rioja et de Navarre. Dans ces régions, les précipitations devraient diminuer à la fin du siècle, ce qui de fait réduira la quantité d'eau disponible pour cette culture. Ces modifications des conditions environnementales pourraient également affecter le développement et la composition des baies de raisin impactant ainsi la qualité des vins produits et toucher toute la filière viti-vinicole. La plupart des recherches menées jusqu'à présent sur les effets du changement climatique sur la qualité de la baie se sont concentrées sur des facteurs uniques. Toutefois, certaines études examinent la combinaison de facteurs permettant de déterminer de quelle manière la production des vignes pourrait être affectée par le changement attendu des conditions environnementales au cours des prochaines années. En outre, différentes approches ont été proposées pour faire face aux éventuels changements non souhaités, notamment l'utilisation de la diversité génétique intra-variétale. Cette stratégie pourrait permettre de conserver la typicité du vin et les variétés régionales dans les conditions de croissance futures en déplaçant la phase de maturation vers des périodes aux conditions environnementales plus favorables à la production de raisins de qualité optimale.

Dans ce contexte, le but de cette thèse était de déterminer la réponse de clones de Tempranillo aux conditions environnementales simulées de 2100, en se concentrant d'une part sur la croissance et le développement des plantes, d'autre part sur la composition des baies. En effet, le potentiel des baies est un facteur important pour la qualité des vins qui en résultent. Ainsi, la concentration finale en sucre détermine le degré de sucrosité et le degré d'alcool des vins, tandis que l'acidité du moût influe sur la croissance et l'activité de la levure pendant la fermentation ainsi que sur l'acidité et la stabilité du vin. Les acides aminés quant à eux, jouent un rôle dans le

goût, l'arôme et l'apparence du vin et représentent la principale source d'azote lors de la fermentation de la levure, bien qu'ils puissent aussi être des précurseurs de composés malsains. Enfin, les anthocyanes jouent un rôle important dans la détermination des caractéristiques organoleptiques du vin et de ses effets bénéfiques pour la santé. La concentration et le profil en anthocyanes dans les baies de raisin diffèrent selon les variétés, en fonction également des conditions de croissance. De plus, le rapport anthocyanes / sucres est un trait sensoriel apprécié considéré dans la production de la vigne.

Cette étude a été conduite sur des boutures fructifères de clones de Tempranillo, caractérisés par des longueurs du cycle de reproduction différentes, qui ont été exposées de la fructification à maturité à différents scénarios de changement climatique au sein de serres à gradient de température (TGG) ou de chambres de croissance (GCG). L'impact de la température élevée (+4 ° C), du CO<sub>2</sub> élevé (700 ppm) et du déficit hydrique, combinés ou non, a été évalué à différents stades du développement des baies.

Les paramètres pris en compte en relation avec la physiologie des plantes comprenaient la croissance, la phénologie, la répartition du carbone, l'activité photosynthétique et la production finale de baies. En ce qui concerne la composition des baies, l'évolution au cours de la période de mûrissement de l'accumulation des composés d'intérêt pour la qualité tels que les sucres, les acides organiques et les acides aminés dans les moûts et les anthocyanes totales de la pellicule a été suivie. Les effets des conditions environnementales testées en relation avec les différents clones sur le ratio final anthocyanes / sucres ainsi que sur le profil en anthocyanes ont également été évalués.

Les résultats montrent une augmentation de la croissance végétative et une réduction du rendement due aux températures élevées. La concentration élevée de CO<sub>2</sub> a également augmenté la croissance végétative et l'activité photosynthétique, même si un processus d'acclimatation a été observé, celle-ci étant plus forte lorsque l'augmentation du niveau de CO<sub>2</sub> ambiant est associée à une température élevée. Le déficit en eau a fortement réduit la conductance stomatique, réduit l'assimilation du C de la feuille et l'activité photosynthétique et, par conséquent, la croissance végétative, occultant ainsi les effets de la température et du CO<sub>2</sub>. La maturation des raisins a également été affectée par le déficit en eau, ce dernier ayant entraîné un retard de maturité.

L'augmentation de la température, que ce soit seule ou combinée à des niveaux élevés de CO<sub>2</sub>, a accéléré l'accumulation de sucres et, par conséquent, réduit la période entre la mi-véraison et la maturité. Cependant, le déficit en eau a atténué ces effets. La température élevée a

également accéléré la dégradation de l'acide malique, en particulier lorsqu'elle est associée à une concentration élevée en CO<sub>2</sub> et à un déficit en eau. L'augmentation de la concentration en CO<sub>2</sub> semble induire le flux anaplérotique au travers du cycle de l'acide tricarboxylique, fournissant une source supplémentaire de sucres. La concentration et le profil des acides aminés ont été influencés différemment par les températures élevées et les concentrations élevées de CO<sub>2</sub> dans l'atmosphère, en fonction de la disponibilité de l'eau et du clone. La combinaison d'une température élevée, de niveaux élevés de CO<sub>2</sub> et d'un déficit en eau a augmenté la concentration en acides aminés. Le profil en acides aminés était fortement affecté par le déficit en eau, conduisant à la diminution des dérivés de shikimate et de phosphoglycérate.

Dans nos conditions expérimentales, la combinaison de températures élevées, de niveaux élevés de CO<sub>2</sub> et de pénurie d'eau a réduit la concentration en anthocyanes dans la pellicule des baies. Le profil des anthocyanes a également été modifié par le changement climatique, la température élevée augmentant l'abondance relative des formes acylées tandis qu'un haut niveau de CO<sub>2</sub> et une limitation de la disponibilité en eau, ont favorisé la teneur relative en malvidine et les formes acylées, méthylées et tri-hydroxylées. La combinaison de température élevée, de CO<sub>2</sub> élevé et de déficit en eau a montré des effets additifs sur le profil des anthocyanes du raisin. Ces résultats suggèrent une moindre concentration en anthocyanes mais une stabilité plus élevée dans les conditions environnementales prévues dans le futur, ce qui était particulièrement visible dans le clone RJ43. Dans les expériences réalisées, une concentration élevée de CO<sub>2</sub> a réduit l'effet de découplage de l'accumulation d'anthocyanes et de TSS dû à la température; cependant, la combinaison de température élevée, de CO<sub>2</sub> élevé et de déficit en eau a entraîné le déséquilibre entre ces deux composants du raisin.

Les clones étudiés ont montré des différences de développement phénologique, de croissance végétative et de reproduction. L'un d'entre eux, 1084, se distinguait des autres clones par son cycle de reproduction le plus long et ses baies plus grosses mais également par son faible taux de sucres, son acidité plus basse et sa concentration en anthocyanes plus faible à maturité. Ces caractéristiques ne sont pas considérées comme appropriées en viticulture et, par conséquent, ce clone pourrait ne pas être considéré comme une alternative intéressante pour la production de vin à l'avenir en termes d'adaptation au changement climatique.

L'effet de la combinaison des niveaux élevés de CO<sub>2</sub>, de température élevée et du déficit en eau sur la composition du raisin a également varié d'un clone à l'autre, notamment, la concentration et le profil en acides aminés ont été modifiés avec une intensité différente en fonction du clone. De plus, les résultats révèlent l'existence d'une réponse différentielle des clones de Tempranillo



aux conditions environnementales prévues pour 2100 en ce qui concerne les réponses physiologiques de la plante et la composition du raisin.

De façon générale, RJ43 était le clone le plus affecté par les futures conditions de croissance (température élevée, haut niveau en CO<sub>2</sub> et déficit en eau élevé) parmi les clones étudiés en termes de phénologie et de concentration et de profil en anthocyanes. Au contraire, VN31 a maintenu le rapport anthocyanes / TSS le plus élevé, alors que 1084 présentait les taux de sucres, d'acide malique et d'anthocyanes les plus bas et un développement particulièrement long des baies. Les différences observées dans la réponse des clones au changement climatique montrent que la longueur du cycle de reproduction n'est pas le seul facteur de réponse. De nouvelles recherches prenant en compte les analyses transcriptomiques aideraient à comprendre les mécanismes à la base de la diversité de réponses observée.

Ce travail révèle l'importance de tester plus largement la performance de différents clones dans les conditions climatiques prévues dans l'avenir et apporte de nouvelles connaissances sur l'utilisation de différents clones de vigne, qui peuvent participer à l'amélioration de l'efficacité viticole dans les futurs scénarios de changement climatique.

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# GENERAL INTRODUCTION





# GENERAL INTRODUCTION

## 1. GRAPEVINE

The genus *Vitis* belongs to the *Vitaceae* family and it comprises 60 species, among which is grapevine (*Vitis vinifera* L.) [1]. Cultivated grapevine is a deciduous perennial plant that presents hermaphroditic flowers. In 2018, 7.4 Mha were cultivated worldwide with grapevine [2] in regions of Africa, America, Asia, Europe and Oceania [3], although the 39 % of the cultivated area in 2016 corresponded to Europe, especially the Mediterranean region (Spain, France, Italy and Turkey) [4].

### 1.1. Berry morphology

The structure of grape berries is divided in three different tissues: exocarp, mesocarp and endocarp. Exocarp is commonly known as “skin”, while mesocarp and endocarp correspond with the fruit pulp. Seeds are located in the interior of the fruit, surrounded by the endocarp (Figure 1). At winemaking, the relationship between skin, flesh and seeds of the berries is of great importance as all of them present different characteristics and some compounds are found mainly in a specific part of the berry [5,6]. Therefore, berry size can affect the composition of grapevine fruit [7].

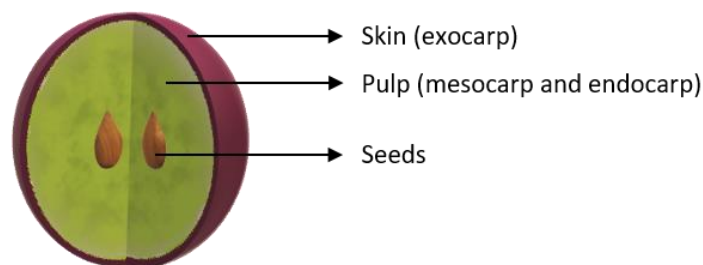


Figure 1. Scheme of the internal structure of a grape berry.

### 1.2. Grape development and composition

Berry biochemistry is modified during the grape development process. The double sigmoidal curve of this process is usually divided into three phases. The first phase, called herbaceous phase, is characterised by intense berry growth (mainly as a consequence of cell division), the accumulation of organic acids and the biosynthesis of tannins and phenolic compounds. The second phase, known as “lag phase”, has a low growth activity and it is the moment when seeds mature and sugars start to accumulate. The third phase, known as “ripening period”, starts with the “veraison” stage. During this third phase, growth continues (mainly through cell expansion) and grapes soften. From a biochemical point of view, berries present high levels of organic acids, which are reduced throughout the ripening of grapes, while sugars, amino acids and anthocyanin concentrations rise changing the colour, aroma

and flavour of grapes [8,9] (Figure 2). The relative composition and metabolic activity that takes place during berry development and ripening may vary among grapevine cultivars [8–10].

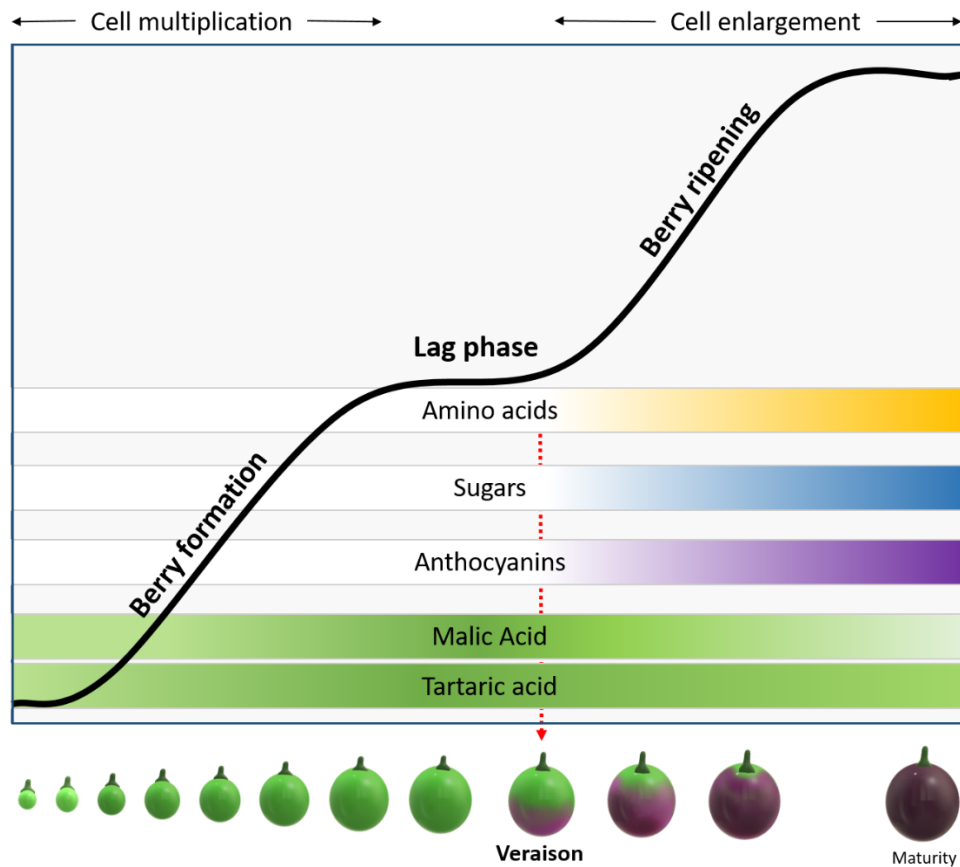


Figure 2. Evolution of berry volume and berry composition. Adapted from Coombe et al. [11].

### 1.2.1. Organic acids

The main organic acids in grapevine are malic and tartaric acid. Acetic, citric, lactic and succinic acids are also present in the berry, but in a lower concentration [8]. While malic acid concentration increases before veraison, it is degraded afterwards, engaging in respiration, biosynthesis of secondary compounds and gluconeogenesis when degraded by PEP-carboxykinase enzyme [8,12,13]. Final must acidity is very important during fermentation in the winemaking process because of its role in the absence of microorganisms contamination [8,14], the yeast activity sensitivity to pH [8] and the relationship between berry acidity and wine colour and flavour [8] (and references therein). The need of having to add compounds to the must to change its acidity might increment the cost of wine production [15]. Besides, acidity can also affect wine stability [8].

### 1.2.2. Sugars

In *V. vinifera*, glucose and fructose are the main compounds that determine the sugar level of grapes while other sugars, such as sucrose, can be present but in a lower proportion [9]. Sugars start to accumulate at veraison, increasing their concentration throughout the ripening process [8,16]. Sugars are mainly obtained as a result of photosynthesis and, under stressing conditions, carbon reserves can be relocated from other plant organs to the berries [17]. In addition, due to the existence of a catalytic pathway of malic acid that can lead to gluconeogenesis, some authors have proposed a possible relation between the degradation of malic acid by PEP-carboxykinase and sugar accumulation during ripening [12,13]. Sugar content determines the sweetness (especially fructose), and the alcoholic degree (mainly glucose) of wines [8], as it is the basis of the alcoholic fermentation [18], and it can play an osmo-regulator role [14].

### 1.2.3. Amino acids

Amino acids are organic molecules that consist of a carbon scaffold and an amino group. They present a high diversity based on the presence of additional groups (as, for instance, aromatic structures). Amino acids are the bricks of proteins but also the precursors of secondary metabolites as phenolic compounds [19] and aromas and aroma precursors [20–22]. In berries, the most predominant amino acids are arginine and proline [23], the later having an osmoprotector role [24]. Concentration of amino acids in berries rises during berry ripening [26], but the accumulation pattern differs among the type of amino acid [23,27]. Amino acid profile and concentration change among varieties [25].

Amino acids have several roles in winemaking: they can determine important characteristics of the final product quality, playing a key role in wine taste, aroma and appearance [8,26,28–30]. Except for proline, amino acids represent the main source of nitrogen for yeast carrying out alcoholic fermentation (preferentially arginine, glutamic acid, glutamine, aspartic acid, asparagine, threonine, alanine and serine) [26,31]. Also, the variability of amino acid profile in wines makes possible to determine and identify wines in great detail (as variety, region of origin and production year) [8,32,33]. However, some amino acids have been reported as precursors of compounds with a known negative impact on humans' health [34]. That is the case of arginine, precursor of putrescine; histidine, precursor of histamine; tyrosine, precursor of tyramine; lysine, precursor of cadaverine; and phenylalanine, precursor of phenylethylamine [34,35].

#### 1.2.4. Phenolic compounds: anthocyanins

Phenolic compounds in grapes can be grouped into non-flavonoids and flavonoids. Non-flavonoids include phenolic acids (mainly accumulated in the pulp, although they can also be found in the skin and the seeds) and stilbenes (mainly present in the skin and the seeds), whereas flavonoids (mainly present in the skin and the seeds) include flavonols, anthocyanins and tannins [8,36,37].

In particular, anthocyanins present a great diversity and they originate from phenylalanine through the phenylpropanoid biosynthetic pathway [38,39]. They have a common basic structure consisting of two benzene rings linked by the flavylum cation (ring C), an oxygenated heterocycle, unsaturated and cationic, which derives from the 2-phenyl-benzopyrilium nucleus. The main anthocyanins in *Vitis vinifera* can be classified into 5 families according to the type of substitution of cycle B and according to the nature and position of the sugar molecule in the structure (Figure 3).

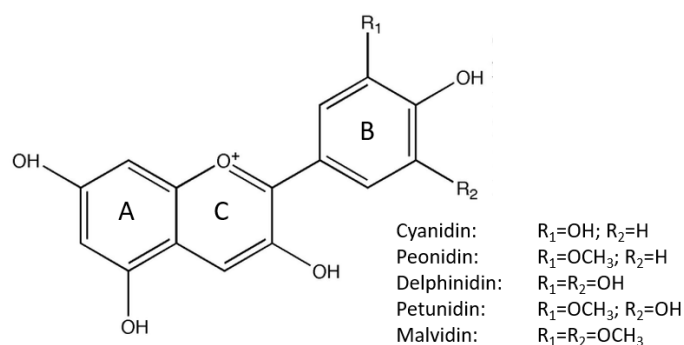


Figure 3. General structure of anthocyanins. Adapted from Castañeda-Ovando et al. [40].

The di-hydroxylated forms include cyanidin and peonidin (the methylated form of cyanidin); the tri-hydroxylated forms contain delphinidin, petunidin and malvidin (the two latter, the methylated forms of delphinidin) (Figure 4).

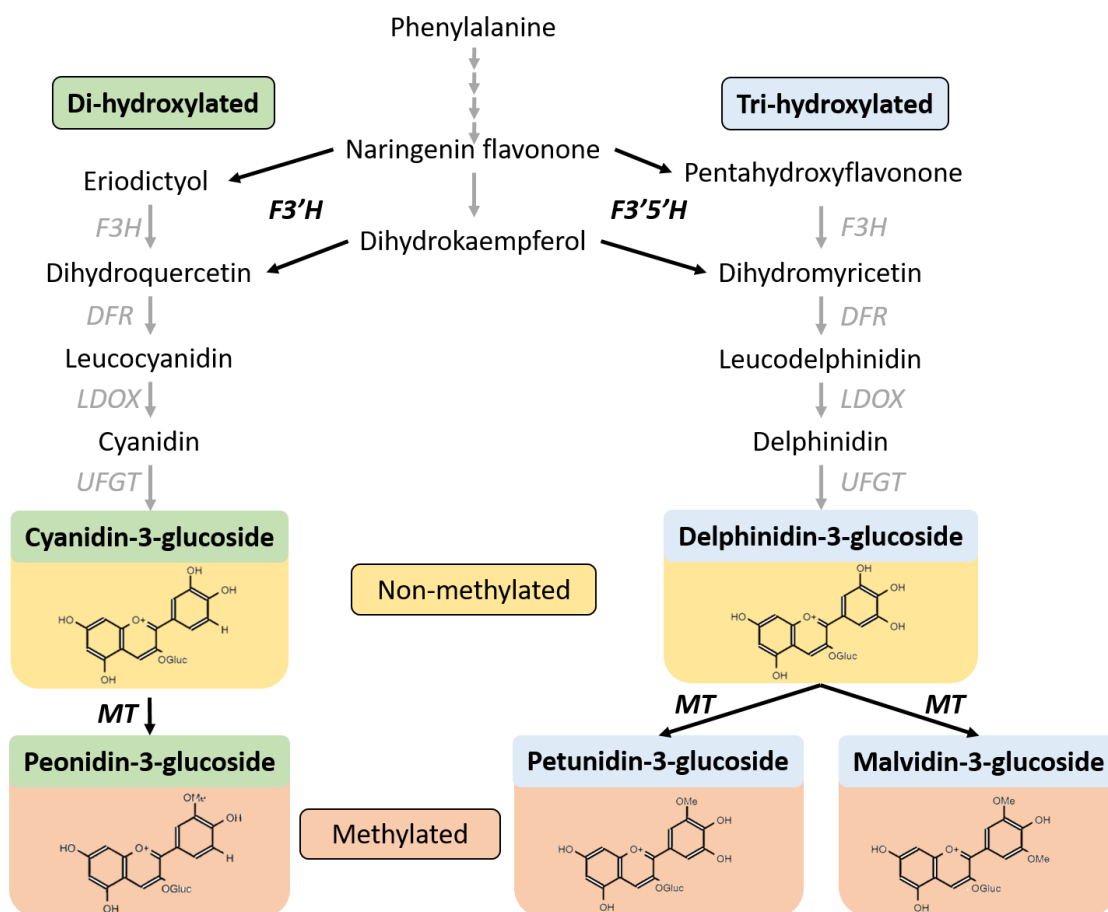


Figure 4. Anthocyanin biosynthetic pathway and the enzymes involved in it: Flavonoid 3'-hydroxylase (F3'H), flavonoid 3'5'-hydroxylase (F3'5'H), flavanone-3-hydroxylase (F3H), di-hydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX), UDP glucose-flavonoid 3-O-glucosyl transferase (UFGT) and methyltransferase (MT). Adapted from Boos et al. [39].

In addition, the degree of acylation of the glucosyl group adds another level of complexity, as every family can be found in its non-acylated (3-monoglucosides) or acylated form (with acetic and coumaric acid, and other residual groups) [41]. Despite this high diversity, malvidin, and especially its form malvidin 3-O-glucoside, is the most abundant anthocyanin in red varieties [41,42]. The accumulation of anthocyanins starts at veraison and continues during berry ripening [9,12,39]. However, some authors have described a decrease in anthocyanin concentration at maturity, as a consequence of degradation processes [43]. Anthocyanins confer colour to grape berries [9], which is of great importance to determine wine colour [39,44,45].

Besides, as the relative abundance of each anthocyanin and their total concentration usually differs among cultivars [42,44,46], these compounds can be used for wine characterisation (reference within [41]). Such variability in anthocyanin composition explains the differences in tonality among varieties [47–49]. In addition, anthocyanins participate in the stability of wine because of their interactions with other constituents such as tannins, proteins or polysaccharides, but also contribute to the health beneficial effects attributed to wine in relation to the prevention of cardiovascular diseases [50] and its antioxidant activity [51].

### *1.3. Economic and cultural value of grapevine*

Wine production, and hence grapevine cultivation, has a strong value both from an economic and cultural point of view. In 2018, world wine production was calculated in 292.3 Mhl, wine consumption in 246 Mhl and wine market valued in 31.3 billion Euro [2]. In Spain, wine exportation, calculated in 20.9 Mhl, was valued in 2.9 million Euro in 2018 (19.4 % of the global market) [2]. Moreover, viticulture in 2013 was estimated to generate 18 million of working positions in Spain [52]. Wine consumption is also deeply integrated in certain cultures, especially in some European countries. In 2018, wine consumption in France was 26.8 Mhl, in Italy 22.4 Mhl, in Germany 20.0 Mhl, in Spain 10.7 Mhl and in Portugal 5.5 Mhl [2]. Moreover, the cultural role of wine is reflected in its incidence in language [53], as well as in its presence throughout history, supported by the references to this beverage in antique texts till nowadays [54–56].

### *1.4. Tempranillo: a red variety*

Tempranillo is a red grapevine variety used for winemaking. It is regarded to have an early budburst, early ripening and a short growth cycle [57,58] and it is considered to have been originated in the area of the Ebro from the varieties Albillo Mayor and Benedicto [59]. Tempranillo has been cultivated for centuries in different regions of Spain, being possible to find it nowadays in different types of soils and climatic conditions [58,60,61]. It is well adapted to southern conditions but sensitive to extreme drought and wind [57,60].

Even though Tempranillo is nowadays grown in 17 countries (Figure 5), the 88 % of the global cultivated area in 2015 was found in Spain [57]. Besides, it was the second variety most cultivated in this country, where it represented the 21 % of the grapevine cultivated area [57], reaching 85 % in the Basque Country, 50 % in Navarre and 76 % in La Rioja (percentages inferred from data published by the Spanish Minister of Agriculture) [62].

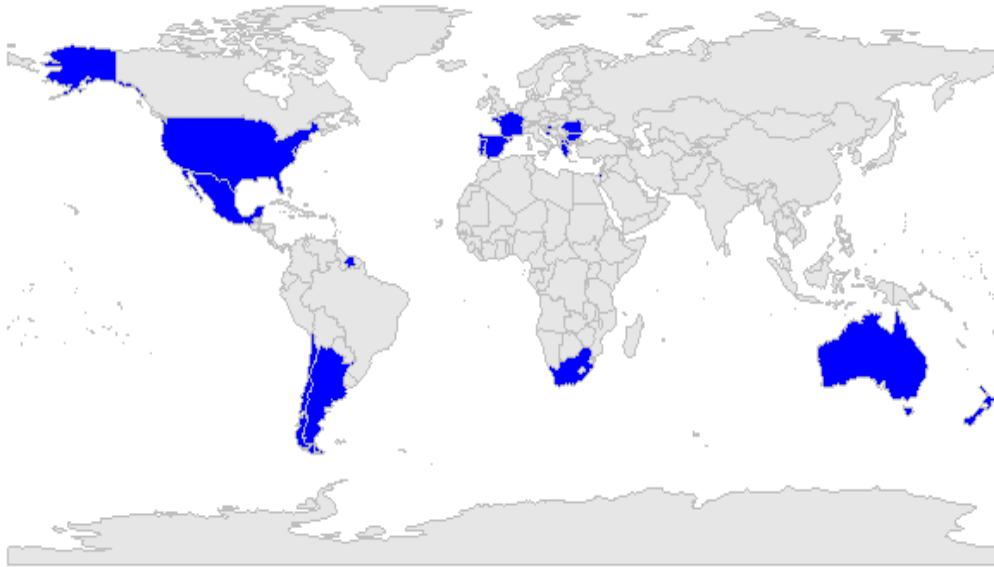


Figure 5. Countries where Tempranillo is cultivated. Adapted from OIV [57].

### *1.5. Somatic variation within grapevine varieties*

Cultivated grapevine has been mainly multiplied by vegetative propagation for the last 5000 years. Despite this propagation method being used to obtain plants that are identical to the original type, spontaneous phenotypic variation occasionally appears as a result of somatic mutations [63]. This somatic variation results from the combination of mutations and cellular events. DNA modification during mitosis is one of the main sources of diversity in this species, which include large deletions, point mutations and repetitions in microsatellite sequences, as well as illegitimate recombinations [1,64]. To manifest a mutant phenotype in a given plant organ, the mutation has to propagate through cell division from the original mutant meristematic cell [65]. The characterisation and well determined phenotype of plants showing different traits within a variety allows to identify clones [66], which can be used in clonal selection [67]. In the case of Tempranillo, there were 49 certified clones of Tempranillo in 2013, from which RJ43, RJ51 and CL306 were among the most distributed ones in Spain [68].



## 2. CLIMATE CHANGE

### 2.1. Origin

The atmospheric gas composition is being strongly modified by human activity since the industrial revolution, due to the emission of greenhouse gases (GHG). These emissions have increased especially during the last years, thus reaching the highest peak in the decade of 2000 to 2010 [69,70]. The atmospheric capacity to regulate the gain and loss of energy, in the form of radiation and global temperature, is strongly determined by human emissions of GHG and, to a lesser extent, to other non-anthropogenic elements as volcanic aerosols [69]. Depending on the degree of future GHG emissions and the expected response of Earth to those emissions, the Intergovernmental Panel on Climate Change (IPCC) has foreseen several scenarios called Representative Concentration Pathways (RCP), which present different possible climatic shifts. The RCP6.0 and RCP8.5 are the least conservative scenarios and consider the highest emission rates [71]. The resulting swifts in environmental conditions have been commonly denominated as “Climate Change”, referring to the rise of air temperature, atmospheric CO<sub>2</sub> levels, changes in precipitation patterns and other modifications (extreme climatic events, swifts in atmospheric circulation or changes in the air relative humidity) [71].

### 2.2. Climatic conditions in RCP6.0 and RCP8.5 scenarios as referred in the IPCC

The IPCC predictions for the atmospheric CO<sub>2</sub> levels at 2100 take into account both the increase of CO<sub>2</sub> due to the GHG emissions, as well as the loss of the CO<sub>2</sub> fixation capacity of the soil and vegetation due to deforestation [71]. The RCP6.0 and RCP8.5 scenarios predict atmospheric CO<sub>2</sub> levels of 669.7 ppm and 935.9 ppm, respectively, for the end of the present century (Figure 6) [71].

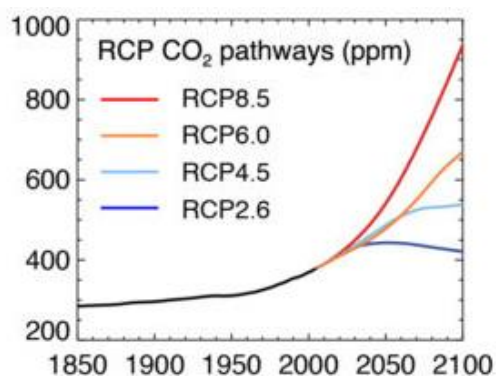


Figure 6. Expected variations in the CO<sub>2</sub> air concentration (expressed in ppm) in coming years according to the RCP2.6, RCP4.5, RCP6.0 and RCP8.5 scenarios. Image obtained from IPCC [71].

The global mean temperature expected for the end of the present century will be  $2.2 \pm 0.5$  °C higher according to the considerations of RCP6.0 scenario, and  $3.7 \pm 0.7$  °C according to the RCP8.5. However, these modifications in mean temperature will not be homogeneous around the globe (Figure 7) [71].

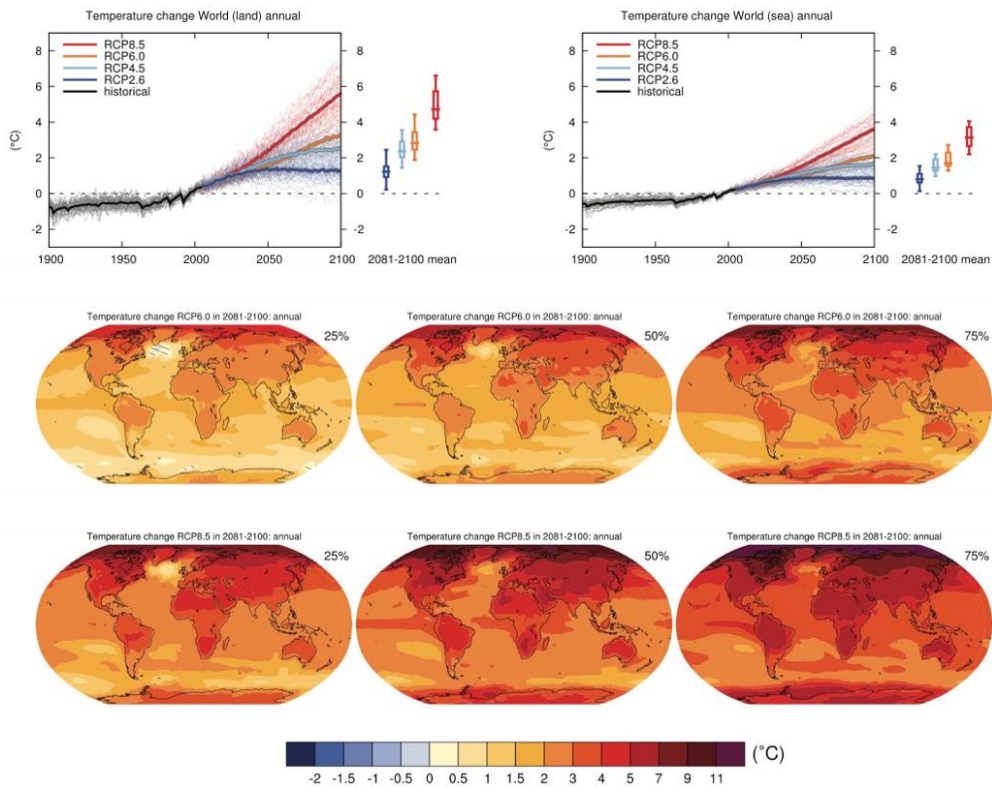


Figure 7. Expected variations in mean annual temperature according to the RCP6.0 and RCP8.5. Images obtained from IPCC [71].

Although, in previous models air relative humidity (RH) had been considered an environmental factor not very affected by climate change, the latest studies predict a decrease in RH in certain areas of the globe in coming years as a consequence of the increase in temperature (Figure 8) [71,72].

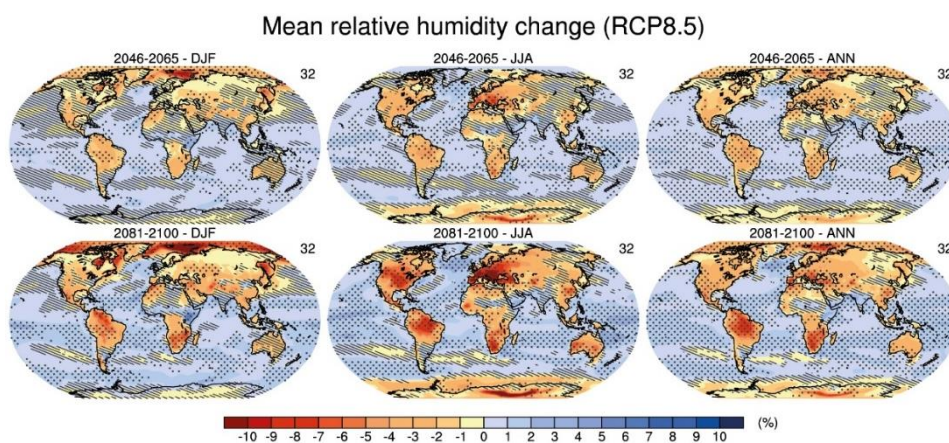


Figure 8. Expected variations in mean relative humidity according to the RCP8.5. Image obtained from IPCC [71].

Regarding water availability, precipitation regimes will change, affecting both the intensity and frequency of rainfall. As a result, droughts are expected to occur more often and in higher intensity in certain regions. In contrast, other regions may experience an increase in precipitations and floods. Finally, some other regions may not suffer significant changes in comparison to the current situation (Figure 9) [71]. However, water deficit experienced by crops will increase even if local rainfall does not decrease because of the impact of the elevated temperature on evapotranspiration [73].

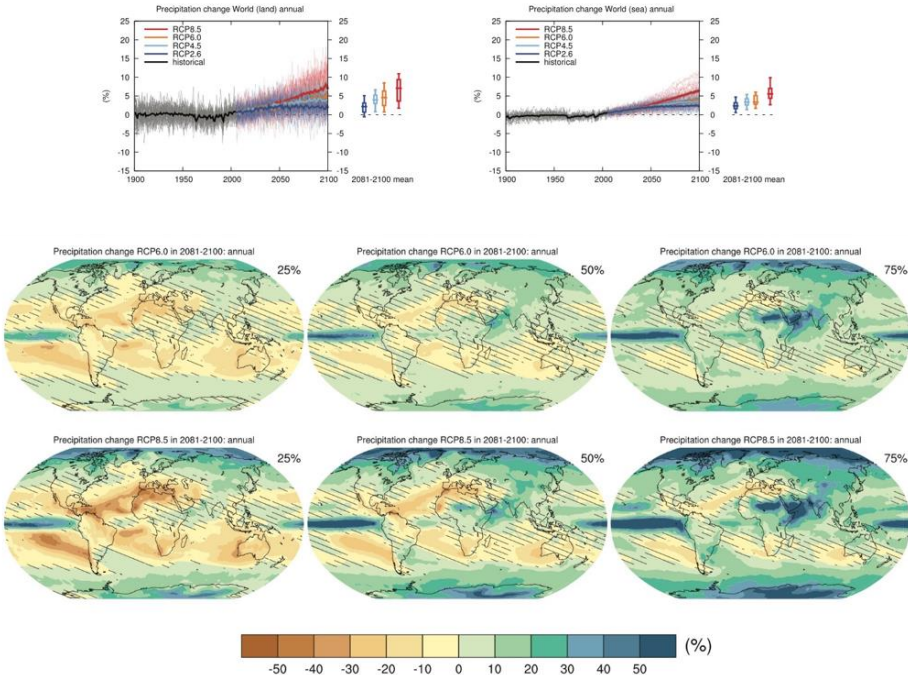


Figure 9. Expected variations in global precipitation at annual level according to the RCP6.0 and RCP8.5. Images obtained from IPCC [71].

*2.3. Climate change effects in agriculture*

Crops performance and their yield are highly dependent on abiotic factors, so changes in climatic conditions will strongly affect agriculture, and most specifically water-intensive agriculture [74]. Besides, climate change is expected to modify the profile and frequency of plant pests and the growth of weeds, affecting crops yield as well [75]. As a result, current food safety and food security are expected to be endangered and socio-economic situation will change as food prices will rise while farmers' income might decrease. For instance, extreme events occurred in the last decades produced losses of billions of \$ in US just in crop damages [75], thus causing migration movements and inducing changes in some cultural and gastronomic features [76].

### 3. GRAPEVINE AND CLIMATE CHANGE

#### 3.1. Grapevine growing area

The Mediterranean region, one of the most productive areas of grapevine in the world, is likely to be strongly affected by climate change in the future [71]. An increase in temperature of 3 °C to 5.8 °C and a reduction in the annual precipitations are projected for this area. Besides, an increase in the length, frequency and/or intensity of both heat waves and drought events is predicted as well (Figure 10) [71]. Also, the Max Planck Institute (MPI-ECHAM5, Roeckner et al. 2003) forecasts a reduction in RH of about 12% during the summer period in comparison to the current values [77,78].

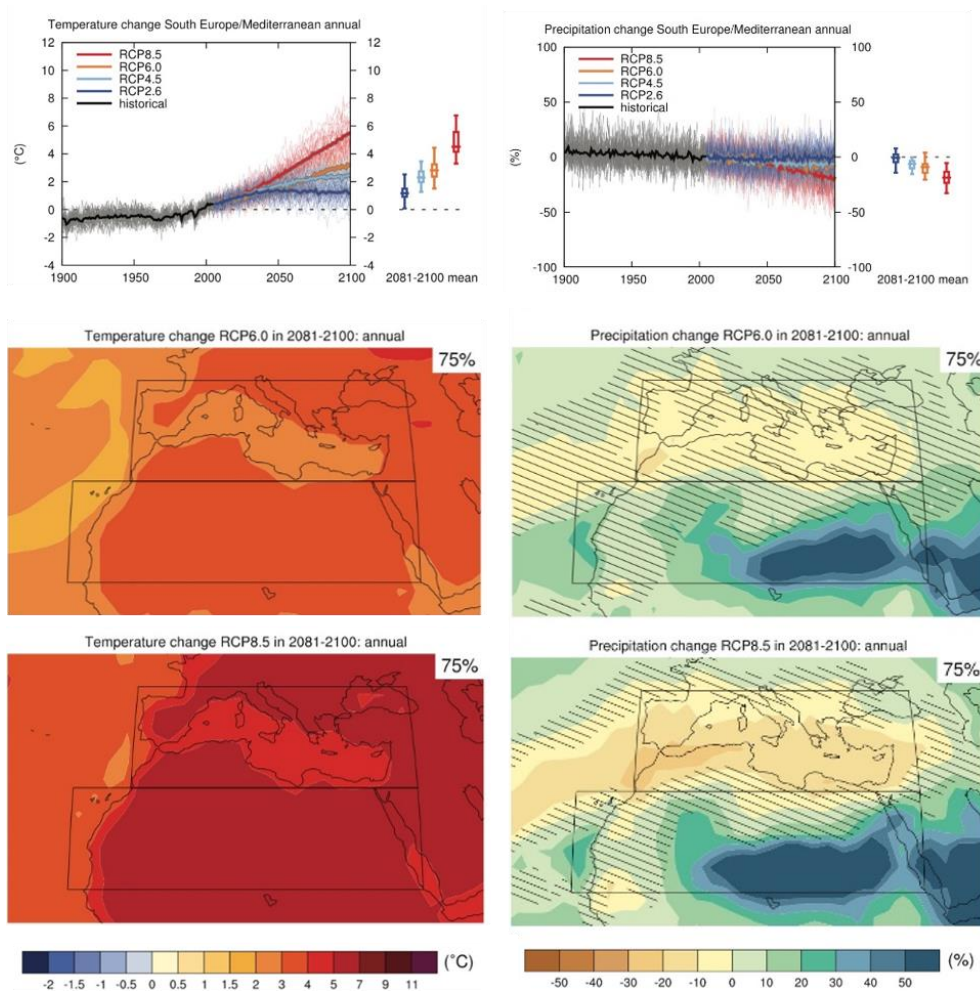


Figure 10. Expected temperature rise and precipitation variation according to RCP6.0 and RCP8.5 for the Mediterranean area. Images obtained from IPCC [71].



### 3.2. *Physiology and must composition*

Regarding the impact of the increase in temperatures on grapevine, a major effect is the advance in the vegetative and reproductive cycles [15,79–83]. Results pointing towards a hastening in grape ripening are reinforced by historic records, which show that dates of harvest were reached earlier in the last years [14]. In addition, warm temperatures have been reported to rise grapevine photosynthetic activity [84,85]. However, when experiments were carried out under more extreme temperature conditions (i.e. 40 °C to 45 °C) photosynthesis was seen to be inhibited [79,86]. Berry composition can also be modified as organic acid degradation [14,87–89] and sugar accumulation [26] usually increase with temperature, while anthocyanin levels are reduced [44,88–91]. Moreover, several authors have referred to a temperature-induced decoupling between sugars and anthocyanin accumulation due to the enhancement in sugar concentration and the decrease in anthocyanins levels [9,92,93].

As a C3 plant, the net photosynthesis of grapevines is limited by CO<sub>2</sub>, so an increase in CO<sub>2</sub> availability may initially enhance photosynthetic activity and CO<sub>2</sub> fixation rates, as already observed by some authors [94–96]. As a consequence of this increased C fixation, a stimulation of vegetative growth at a plant level [97,98] and a rise in grape yield [97] have been reported. However, after medium or long-term exposures to elevated CO<sub>2</sub>, usually a down-regulation of photosynthesis happens, pointing towards a photosynthetic acclimation process to high CO<sub>2</sub> [77,95,96,99,100]. Regarding grape composition, Bindi et al. detected an increase in organic acids and sugars in berries at early stages of development when plants were grown under high atmospheric CO<sub>2</sub> concentration, these effects disappearing at maturity [97]. However, there is not consensus on the effects of high CO<sub>2</sub> on anthocyanin concentration, as some studies have reported an increment of these compounds [98], not observed in other cases [101].

The effect of water scarcity on grapevine physiology and grape composition has been well documented. Drought can modify grapevine phenology depending on its intensity [102]. In addition, severe drought can reduce the rate of C assimilation [103], vegetative growth [25,95,98] grape yield [25] and berry size [7,104]. Berry composition is also affected by water scarcity, resulting in berries with lower concentration of organic acids [105] and higher levels of sugars [106], amino acids [25,107] and anthocyanins [29,108].

Due to the technical complexity, most of the research on the effects of climate change on grapevine growth and berry composition have analysed the impact of single environmental factors, as previously described. There is scarce research carried out considering the interaction among factors, specially temperature and CO<sub>2</sub> [98,109,110]. However, these studies are of high interest since the impact of combined environmental stress factors cannot be inferred from the addition of the individual responses. Indeed, the impact of a particular factor of climate change often depends upon the presence of other climate change factors [111–113].

The studies on the combined effect of climate change related factors have pointed towards a shortening of the phenological development [83,92,100,114] in plants exposed to high temperature combined with elevated CO<sub>2</sub>. Under this condition, photosynthetic activity seemed to be initially stimulated, although this effect disappeared under extreme temperature conditions, getting inhibited [85,86]. Berry composition was also affected as malic acid degradation and sugars accumulation were enhanced [92,114], while the concentration of anthocyanins decreased in some cases [83,114] and rose in others [92]. Besides, Torres et al. observed an increase in amino acids concentration when considering the combination of high temperature and drought [18].

Consequently, climate change might affect wine production by different means. Firstly, yield may be reduced, mainly due to water deficiency [25,104]. The modifications of berry composition are expected to have significant impact on wine characteristics as the increase in sugar content will probably produce a higher alcohol content, being a threaten to microbiota during fermentation (including yeast performance) [115]. The reduction in the levels of aroma precursors and coloured compounds may make wines to be less intense (both in taste and in colour) and concentration of acetic acid is expected to rise, even over the legal value [14]. Above that, the cost of fermentation process might rise due to the need of stronger cooling systems. Eventually, prices of wine might increase in those markets where alcohol volume determines the taxes percentage on the product [14]. Other effects of climate change on wine final characteristics might arrive through changes in oak wood quality used for making the barrels [14,116].

Therefore, the socio-economic and cultural value of grapevine, and especially when related to wine production, makes worthy to take into consideration the potential effects of climate change on this crop. The understanding of the impact that the expected environmental conditions may have on it can provide useful information to design adaptation strategies, thus ensuring the sustainability of grapevine and wine production in the future.

### *3.3. Adaptive strategies to a changing environment*

In order to mitigate the potential negative effects that climate change may have in viticulture, diverse approaches have been proposed in the last years. Some of them are focused on agricultural practises such as the guiding system of the vines, looking for training systems and other field architecture designs (such as higher trunks or row direction) that favour the reduction of water loss and the impact of extreme temperature [15,73,117]. Strategies involving pruning practises and low leaf to fruit ratio have been also proposed to delay ripeness to the end of the season when temperatures start to decline [15,118]. Other approaches consider the possibility of modifying the plant material used. In this way, some authors propose the use of varieties cultivated in more septentrional areas or at lower latitudes, because of being more adapted to higher temperatures [15], late-ripening varieties [119] or different rootstocks [73,120]. Finally, the selection of clones within the same variety that are more adapted to future climate conditions in the areas where they are currently cultivated has also been proposed [15,121].

Some of these approaches can be applied individually or combined. The choice of one or another one should take into account: the specific characteristics of the area where it is expected to be implemented, considering the varieties historically cultivated in the zone (their adaptive capacity, cultural meaning and intra-varietal diversity), the specific future climate previsions and the socio-economic characteristics of the agriculture in the area.

#### Use of clones

Genetic diversity among clones can represent a source of potential candidates with suited performances under future environmental conditions. This diversity may allow the selection of clones with traits that are expected to reduce the potential negative impact of climate change as could be, for instance, late-ripening clones [73]. One of the advantages of this approach in comparison to other strategies focused on plant material, such changes of grapevine varieties, is the possibility of keeping the cultivation of a variety in its traditional region [122,123], as would be the case for the cultivation of Tempranillo in Spain, Merlot in France, Sangiovese in Italy or Fernao Pires in Portugal [124]. Also, it allows the production of wine with the same typicity and already recognised by the corresponding protection figure [68], avoiding problems related to consumer recognition [125]. In the case of Tempranillo, the high number of clones selected and the fact that many of them are already commercialised makes the use of the intra-varietal diversity a highly suited approach that may permit to keep the production of well-recognised grapevine varieties into their traditional area.

#### **4. STUDIES OF GRAPEVINE UNDER CONTROLLED ENVIRONMENTAL CONDITIONS**

Replication of foreseen environmental conditions is a useful approach to study the response of plants to predicted climate scenarios and to determine their performance in the future. However, this type of studies is difficult to achieve in the field because of their technical complexity and high economic cost. For this reason, other approaches have been developed to carry out these experiments.

A plant model system that facilitates these studies is the fruit-bearing cutting system, which allows obtaining small grapevine plants with grape bunches from cuttings. This method of handling and processing plant material was described by Mullins and Rajasekaran [126] and its capacity to produce plants with a high resemblance to field grapevines has been confirmed in several studies that include phenological, physiological and transcriptomic considerations [87,127–131].

The growth of plants under controlled conditions can be done by using special facilities as Temperature Gradient Greenhouses (TGGs) (Figure 11) or Growth Chamber-Greenhouses (GCGs) (Figure 12) [132] which allow not only to modify the growing environmental conditions but also to combine different environmental factors in multi-stress studies. The TGGs consist of three continuous modules, the first one maintained at the same temperature as outside, while the third one keeping a constant increment of the temperature respect to the first one. Such facility allows to study the impact of high temperatures under more realistic conditions. In contrast, GCGs keep a constant temperature, independently of the external ambient temperature, with a higher level of control. Moreover, treatments based on the increase of air CO<sub>2</sub> concentration can be applied in the TGGs as well as in the GCGs. Both infrastructures have been proved by previous studies carried out in our research group to be suitable for the study of the effect of multivariable environmental conditions on different plant species, including grapevine [18,83,92,98,110].





Figure 11. Picture of the Temperature Gradient Greenhouses (TGGs) at *Universidad de Navarra*. (Source: A. Urdiain).



Figure 12. Picture of the Growth Chamber-Greenhouses (GCGs) at *Universidad de Navarra*. (Source: A. Urdiain).

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## **OBJECTIVES**



## OBJECTIVES

The general aim of the present thesis was to assess the response of different clones of Tempranillo, characterised with diverse phenological cycle length, to 2100-expected-environmental conditions and consider their potential for being used in grapevine production in the future.

The following partial objectives were established in order to achieve the general aim:

- To determine the effect of individual and combined environmental factors (elevated air temperature, high atmospheric CO<sub>2</sub> concentration and drought) on grapevine physiology, growth and phenological development (**Chapters 2 and 3**)
- To determine the effect of individual and combined environmental factors (elevated air temperature, high atmospheric CO<sub>2</sub> concentration and drought) on grape composition (sugars, organic acids, amino acids, anthocyanins and the anthocyanin:sugar ratio) (**Chapters 1, 4 and 5**)
- To determine the effect of individual and combined environmental factors on the anthocyanin profile of grapes (**Chapter 6**)
- To evaluate the response of different clones of the cultivar Tempranillo to the projected environmental conditions, thus contributing to increase the knowledge about the existing genetic diversity of Tempranillo, and the suitability of using it as a mitigation strategy in the future (**Chapters 1 and 6**)
- To evaluate the adequacy of the use of clones with a longer reproductive cycle as a potential approach to face with future growing conditions originated by climate change (**Chapters 1 to 6**)



## **CHAPTER 1**

Tempranillo clones differ in the response of berry sugar and anthocyanin accumulation to elevated temperature

M. Arrizabalaga, F. Morales, M. Oyarzun, S. Delrot, E. Gomès, J.J. Irigoyen, G. Hilbert, I. Pascual, Tempranillo clones differ in the response of berry sugar and anthocyanin accumulation to elevated temperature, *Plant Sci.* 267 (2018) 74–83.





# Tempranillo clones differ in the response of berry sugar and anthocyanin accumulation to elevated temperature

## ABSTRACT

The intra-varietal genetic diversity of grapevine (*Vitis vinifera* L.) may be exploited to maintain grape quality under future warm conditions, which may alter grape berry development and composition. The present study assesses the effects of elevated temperature on the development of berry, grape composition and anthocyanins:sugars ratio of thirteen clones of *V. vinifera*. cv. Tempranillo that differed in length of the ripening period (time from veraison to berry total soluble solids, mainly sugars, of ca. 22 °Brix). Two temperature regimes (24 °C/14 °C or 28 °C/18 °C, day/night) were imposed to grapevine fruit-bearing cuttings from fruit set to maturity under greenhouse-controlled conditions. Elevated temperature hastened berry development, with a greater influence before the onset of ripening, and reduced anthocyanin concentration, colour intensity and titratable acidity. The clones significantly differed in the number of days that elapsed between fruit set and maturity. At the same concentration of total soluble solids, the anthocyanin concentration was lower at 28 °C/18 °C than 24 °C/14 °C, indicating a decoupling effect of elevated temperature during berry ripening. Thermal decoupling was explained by changes in the relative rate of response of anthocyanin and sugar build-up, rather than delayed onset of anthocyanin accumulation. Clones differed in the degree of thermal decoupling, but it was directly associated with differences neither in the length of their ripening period nor in plant vigour.

**Keywords:** Anthocyanins:sugars decoupling; Berry development; Clone; Grapevine (*Vitis vinifera* L.); Intra-varietal variability; Temperature

## 1. INTRODUCTION

Climatic conditions have changed over the past decades, and simulations with different scenarios of greenhouse gas emissions show that the observed tendencies will continue in the near future [1]. According to the Intergovernmental Panel on Climate Change (IPCC), the increase of global mean surface temperature by the end of the 21<sup>st</sup> century (2081-2100), relative to the current reference period (1986-2005), is likely to be from 0.3 °C to 4.8 °C depending on the mitigation scenario [1]. In addition, it is likely that heat waves will occur with higher frequency and longer duration as a consequence of the increase in temperature variability [1,2].

Among human activities, agriculture -in particular viticulture- is highly dependent upon climatic conditions during the growing season [3]. The grape berry is one of the fruits whose composition is highly sensitive to the environment [4], temperature being an important environmental factor during berry development and ripening [5]. Many studies have linked increases in temperature to accelerated phenology, with the potential to greatly affect grape attributes for the production of red table wines. For example, increased background temperature has been reported to advance budburst, flowering and to hasten berry development [6–8]. Simulations using a model for the developmental stages of Riesling and Gewurztraminer predict an earlier onset of veraison (up to 23 days before), by the end of the present century compared with its timing in 1976-2008, resulting in an important increase in mean temperatures (more than 7 °C) during the ripening period [9]. Such changes will likely impact on grape and wine quality. One of the clearest relationships between temperature and fruit quality concerns grape berry acidity, as high temperatures reduce the concentration of organic acids, especially malic acid [10–12], desynchronizing sugar and organic acid metabolisms [13,14]. In addition, high temperatures during ripening decrease anthocyanin concentration in grapes [15–17], due to the inhibition of anthocyanin biosynthesis, chemical or enzymatic degradation and/or the imbalance in the expression and function of specific transmembrane transporters [16,18,19]. Elevated temperature can also uncouple berry traits, leading to an unbalanced wine. In previous studies, seed ripening was advanced in relation to other berry tissues, and this asynchrony may have direct oenological implications affecting the resultant phenolic composition and sensory attributes of wines [15,20]. In addition, a consistent thermal decoupling of anthocyanins and sugars was observed in berries of cv. Cabernet Sauvignon and Shiraz regardless of the irrigation regime and source:sink ratio, with consequences for the colour-alcohol balance of wine [21].

In order to avoid quality alterations caused by high temperatures during fruit ripening, phenology should be delayed [3]. With this aim in mind, besides changes in vineyard location (higher latitudes and altitudes) or modifications of training systems (higher trunks, late pruning, minimal pruning of

reduced leaf area to fruit weight ratios) [3,22–24], plant material is a major tool for adapting vineyards to warm temperatures. Thus, ripeness can be delayed by the use of late-ripening varieties. However, if the objective is to maintain wine typicity, one alternative is to explore in depth the existing intra-varietal genetic variability. Because much of grapevine plants are reproduced by vegetative propagation, spontaneous mutations can accumulate over time [25]. When these natural events have significant phenotypical effects, the new plant can bear interesting traits, thus leading to clones within a variety that can be exploited for clonal selection and propagation [26–28]. During the last two centuries, clonal selections were performed to improve vineyard health and production traits (yield, precocity, flavour and colour among others) [29]. The existing clone collections worldwide can be explored to detect any phenotypic variation that could be powerful means of adaptation to climate change, looking for either late-ripening clones or clones with a high ability to maintain some required characteristics under warm conditions.

The objective of this study was to evaluate the response of thirteen clones of Tempranillo to elevated temperature, focusing on the phenology of grape development, berry composition and the thermal decoupling of sugar and anthocyanin accumulations. The study tries to explore the possibility of using the intra-varietal variation of the Tempranillo cultivar to maintain high quality standards in berries under future warmer conditions.

## **2. MATERIAL AND METHODS**

### *2.1. Plant material and growth conditions*

Dormant cuttings of thirteen clones of grapevine (*V. vinifera* L.) cv. Tempranillo were obtained from the germplasm bank of the Institute of Sciences of Vine and Wine (Rioja Government, Spain) located in “La Grajera” (La Rioja, Spain). The studied clones were: 86, 1052, 336, 518, 501, 349, 280, 825, 807, 814, 318, 56, and 1084. They had been previously characterized in the clone bank for three years (2009-2011) focusing on phenological development (dates of budburst, flowering, veraison and maturity), grape production and must composition at harvest time, and had showed differences in the length of the ripening period (number of days between veraison and berry with total soluble solids (TSS, mainly sugars) of ca. 22 °Brix) (unpublished data). Cuttings 400-500 mm-long were selected to maximize the chances of them bearing fruit, as described in Mullins and Rajasekaran (1981) [30], with slight modifications. Briefly, rooting was induced using indole butyric acid (400 mg L<sup>-1</sup>) in a heat-bed (27 °C) kept in a cold room (5 °C). Once cuttings had developed roots, they were transplanted to 6.5 L pots containing 2:1 peat:perlite (v/v) and transferred to growth chamber- greenhouses (GCGs). Initial growth temperature conditions until fruit set were 25 °C/15 °C (day/night). Plants grew with natural

light supplemented with a system of high-pressure sodium lamps (HQT-TS 400W/D Osram, Augsburg, Germany), which was triggered when photosynthetically active radiation (PAR) dropped below a photon flux density of  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  and used to maintain a photoperiod of 14h (more details about GCGs in Morales et al.[31]). Plants were irrigated with the nutritive solution described by Ollat et al. [32]. Under these conditions, the bud-break took place after 1 week. Only one leaf was allowed to grow in the developing shoot, manually removing the rest of the leaves. The tip of the shoot was manually excised above the inflorescence. A new shoot (lateral) was then allowed to develop and support the vine. Only one inflorescence was allowed to develop on each plant, manually excising the rest (when and if they appeared). Until fruit set, vegetation was controlled and only 4 leaves per plant were allowed to grow.

## *2.2. Experimental design*

When fruit set was complete, plants with similar flowering and fruit set dates were selected to perform the experiment. Plants of each clone were divided into two homogeneous groups (4-6 plants each) in terms of fruit set date and grape bunch size. One group of plants grew in a GCG at 24 °C/14 °C and the other group at 28 °C/18 °C (day/night) in a second GCG. Light conditions were as those described above.

## *2.3. Grape sampling*

Grape berry sampling was performed at four developmental stages: i) the onset of veraison, when berries had already softened and just began to colour; ii) mid-veraison, half of the berries in the bunch had turned red. At this stage, berries with the same proportion of coloured skin surface (ca. 50 %) were sampled; iii) two weeks after mid-veraison; and iv) maturity, when the grapes in the bunch reached a TSS content of ca. 22 °Brix. Phenological stages were assessed and defined individually for each plant. The onset of veraison and mid-veraison stages were visually assessed through daily observations. To determine the maturity stage, each bunch was assessed individually and the TSS content was periodically monitored from two weeks after mid-veraison by taking samples of 2-3 berries until the sample reached a level of TSS of at least 22 °Brix. Each plant was sampled individually when it reached the desired stage. Berry samples contained 3-4 berries per bunch (one bunch per plant). Berries were always taken from the top and middle portion of the bunch, which allocate the highest number of berries. At maturity, bunches were weighed and all berries from each bunch were separated, counted and weighed to determine berry weight. Berry diameter was measured in 10 berries per bunch with a caliper. These berries were also weighed, and the peel was manually separated and weighed to

determine the relative skin mass ratio. The rest of the grapes were immediately frozen with liquid N<sub>2</sub> and kept at -80 °C until analysis.

#### *2.4. Phenological development and leaf area*

Three events, fruit-set, mid-veraison and maturity, were selected for the study of phenological development, which was determined as the elapsed time between fruit-set and mid-veraison, and between mid-veraison and maturity (TSS of ca. 22 °Brix). Fruit-set and mid-veraison were visually assessed through daily observations, and maturity was determined as described in Section 2.3. Each event was determined for each plant individually. Plant leaf area was estimated using a model based on Costanza et al. [33] and adapted for cv. Tempranillo, which relates actual leaf area, 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 was built over shoot samplings taken throughout the season from extra plants.

#### *2.5. Evolution of total soluble solids (TSS) and total skin anthocyanins*

Grapes collected at the onset of veraison, mid-veraison, two weeks after mid-veraison and maturity were manually separated into skin, pulp and seeds. Skins were freeze dried, ground and extracted in methanol containing HCl (0.1 %, v/v) for 40 min according to Martínez-Lüscher et al. [34]. Extracts were centrifuged and the supernatants were analysed by UV-B visible spectrophotometer (UV mini-1240, Shimadzu, Tokyo) in a range from 230 nm to 700 nm. Maximal absorption signal (peak around 536 nm) was used to calculate the concentration of total anthocyanins. A calibration curve was prepared with malvidin-3-O-glucoside (Sigma-Aldrich Quimica SL, Madrid, Spain). Total soluble solids (TSS) were measured in the must extracted from the pulp, using a refractometer (Abbe Digital 315RS, Zuzi, Beriain).

#### *2.6. Phenolic and technological maturity parameters at ripeness*

At maturity, samples of 20 berries per bunch were crushed to extract the juice and centrifuged to analyze the following technological maturity parameters: TSS content; total acidity by titration with NaOH according to the OIV [35]; and L-malic acid by an enzymatic method (Enzytec L-Malic Acid, Boehringer Mannheim/R-Biopharm, Darmstadt). Another 20 berries were homogenized with a laboratory batch ball mill (Retsch MM400, Germany) for 2 minutes for the determination of the phenolic maturity parameters: colour density, tonality, extractable anthocyanins and total polyphenol index (TPI). Part of the crushed sample was centrifuged and diluted 10 times to determine colour density and tonality [36,37]. Absorbance was measured at 420, 520 and 620 nm using

spectrophotometer. Colour density was calculated as the sum of the absorbances at 420, 520 and 620 nm. Tonality was determined by the ratio between the absorbances at 420 and 520 nm. Another part of the crushed sample was macerated with tartaric acid (pH 3.2) for 4 h. The macerated samples were centrifuged and used for the determinations of extractable anthocyanins [38] and total polyphenol index, as the absorbance at 280 nm, according to the Glories method [37,39].

### *2.7. Statistical analysis*

Statistical analysis was performed with XLstat-Pro (Addinsoft). Data were subjected to a two-way ANOVA (13x2) in order to partition the variance into the main effects, clone and temperature regime, as well as the interaction between them. When the F ratio was significant, ( $P < 0.05$ ) differences among treatments were tested with Least Significant Difference (LSD) post-hoc test. Phenological parameters and grape composition measurements were also analyzed using a principal component analysis (PCA). Linear regressions were performed to analyse the relationship between total skin anthocyanins and TSS [21]. An analysis of residuals was used to test the effects of temperature. For that, the equation obtained after including the thirteen clones and two temperature regimes (24 °C/14 °C and 28 °C/18 °C) was fitted to the data, and the effect of temperature on residuals was tested with analysis of variance. The null hypothesis was that the residuals of the 24 °C/14 °C versus 28 °C/18 °C treatments are statistically undistinguishable within each clone.

## **3. RESULTS**

### *3.1. Phenological development, leaf area and grape berry yield*

Elevated temperature significantly shortened, in 13 days on average, the period comprised between fruit set and maturity, defined as TSS of ca. 22 °Brix ( $P < 0.0001$ ). Temperature had a greater influence before veraison (Fig. 1A). The clones studied significantly differed (up to 32 days,  $P < 0.0001$ ) in the number of days that elapsed between fruit set and maturity. Significant differences among clones were mainly observed in the period comprised between mid-veraison and maturity (Fig. 1B). The 349, 807, 814, 56 and 1084 accessions had the longest ripening periods. There was no significant interaction between clone and temperature for phenological development.

Plants of the studied clones had significant differences in their leaf area throughout the experiment, especially at maturity (Fig. 2). In general, under 24 °C/14 °C, the clones that showed a later maturity in the present study (349, 807, 814, 56 and 1084) had larger leaf areas at maturity compared with those with short ripening behaviour. Taking the thirteen clones into consideration, elevated temperature

significantly reduced total leaf area at the onset of veraison and maturity, regardless of the clone, although more evident in the later maturing clones (Fig. 2).

The clones of Tempranillo studied significantly differed in bunch size (bunch weight and number of berries per bunch) and berry characteristics (berry weight and diameter) (Table 1). The 86 and 501 accessions were among the clones with the highest bunch weight at 24 °C/14 °C. In contrast, the 336 one had the lowest bunch size both under 24 °C/14 °C and 28 °C/18 °C. Elevated temperature did not affect grape yield but significantly reduced berry weight and diameter, taking all clones into consideration.

### 3.2. Evolution of total soluble solids (TSS) and total skin anthocyanins

A significant interaction between clone and temperature was observed at the onset of veraison and mid-veraison for TSS (Fig. 3). Nevertheless, these differences, although statistically significant, were probably not significant from a physiological point of view, due to the low TSS content at these two stages. In contrast, significant differences among clones were observed two weeks after mid-veraison and maturity, irrespective of the temperature regime. In this way, the clones that had showed a short ripening behaviour had, on average, higher TSS than those with a long ripening behaviour, both two weeks after mid-veraison ( $18.7 \pm 0.2$  and  $16.7 \pm 0.4$ , earlier and later maturing clones, respectively) and at maturity ( $22.5 \pm 0.2$  and  $20.7 \pm 0.3$ , earlier and later maturing clones, respectively) (the means include both 24 °C/14 °C and 28 °C/18 °C treatments). Among the earlier maturing clones, the 280 and 518 accessions showed the highest TSS at two weeks after mid-veraison, reaching the maximal sugar content at this stage, whereas the 1084 (later maturing) accession showed the lowest TSS both two weeks after mid-veraison and at maturity, irrespective of the temperature. The rate of increase in TSS between the onset of veraison and maturity was significantly higher ( $P < 0.0001$ ) in the earlier maturing clones ( $0.38 \pm 0.02$  °Brix day<sup>-1</sup>) than in the later maturing ones ( $0.23 \pm 0.01$  °Brix day<sup>-1</sup>), and this rate significantly increased with temperature ( $P < 0.0001$ ) from  $0.28 \pm 0.02$  °Brix day<sup>-1</sup> in the treatment with 24 °C/14 °C to  $0.36 \pm 0.01$  under 28 °C/18 °C. The concentration of total skin anthocyanins at mid-veraison did not significantly differ among clones, and it was not influenced by temperature (Fig. 4). In contrast, two weeks after mid-veraison and at maturity, clones differed in their anthocyanin levels, with the 280 (early maturing) and 1084 (later maturing) accession being among those with the highest and lowest anthocyanin concentrations, respectively. Elevated temperature significantly reduced the concentration of anthocyanins two weeks after mid-veraison, taking all clones into consideration. Although this trend was also observed at maturity, the significant interaction between clone type and temperature reveals that not all the clones were affected by temperature to the same extent.



### *3.3. Principal component analysis of phenological development and grape berry technological and phenolic maturity at ripeness*

Both grape juice analyses (technological and phenolic maturity) and phenological development were analyzed by principal component analysis (PCA). The first two principal components explained about 75 % of the total variability and clearly separated samples according to the temperature regime (Fig. 5A). The loading plot reveals that the distinction between plants grown at 24 °C/14 °C and 28 °C/18 °C observed along PC1 was associated with an increased tonality, as well as lower anthocyanin concentration, total polyphenol index (TPI), intensity and titratable acidity under elevated temperature (Fig. 5B, Table S1). PC2 discriminated clone types according to their phenological pattern (Fig. 5B, 1A and B) and TSS level (Fig. 5B and 3). The 1084, 807, 814, 349 and 56 accessions grown at 24 °C/14 °C were separated along PC2 due to a longer ripening period (elapsed days from mid-veraison to maturity, TSS= ca. 22 °Brix). Under 28 °C/18 °C, only the 1084, 807 and 814 accessions maintained a long ripening period.

### *3.4. Relationship between the dynamics of TSS and anthocyanins*

The pooled data supported a strong linear relationship ( $P < 0.001$ ) between TSS and anthocyanins from mid-veraison to maturity (approximately linear phase when TSS and anthocyanins increased in parallel) (Fig. 6A). The slope of this correlation was higher for plants grown at 24 °C/14 °C than those grown at 28 °C/18 °C ( $P = 0.001$ ) (Fig. 6B). The positive mean residual for the treatment 24 °C/14 °C ( $2.95 \pm 0.75$ ) compared with the negative residual for 28 °C/18 °C ( $-2.7 \pm 0.58$ ) (significantly different  $P = 0.001$ ) indicates a lower concentration of anthocyanins at the same concentration of soluble solids in the warmest treatment, thus supporting a thermal decoupling between these two parameters. Nevertheless, after analyzing the correlation between TSS and anthocyanins and calculating the residuals for each clone individually, the results indicate that the magnitude of this decoupling differed among clones (Fig. 7). The thermal decoupling between TSS and anthocyanins was clearly noticeable in the 518, 501, 280, 349, 814 and 56 accessions (Fig. 7), in which the mean residual for 24 °C/14 °C was significantly higher than that for 28 °C/18 °C (Fig. 8). In contrast, the 86, 1052, 336, 825, 318, 807 and 1084 ones were the less affected clones.

## **4. DISCUSSION**

One of the challenges of viticulture in the future is to cope with the effects of global warming on the environmental conditions. In this context, the existing genetic intra-varietal diversity, particularly with regard to phenological diversity, can be a valuable tool to mitigate the effect of increased temperatures on grape quality when the typicity of wines derived from one particular cultivar needs

to be preserved. Nevertheless, clone performances vary with environmental conditions due to clone-environment interactions [40]. The present study tries to assess the effect of elevated temperature on grape berry ripening and composition, focusing on the unbalance of sugars and anthocyanins, of thirteen clones of Tempranillo that differed in the length of the ripening period. The assessment of the impact of climate change factors, such as temperature, requires the use of direct methods that involve the experimental manipulation of environmental factors under fine control conditions. In this regard, the growth-chamber greenhouses used in this work have been proposed as suitable tools for such kind of studies [31]. Although the results obtained need to be considered in the light of the limitations of the study (i.e. only one-season experiment), enough samplings and replicates under greenhouse conditions should be reliable and the results may be used as the basis for future studies under field conditions.

#### *4.1. Grape development of Tempranillo clones under elevated temperature*

Grapevines are multiplied by vegetative propagation, which is a conservative strategy to obtain clones that are genetically identical copies of an original seedling. Nevertheless, somatic mutations may occur naturally and accumulate during this process [25]. Therefore, grapevine varieties are not genetically homogeneous [41] and precocity of phenological cycle can vary among clones of the same variety [40]. Despite the narrow intra-varietal genetic diversity described for Tempranillo in previous studies [42], the clones of Tempranillo evaluated in the present study clearly differed in the time from fruit set to maturity (TSS of ca. 22 °Brix), particularly in the length of the ripening period (veraison to maturity) (Fig. 1). In this way, those clones that had showed a later maturity in the present study (807, 814, 56, 1084, and 349) clearly showed lower levels of TSS two weeks after mid-veraison compared with the clones with early ripening behaviour (Fig. 3). These differences among clones were not associated with differences in the bunch size as indicates the weak correlation between these two parameters ( $R^2 = 0.015$ ,  $P = 0.180$ ). In addition, a significant but positive correlation was observed between the ripening time (number of days from mid-veraison to maturity) and the total leaf area measured at maturity ( $R^2 = 0.232$ ,  $P < 0.0001$ ). That is, the longer the ripening period, the greater the final leaf area. This result indicates that the short ripening period of some clones was not associated with a higher potential source of photosynthates, represented by the leaf area.

Grapevine phenology and berry ripening are traditionally assessed as temperature-dependent processes, and climate change is expected to advance grapevine phenological stages [6,8,9,43]. In agreement with this, elevated temperature applied from fruit set to maturity significantly hastened the phenological development of Tempranillo fruit-bearing cuttings (Fig. 1). This temperature effect was more evident at the onset of veraison, thus shortening to a greater extent the time elapsed

between fruit set and mid-veraison compared with the period comprised from mid-veraison to maturity. The results suggest that the effect of temperature depends on the phenological stages. In a field experiment using open top chambers, Sadras and Moran [7] reported that the temperature effect on grapevine phenology increased around bunch closure and declined gradually between the onset of rapid sugar accumulation and maturity. In line with this, Sadras and Petrie [8] concluded that the early maturity associated with higher temperatures was primarily driven by early onset of ripening, rather than higher rates of increase in TSS during the ripening period. In contrast, in experiments under controlled conditions using the microvine model, Rienth et al. [44] reported a delay in the onset of veraison, whereas the ripening period was favoured by high temperatures. In our case, besides the advancement of the onset of ripening (on a temporary basis), a clear increase in the rate of TSS accumulation between the onset of veraison and maturity was also observed, thus also contributing to the advanced maturity. A general acceleration of sugar accumulation under high temperature conditions was reported by Pastore et al. [45], whereas other experiments showed that sugar accumulation is not or only slightly affected, or sometimes even reduced [16,46]. The ripening of non-climateric fruits relies to a great extent on photoassimilation in the leaves, translocation and storage of these photoassimilates, which are reactions greatly affected by temperature, and the effect of temperature on sugar accumulation may depend on the amount of temperature increase. In this way, temperatures close to 40 °C have been reported to negatively impact on the photosynthetic supply of sugar to the berry [46] and on the amount of sugar transporter transcripts [47]. On the contrary, the milder conditions of our experiment led to an increased sugar accumulation. Leaf area is the main source of photosynthates, and canopy management is often considered to affect grape berry ripening. Restricting potential carbohydrate sources through a reduction of leaf area has been reported to delay the time of veraison [24] and maturity [43]. In the present study, total leaf area of plants grown under 28 °C/18 °C was, in general, lower compared with that of plants grown at 24 °C/14 °C, both at the onset of veraison and maturity (Fig. 2), and the leaf area to fruit mass ratio did not change significantly with temperature ( $P = 0.363$ , data not shown). Therefore, the advancement in phenology observed with elevated temperature cannot be explained by changes in leaf area or leaf area to fruit mass ratio induced by this treatment.

#### *4.2. Elevated temperature affected grape composition of Tempranillo clones*

Climate changes are particularly important for viticulture. Temperature is one of the environmental factors that dramatically affect grape chemical composition, and moderately higher temperatures usually lead to higher sugar concentration, lower total acidity, anthocyanin concentration and grape colour [48,49]. In the present study, grape technological and phenolic maturity, as well as phenological

development analyses helped to clearly discriminate samples under different temperature regimes in all the clones studied (Fig. 5). This discrimination was based on a higher tonality under elevated temperature, which may suggest changes in the anthocyanin composition, and lower titratable acidity of these berries (Fig. 5; Table S1). Warmer temperatures during ripening are responsible for a faster decrease of berry acidity, due to higher losses of malic acid [12]. Carbonell et al. [16] suggested that the higher malate catabolism under high temperature might partially result from imbalance in the expression and function of its specific tonoplasmic transporters, thus resulting in a higher malate export rate from the vacuole to the cytosol that may accelerate its catabolism. In addition, thermal up-regulation of genes encoding enzymes involved in malate degradation (malic enzyme and malate dehydrogenase) and mitochondrial transporters have been described in grape berries [44]. Acidity is not only important for flavour balance and organoleptic properties of wine, but also contribute to wine stability [3]. Grapes ripened under elevated temperature had also lower phenolic content, measured as total polyphenol index, and lower anthocyanin concentration, measured both two weeks after mid-veraison and at maturity (Table S1 and Fig. 4). High temperatures decrease anthocyanin concentration in grapes [15–17], and it has been associated with the inhibition of mRNA transcription of the anthocyanin biosynthetic genes, as well as chemical and/or enzymatic degradation [18,19,47]. Furthermore, temperature may also reduce the anthocyanin content, affecting its subcellular transport through the down-regulation of several transmembrane transporter-encoding genes involved in the import of anthocyanins in the vacuole [16].

#### *4.3. Tempranillo clones differed in the degree of total soluble solids and anthocyanin thermal decoupling*

Anthocyanin content increases linearly with sugar content from certain sugar concentration values (which can range from 6.8 to 13.3 °Brix, depending on the water availability, source:sink ratio or cultivar) onwards, and high temperatures can uncouple these traits [4,21]. In a field experiment with different grapevine cultivars, irrigation regimes and fruit loads, Sadras and Moran [21] reported that elevated temperature decoupled anthocyanins and sugars. In the same line, Martínez de Toda and Balda [50], who studied two vineyards located in two different climatic areas, described a decrease in the anthocyanin:sugar ratio of grapes ripened in the warmest area. In agreement with these works, a clear imbalance between these two berry traits was observed in the present study in the treatment with 28 °C/18 °C. However, in our case the decoupling was more likely to be caused by relative changes in the accumulation rates of these compounds with the temperature, rather than a delayed onset in the accumulation of anthocyanins [21]. In addition, the results indicate that, despite the narrow intra-varietal genetic variation described for Tempranillo cultivar in previous studies [42], the thermal

disruption of the anthocyanin:sugar relationship was different among clones, with the 501, 349 and 56 accessions being among the most affected, as shows the significant differences between the residual values at 24 °C/14 °C and 28 °C/18 °C. In contrast, the imbalance between these traits was less evident in the 86, 1052, 336, 318 and 1084 accessions. Differences in the degree of decoupling seem to be associated neither with plant phenology nor leaf area, as shows the weak correlations between the ratio TSS:anthocyanins at maturity and the elapsed time between mid-veraison and maturity ( $R^2 = 0.021$ ,  $P = 0.114$ ), total leaf area ( $R^2 = 0.028$ ,  $P = 0.070$ ) and source:sink ratio ( $R^2 = 0.012$ ,  $P = 0.237$ ). Similarly, Sadras and Moran [21] found no effect of changes in the source:sink ratio on the degree of thermal decoupling of anthocyanins and sugars in cv. Shiraz.

The intra-varietal diversity expressed by the clones of Tempranillo studied may be helpful to select clones more adapted to future climate conditions. In this way, among the less affected clones, probably the 86 and 336 would be the most interesting accessions from an adaptation point of view, since this clones not only did not show a marked thermal sugar:anthocyanin decoupling, but also they were able to maintain high concentrations of total skin anthocyanins, extractable anthocyanins and colour intensity under 28 °C/18 °C.

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## TABLES

Table 1. Yield, yield components and berry traits of thirteen clones of *Vitis vinifera* cv. Tempranillo grown from fruit set to maturity under two temperature regimes: 24 °C/14 °C and 28 °C/18 °C day/night. Values are means ± SE (n = 4-6). Probability values (P) for main effects of clone  $P_{(clone)}$ , temperature  $P_{(T)}$  and their interaction  $P_{(clone \times T)}$ .\*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant. Means with letters in common within the same parameter are not significantly different ( $P < 0.05$ ) according to LSD test. Values in bold highlight the highest values at 24 °C/14 °C and the lowest values at both temperatures.

| Accession               | Temperature      | Bunch weight (g) | Number of berries | Berry weight (g) | Diameter (mm)  | Relative skin mass (%) |
|-------------------------|------------------|------------------|-------------------|------------------|----------------|------------------------|
| Earlier maturing clones | 86 24 °C/14 °C   | 316.0 ± 25.2 a   | 257.4 ± 34.5 ab   | 1.24 ± 0.05 ab   | 12.0 ± 0.4 b-d | 27.4 ± 4.4             |
|                         | 28 °C/18 °C      | 244.3 ± 26.0 a-e | 224.2 ± 34.6 a-c  | 1.19 ± 0.17 ab   | 11.6 ± 0.5 b-f | 30.7 ± 1.0             |
|                         | 1052 24 °C/14 °C | 271.3 ± 11.7 a-c | 257.8 ± 15.8 ab   | 1.11 ± 0.06 b    | 11.4 ± 0.3 b-f | 26.3 ± 1.2             |
|                         | 28 °C/18 °C      | 265.3 ± 38.9 a-e | 230.0 ± 38.7 a-c  | 1.25 ± 0.08 ab   | 12.0 ± 0.3 b-d | 28.8 ± 2.3             |
|                         | 336 24 °C/14 °C  | 161.8 ± 46.8 de  | 114.0 ± 39.9 d    | 1.50 ± 0.13 a    | 12.8 ± 0.8 ab  | 32.1 ± 3.6             |
|                         | 28 °C/18 °C      | 156.7 ± 88.3 e   | 151.3 ± 82.1 cd   | 1.05 ± 0.03 b    | 10.5 ± 0.8 f   | 26.6 ± 7.4             |
|                         | 518 24 °C/14 °C  | 251.1 ± 41.7 a-e | 185.7 ± 48.0 b-d  | 1.33 ± 0.06 ab   | 12.5 ± 0.3 a-c | 24.6 ± 2.2             |
|                         | 28 °C/18 °C      | 313.9 ± 49.4 ab  | 313.3 ± 34.4 a    | 0.99 ± 0.04 b    | 10.6 ± 0.1 ef  | 19.9 ± 0.5             |
|                         | 501 24 °C/14 °C  | 300.3 ± 40.5 ab  | 255.4 ± 54.1 ab   | 1.23 ± 0.11 ab   | 12.1 ± 0.4 a-d | 24.9 ± 0.8             |
|                         | 28 °C/18 °C      | 310.4 ± 26.9 ab  | 275.2 ± 13.8 ab   | 1.19 ± 0.05 ab   | 11.2 ± 0.4 c-f | 30.9 ± 4.5             |
|                         | 280 24 °C/14 °C  | 219.9 ± 17.0 b-e | 183.2 ± 24.5 b-d  | 1.26 ± 0.06 ab   | 12.7 ± 0.7 ab  | 28.1 ± 1.6             |
|                         | 28 °C/18 °C      | 179.5 ± 31.9 b-e | 159.8 ± 28.1 cd   | 1.06 ± 0.16 b    | 11.1 ± 0.8 c-f | 31.9 ± 4.7             |
|                         | 825 24 °C/14 °C  | 262.3 ± 56.5 a-e | 195.4 ± 43.2 b-d  | 1.31 ± 0.08 ab   | 12.1 ± 0.3 a-d | 26.0 ± 3.5             |
|                         | 28 °C/18 °C      | 279.9 ± 37.2 a-b | 238.4 ± 44.5 a-c  | 1.22 ± 0.08 ab   | 12.0 ± 0.3 b-d | 26.8 ± 2.3             |
|                         | 318 24 °C/14 °C  | 226.8 ± 25.8 b-e | 160.0 ± 20.9 cd   | 1.42 ± 0.09 a    | 13.3 ± 0.3 a   | 28.3 ± 0.6             |
|                         | 28 °C/18 °C      | 219.2 ± 31.7 b-e | 166.0 ± 29.5 cd   | 1.30 ± 0.07 ab   | 11.7 ± 0.3 b-f | 27.3 ± 1.8             |
| Later maturing clones   | 807 24 °C/14 °C  | 224.2 ± 24.9 b-e | 207.4 ± 43.2 a-d  | 1.10 ± 0.12 b    | 11.8 ± 0.2 b-e | 33.9 ± 1.9             |
|                         | 28 °C/18 °C      | 262.9 ± 52.1 a-e | 275.2 ± 44.5 ab   | 1.18 ± 0.11 ab   | 11.1 ± 0.6 c-f | 25.3 ± 1.8             |
|                         | 814 24 °C/14 °C  | 260.9 ± 35.5 a-e | 291.0 ± 37.2 a    | 1.13 ± 0.08 b    | 11.1 ± 0.6 c-f | 30.8 ± 0.9             |
|                         | 28 °C/18 °C      | 272.3 ± 31.4 ab  | 285.8 ± 52.9 a    | 1.03 ± 0.07 b    | 10.6 ± 0.5 f   | 29.7 ± 3.3             |
|                         | 56 24 °C/14 °C   | 173.0 ± 39.2 c-e | 165.0 ± 15.2 cd   | 1.14 ± 0.09 ab   | 11.5 ± 0.3 b-f | 32.6 ± 1.0             |
|                         | 28 °C/18 °C      | 262.9 ± 28.8 a-e | 196.3 ± 9.0 a-d   | 1.11 ± 0.07 b    | 11.3 ± 0.5 b-f | 23.1 ± 1.7             |
|                         | 1084 24 °C/14 °C | 240.4 ± 15.2 a-e | 240.5 ± 13.4 a-c  | 1.21 ± 0.20 ab   | 11.1 ± 0.3 c-f | 32.3 ± 2.3             |
|                         | 28 °C/18 °C      | 216.8 ± 12.0 b-e | 219.8 ± 12.2 a-c  | 1.06 ± 0.08 b    | 10.7 ± 0.4 ef  | 30.6 ± 3.1             |
|                         | 349 24 °C/14 °C  | 162.1 ± 14.8 de  | 152.7 ± 24.0 cd   | 1.18 ± 0.09 ab   | 11.7 ± 0.7 b-f | 31.8 ± 2.8             |
|                         | 28 °C/18 °C      | 219.9 ± 9.6 b-e  | 209.3 ± 20.7 a-d  | 1.01 ± 0.09 b    | 10.7 ± 0.5 d-f | 35.0 ± 5.4             |
| $P_{(clone)}$           |                  | **               | ***               | *                | ***            | ns                     |
| $P_{(T)}$               |                  | ns               | ns                | *                | *              | ns                     |
| $P_{(clone \times T)}$  |                  | ns               | ns                | ns               | ns             | ns                     |

**FIGURES**

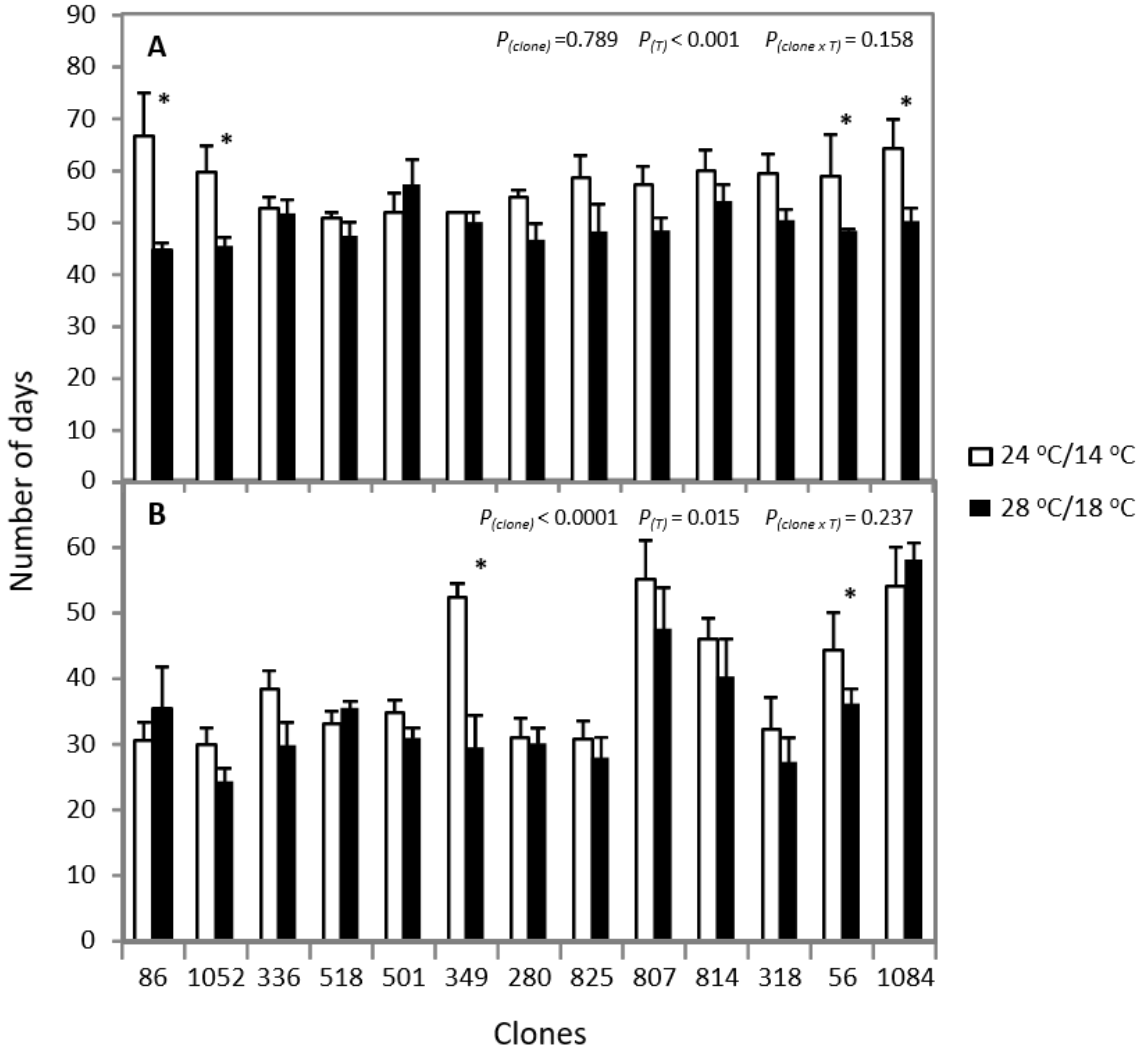


Fig. 1. Elapsed days from fruit set to mid-veraison (A) and from mid-veraison to maturity (B) of fruit-bearing cuttings of thirteen clones of Tempranillo grown at 24 °C/14 °C and 28 °C/18 °C (day/night) from fruit set to maturity. Bars are means ± SE, n = 4-6. Probability values (*P*) for main effects of clone  $P_{(clone)}$ ; temperature,  $P_{(T)}$ ; and their interaction  $P_{(clone \times T)}$ . Asterisks indicate significant differences between the two temperature regimes within each clone.

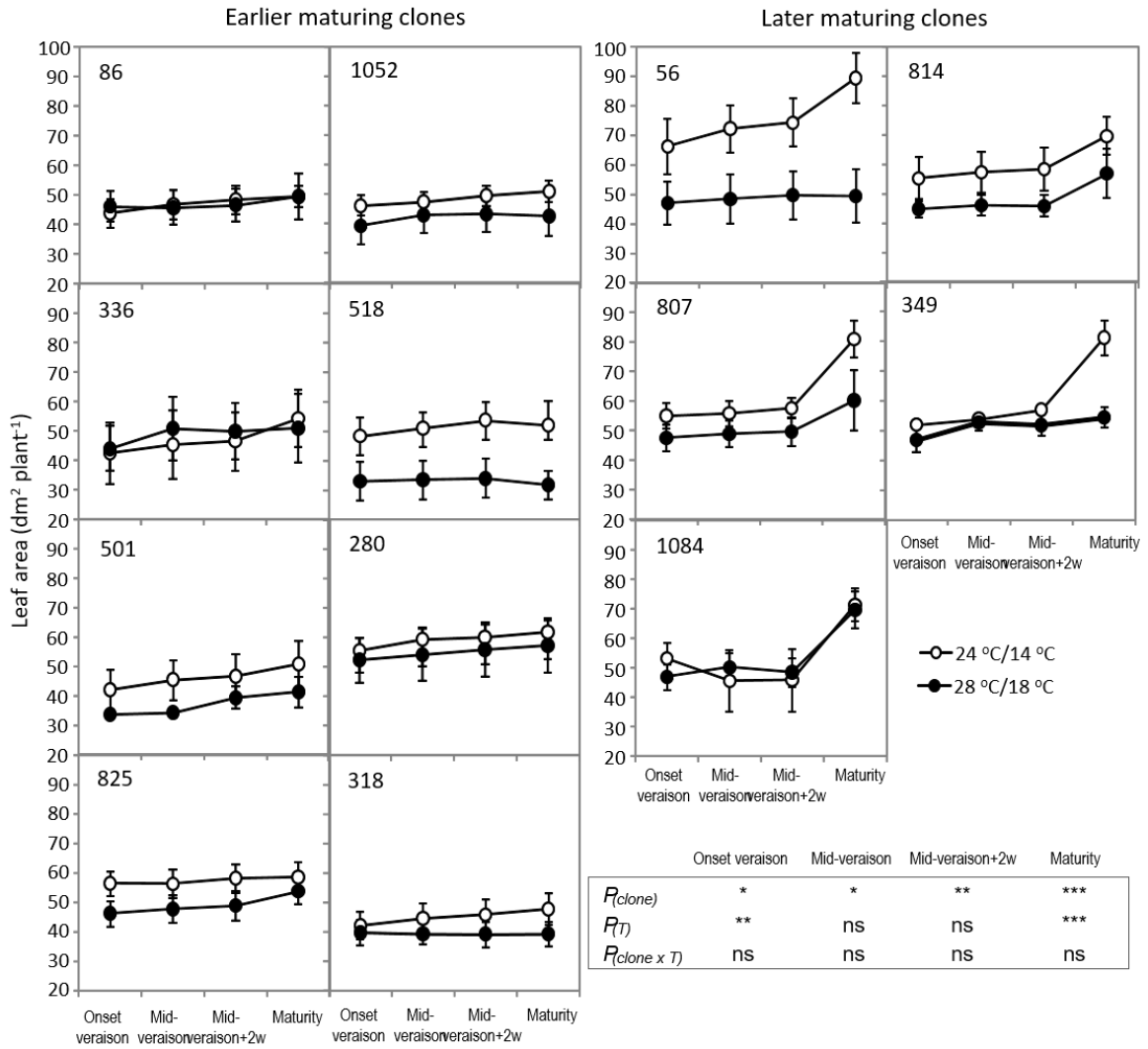


Fig. 2. Evolution of total leaf area of thirteen clones of Tempranillo grown at 24 °C/14 °C (day/night) and 28 °C/18 °C from fruit set to maturity. Points are means  $\pm$  SE, n = 4-6. Probability values ( $P$ ) for main effects of clone,  $P_{clone}$ ; temperature,  $P_T$ ; and their interaction  $P_{clone \times T}$ . \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant.

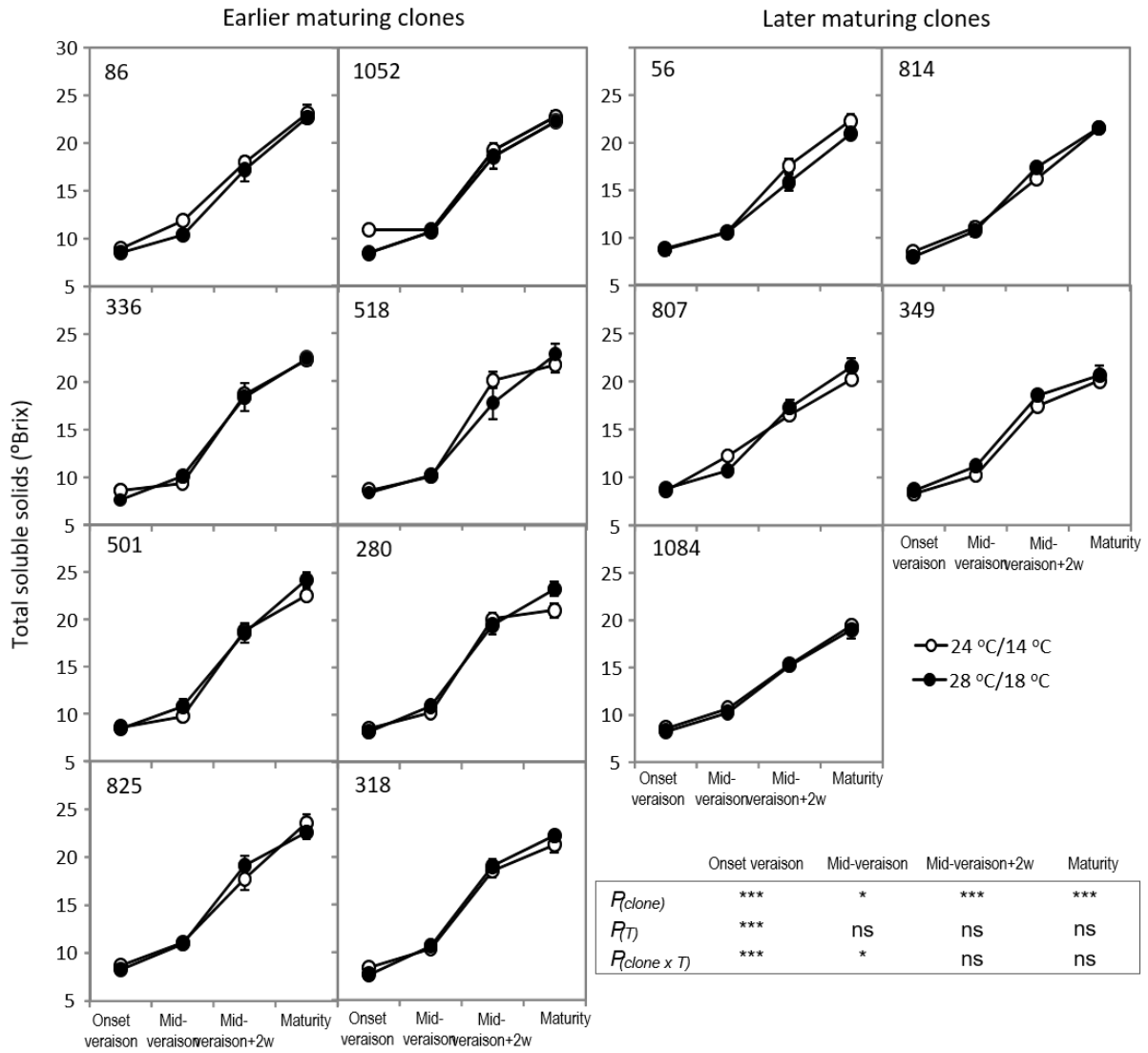


Fig. 3. Evolution of total soluble solid concentration throughout berry development of thirteen clones of Tempranillo grown at 24 °C/14 °C and 28 °C/18 °C (day/night) from fruit set to maturity. Points are means  $\pm$  SE,  $n = 4-6$ . Probability values ( $P$ ) for main effects of clone,  $P_{(clone)}$ ; temperature,  $P_{(T)}$ ; and their interaction  $P_{(clone \times T)}$ . \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant.

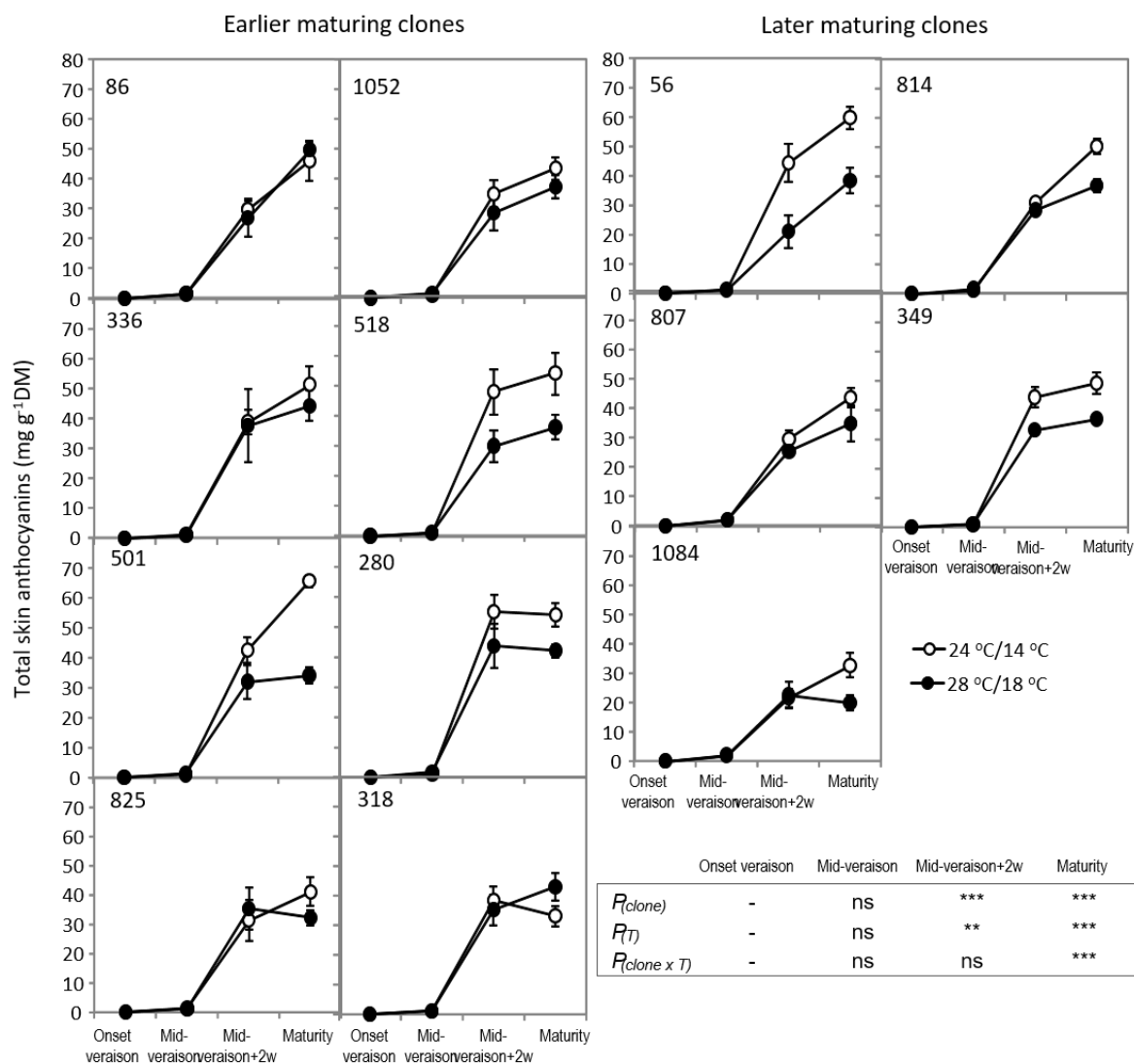


Fig. 4. Evolution of total skin anthocyanin concentration throughout berry development of thirteen clones of Tempranillo grown at 24 °C/14 °C and 28 °C/18 °C (day/night) from fruit set to maturity. Points are means  $\pm$  SE,  $n = 4-6$ . Probability values ( $P$ ) for main effects of clone,  $P_{(clone)}$ ; temperature,  $P_{(T)}$ ; and their interaction  $P_{(clone \times T)}$ . \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant.

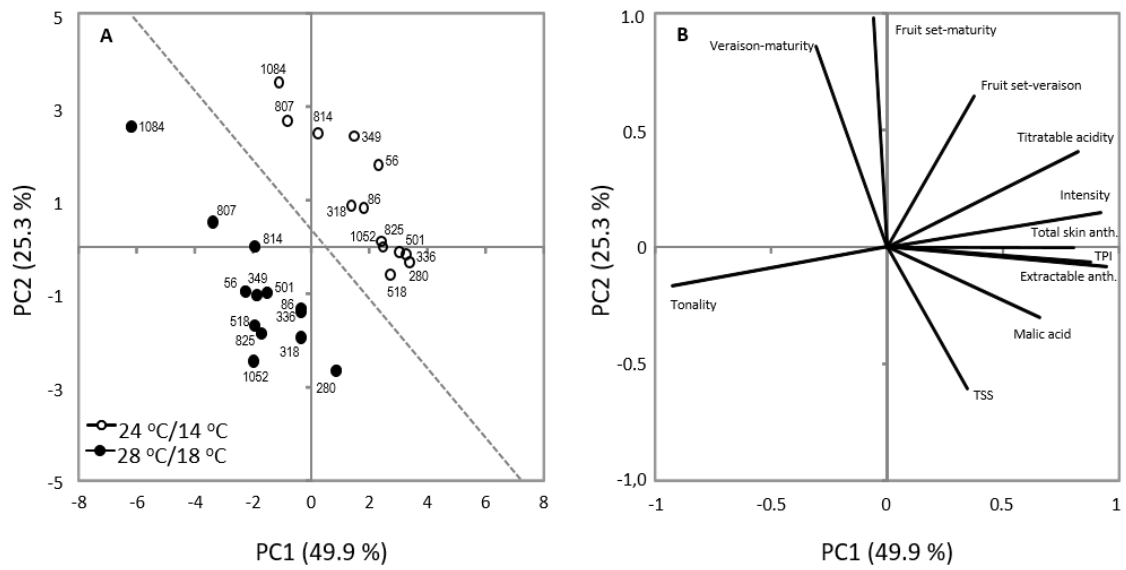


Fig. 5. Principal component analysis of phenological development and grape technological and phenolic maturity: score (A) and loading plot (B). TPI, total polyphenol index; anth, anthocyanins. The dash line indicates the separation between temperature regimes along PC1.

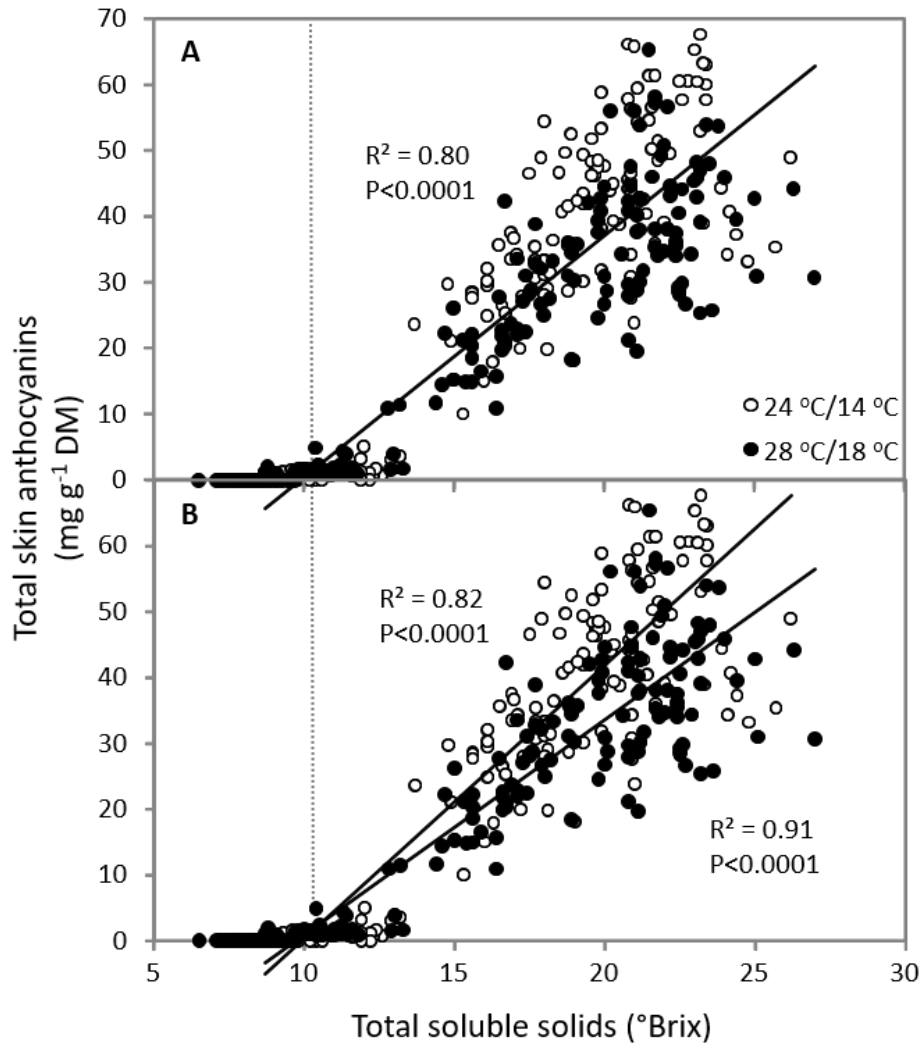


Fig. 6. Relationship between total soluble solids and total skin anthocyanins in berries of thirteen clones of Tempranillo under two temperature regimes: 24 °C/14 °C and 28 °C/18 °C (day/night) (A) and effect of temperature on the TSS:anthocyanins relationship (B). The continuous lines represent the fitted regressions, taking into account only those values within or close to the approximately linear phase where TSS and anthocyanins increased in parallel (samples taken at mid-veraison, two weeks after mid-veraison and maturity). Values of the sampling point corresponding to the onset of veraison (those on the left of the dash line) were not included in the linear regression analysis.



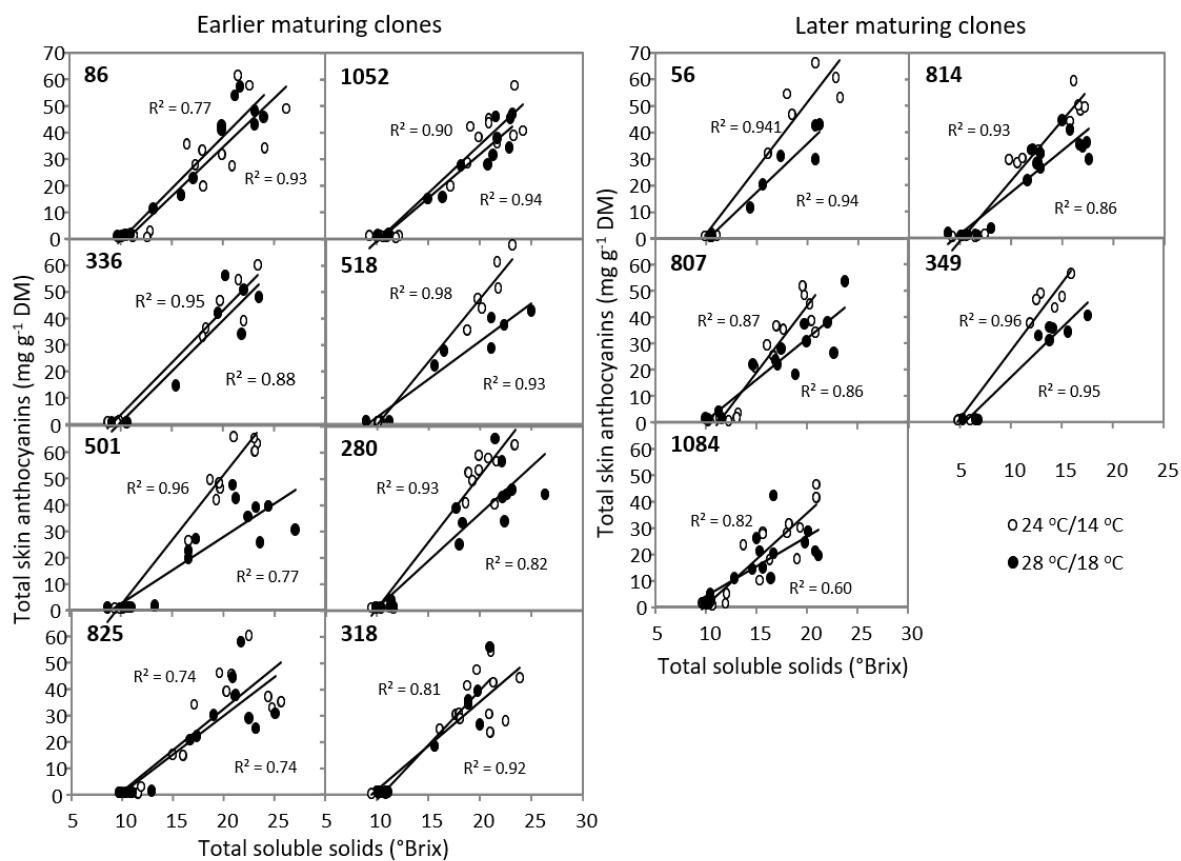


Fig. 7. Effect of temperature on the relationship between total soluble solids and total skin anthocyanins within the thirteen clones of Tempranillo. The lines represent the fitted regressions ( $P < 0.0001$  in all the cases), taking into account only those values within or close to the approximately linear phase where TSS and anthocyanins increased in parallel (samples taken at mid-veraison, two weeks after mid-veraison and maturity).

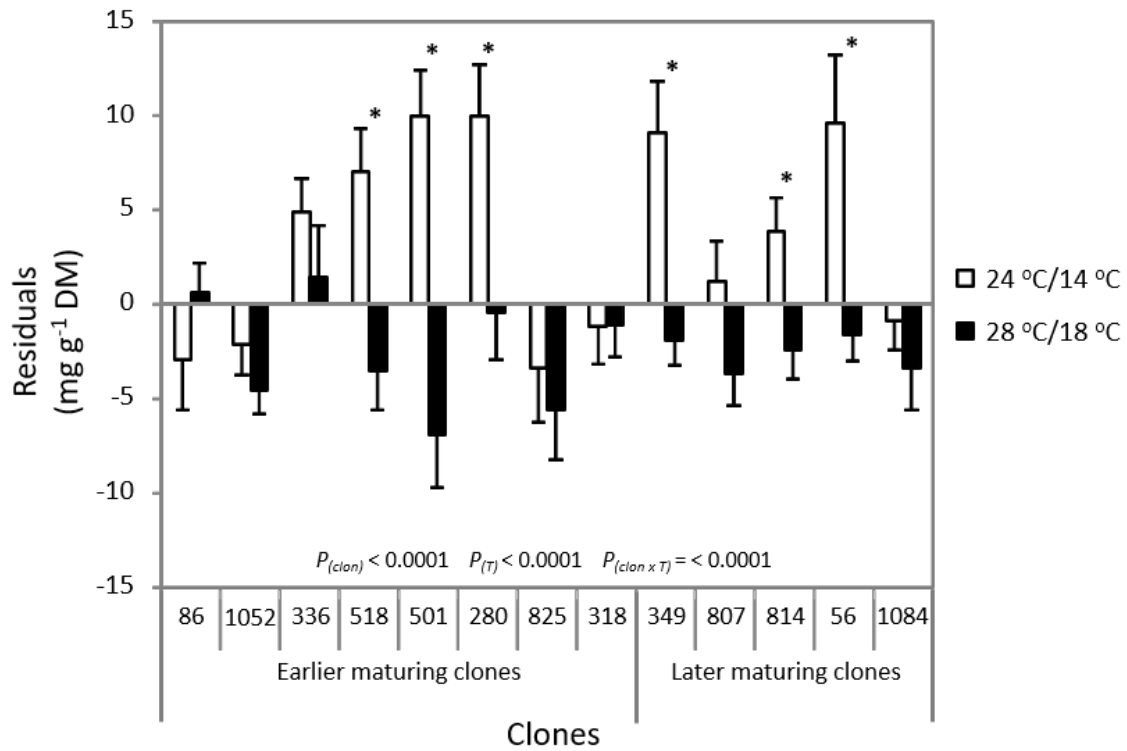


Fig. 8. Residuals of regressions comparing temperature regimes (24 °C/14 °C and 28 °C/18 °C, day/night) for the thirteen clones of Tempranillo. Bars are means  $\pm$  SE,  $n = 3-6$ . Probability values ( $P$ ) for main effects of clone,  $P(\text{clone})$ ; temperature,  $P(T)$ ; and their interaction  $P(\text{clone} \times T)$ . Asterisks indicate significant differences between the two temperature regimes within each clone.

## SUPPLEMENTARY MATERIAL

Table S1. Phenolic and technological quality parameters of mature grapes of thirteen clones of *Vitis vinifera* cv. Tempranillo grown from fruit set to maturity under two temperature regimes: 24 °C/14 °C and 28 °C/18 °C day/night. Values are means ± SE (n = 4-6). Probability values (*P*) for main effects of clone *P*(clone), temperature *P*(T) and their interaction *P*(clone × T).\*\*\*, *P* < 0.001; \*\*, *P* < 0.01; \*, *P* < 0.05; ns, not significant. Means with letters in common within the same parameter are not significantly different (*P* < 0.05) according to LSD test.

| Accession               | Temperature          | Titratable acidity (g L <sup>-1</sup> ) | Malic acid (g L <sup>-1</sup> ) | Extractable anthocyanins (mg L <sup>-1</sup> ) | Total polyphenol index | Colour intensity (OD <sub>420nm+520nm+620nm</sub> ) | Tonality (OD <sub>420nm/520nm</sub> ) |                 |
|-------------------------|----------------------|---|---------------------------------|--|------------------------|---|---------------------------------------|-----------------|
| Earlier maturing clones | 86                   | 24 °C/14 °C                             | 7.2 ± 0.4 b-d                   | 6.3 ± 0.6 a-c                                  | 324.8 ± 31.6 c-g       | 52.4 ± 1.5 a-e                                      | 1.8 ± 0.2 d-i                         | 0.48 ± 0.01 e-j |
|                         |                      | 28 °C/18 °C                             | 5.2 ± 0.5 ef                    | 4.5 ± 0.6 c-g                                  | 296.1 ± 33.1 b-f       | 48.1 ± 4.7 b-f                                      | 1.7 ± 0.2 e-j                         | 0.52 ± 0.02 h-j |
|                         | 1052                 | 24 °C/14 °C                             | 7.1 ± 0.4 b-d                   | 5.1 ± 0.4 a-f                                  | 390.4 ± 35.4 ab        | 56.0 ± 1.5 a-c                                      | 2.5 ± 0.2 a-c                         | 0.47 ± 0.01 h-j |
|                         |                      | 28 °C/18 °C                             | 4.9 ± 0.2 f                     | 4.7 ± 0.3 a-g                                  | 223.5 ± 33.9 gh        | 44.2 ± 4.0 ef                                       | 1.3 ± 0.2 g-k                         | 0.61 ± 0.05 b-e |
|                         | 336                  | 24 °C/14 °C                             | 8.5 ± 1.5 ab                    | 6.1 ± 0.4 a-d                                  | 330.8 ± 8.6 a-f        | 60.3 ± 4.4 a  | 2.8 ± 0.1 ab                          | 0.46 ± 0.01 i-j |
|                         |                      | 28 °C/18 °C                             | 4.5 ± 0.4 f                     | 5.3 ± 0.4 a-f                                  | 299.0 ± 33.5 b-g       | 49.0 ± 4.5 a-f                                      | 1.6 ± 0.4 e-k                         | 0.56 ± 0.03 d-h |
|                         | 518                  | 24 °C/14 °C                             | 7.8 ± 0.4 a-c                   | 5.6 ± 0.3 a-e                                  | 387.6 ± 35.4 a-c       | 60.8 ± 3.9 a  | 2.1 ± 0.3 b-f                         | 0.49 ± 0.03 g-j |
|                         |                      | 28 °C/18 °C                             | 4.1 ± 0.3 f                     | 5.0 ± 0.3 a-f                                  | 243.8 ± 65.4 e-h       | 47.1 ± 4.7 c-f                                      | 1.2 ± 0.7 h-l                         | 0.63 ± 0.02 b-d |
|                         | 501                  | 24 °C/14 °C                             | 7.9 ± 0.6 ab                    | 5.7 ± 0.8 a-e                                  | 381.0 ± 41.8 a-c       | 54.9 ± 2.8 a-d                                      | 2.3 ± 0.3 b-d                         | 0.45 ± 0.01 j   |
|                         |                      | 28 °C/18 °C                             | 4.2 ± 0.2 f                     | 4.5 ± 0.1 c-g                                  | 229.3 ± 24.1 f-h       | 48.9 ± 3.3 b-f                                      | 1.2 ± 0.2 i-l                         | 0.66 ± 0.04 b   |
|                         | 280                  | 24 °C/14 °C                             | 7.2 ± 0.6 b-d                   | 5.4 ± 0.4 a-e                                  | 416.0 ± 36.6 a         | 60.1 ± 2.1 a  | 3.0 ± 0.5 a                           | 0.49 ± 0.01 h-j |
|                         |                      | 28 °C/18 °C                             | 5.3 ± 0.3 ef                    | 5.3 ± 0.3 a-e                                  | 354.6 ± 48.6 a-d       | 56.9 ± 2.3 ab                                       | 1.9 ± 0.3 c-h                         | 0.58 ± 0.02 b-f |
|                         | 825                  | 24 °C/14 °C                             | 8.8 ± 1.2 a                     | 6.5 ± 1.2 a                                    | 349.7 ± 57.2 a-d       | 53.0 ± 1.7 a-e                                      | 2.3 ± 0.3 b-e                         | 0.47 ± 0.02 i-j |
|                         |                      | 28 °C/18 °C                             | 5.0 ± 0.2 f                     | 4.7 ± 0.6 b-g                                  | 263.6 ± 16.5 d-g       | 45.6 ± 2.9 d-f                                      | 1.1 ± 0.1 j-l                         | 0.57 ± 0.03 c-g |
|                         | 318                  | 24 °C/14 °C                             | 8.3 ± 0.6 a                     | 6.4 ± 0.8 ab                                   | 329.6 ± 31.2 a-f       | 51.8 ± 5.7 a-e                                      | 1.9 ± 0.2 c-g                         | 0.49 ± 0.01 h-j |
|                         |                      | 28 °C/18 °C                             | 5.5 ± 0.4 ef                    | 5.7 ± 0.2 a-e                                  | 270.5 ± 31.7 d-g       | 48.7 ± 2.6 b-f                                      | 1.6 ± 0.1 f-k                         | 0.59 ± 0.05 b-e |
| Later maturing clones   | 807                  | 24 °C/14 °C                             | 7.5 ± 0.9 a-c                   | 3.4 ± 0.9 fg                                   | 236.1 ± 17.2 f-h       | 47.2 ± 6.2 c-f                                      | 1.6 ± 0.2 f-k                         | 0.52 ± 0.03 f-j |
|                         |                      | 28 °C/18 °C                             | 4.5 ± 0.2 f                     | 2.9 ± 0.6 g                                    | 223.7 ± 22.6 gh        | 45.4 ± 2.6 d-f                                      | 1.0 ± 0.1 kl                          | 0.65 ± 0.04 bc  |
|                         | 814                  | 24 °C/14 °C                             | 7.0 ± 0.5 b-d                   | 4.0 ± 0.6 e-g                                  | 301.2 ± 26.9 b-g       | 48.1 ± 2.1 b-f                                      | 1.9 ± 0.2 c-g                         | 0.50 ± 0.02 g-j |
|                         |                      | 28 °C/18 °C                             | 4.5 ± 0.2 f                     | 4.2 ± 0.7 d-g                                  | 267.5 ± 19.1 d-g       | 47.5 ± 3.1 c-f                                      | 1.2 ± 0.1 h-k                         | 0.64 ± 0.02 b-d |
|                         | 56                   | 24 °C/14 °C                             | 7.7 ± 0.9 a-c                   | 6.1 ± 0.6 a-d                                  | 356.1 ± 75.7 a-d       | 49.3 ± 5.9 a-f                                      | 2.5 ± 0.5 a-c                         | 0.49 ± 0.02 h-j |
|                         |                      | 28 °C/18 °C                             | 5.1 ± 0.4 ef                    | 5.7 ± 0.6 a-e                                  | 231.9 ± 31.0 f-h       | 40.2 ± 1.3 f  | 1.1 ± 0.1 j-l                         | 0.64 ± 0.04 b-d |
|                         | 1084                 | 24 °C/14 °C                             | 6.4 ± 0.2c-e                    | 4.9 ± 0.9 a-f                                  | 244.0 ± 20.5 e-g       | 48.2 ± 3.1 b-f                                      | 1.5 ± 0.2 f-k                         | 0.54 ± 0.02 e-i |
|                         |                      | 28 °C/18 °C                             | 4.2 ± 0.2 f                     | 3.4 ± 0.5 fg                                   | 143.9 ± 9.3 gh         | 40.3 ± 1.5 f  | 0.6 ± 0.0 l                           | 0.81 ± 0.02 a   |
|                         | 349                  | 24 °C/14 °C                             | 7.6 ± 0.5 a-c                   | 4.0 ± 0.7 d-g                                  | 343.0 ± 32.9 a-e       | 54.3 ± 2.8 a-e                                      | 2.9 ± 0.3 ab                          | 0.46 ± 0.01 i-j |
|                         |                      | 28 °C/18 °C                             | 5.5 ± 0.4 d-f                   | 5.1 ± 0.9 a-f                                  | 276.5 ± 14.0 c-g       | 47.0 ± 1.1 c-f                                      | 1.1 ± 0.1 j-l                         | 0.61 ± 0.02 b-e |
|                         | <i>P</i> (clone)     |   | ns                              | **   | ***                    | **  | ***                                   | ***             |
|                         | <i>P</i> (T)         |   | ***                             | **   | ***                    | ***   | ***                                   | ***             |
|                         | <i>P</i> (clone × T) |   | ns                              | ns   | ns                     | ns  | ns                                    | *               |

## **CHAPTER 2**

Growth performance and carbon partitioning of grapevine Tempranillo clones under simulated climate change (elevated CO<sub>2</sub> and temperature) scenarios

*Article under revision in the Journal of Plant Physiology*



# Growth performance and carbon partitioning of grapevine Tempranillo clones under simulated climate change (elevated CO<sub>2</sub> and temperature) scenarios

## ABSTRACT

Atmospheric CO<sub>2</sub> levels and global temperatures are expected to rise in the next decades and viticulture must face these changes. Within this context, exploiting the intra-varietal diversity of grapevine (*Vitis vinifera* L.) can be a useful tool for the adaptation of this crop to climate change. The aim of the present work was to study the effect of elevated temperature and high levels of atmospheric CO<sub>2</sub>, both individually and combined, on growth, phenology and carbon partitioning of five clones of the cultivar Tempranillo (RJ43, CL306, T3, VN31 and 1084). The hypothesis that clones within the same variety that differ in their phenological development may respond in a different manner to the above mentioned environmental factors from a physiological point of view was tested. Grapevine fruit-bearing cuttings were grown from fruit set to maturity under two temperature regimes: ambient (T) vs. elevated (ambient + 4 °C; T+4), combined with two CO<sub>2</sub> levels: ambient (ca. 400 ppm; ACO<sub>2</sub>) vs. elevated (700 ppm; ECO<sub>2</sub>), in temperature-gradient greenhouses (TGGs). Considering all the clones, elevated temperature hastened grape development and increased vegetative growth, but reduced grape production, the latter being most likely associated with the heat waves recorded during the experiment. Plants in the elevated CO<sub>2</sub> treatments showed a higher photosynthetic activity at veraison and an increased vegetative growth, but they showed signs of photosynthetic acclimation to ECO<sub>2</sub> at maturity according to the C:N ratio, especially when combined with high temperature. The combination of ECO<sub>2</sub> and T+4, mimicking climate change environmental conditions, showed additive effects in some of the parameters analysed. Clones showed differences in their phenological development, which conditioned some responses to elevated CO<sub>2</sub> and temperature in terms of vegetative production and C partitioning into different organs. The work adds new knowledge on the use of different grapevine clones, that can be useful to improve the viticultural efficiency in future climate change scenarios.

**Keywords:** Climate change; Grapevine (*Vitis vinifera*); Genetic variability; <sup>13</sup>C isotopic composition; Phenology; Vegetative and reproductive growth

## 1. INTRODUCTION

As a result of human activity, emissions of greenhouse gases (GHG) have greatly risen during the last centuries, and especially during the last decades [1]. It has been proved that these atmospheric modifications will have climatic consequences. The combination of these effects is normally referred as “Climate Change” and it includes, among others, changes in the temperature regime and the atmospheric CO<sub>2</sub> concentration [1]. The rise in the concentration of GHG in the atmosphere reduces the capacity to lose heat, increasing the global mean temperature. According to the Intergovernmental Panel on Climate Change (IPCC), some of the worst scenarios for the mean global temperature and the air CO<sub>2</sub> concentration (RCP 6.0 and RCP 8.5 of the IPCC) predict for 2100 a rise between  $2.2 \pm 0.5$  °C and  $3.7 \pm 0.7$  °C of the average global surface temperature and an atmospheric CO<sub>2</sub> concentration between 669.7 ppm and 935.9 [2]. The fact that plant performance is highly dependent on environmental conditions makes plants especially sensitive to climate change in terms of photosynthetic efficiency, reproductive development and plant architecture [3,4]. From an agricultural perspective, the key point is to understand how simultaneous increases of CO<sub>2</sub> and temperature will affect different groups of plants [5].

Grapevine is one of the most widely cultivated crops in the world, and it is grown on nearly every continent [6]. The impact of the predicted global change on grapevine growth and development has been studied due to its economic and cultural value. The increase of average temperatures has been reported to rise grapevine photosynthetic activity [7,8], although at extreme temperatures, photosynthesis can be inhibited [9,10]. Warmer temperatures also affect grapevine growth, phenological timing [9,11], and can impact the grape quality balance [12]. Rising CO<sub>2</sub> levels, in turn, can increase vegetative growth at ambient temperature [13,14], as they enhance photosynthetic activity. Nevertheless, long-term exposures to elevated CO<sub>2</sub> concentration often lead to biochemical and molecular changes resulting in photosynthetic down-regulation (referred also as photosynthetic acclimation) [15–17]. In the literature, the effects of climate change (elevated CO<sub>2</sub> and temperature) on grapevine physiology have been investigated mainly as individual effects rather than in combination. However, in the future, plants will not experience climate change factors individually [3] but simultaneously. Besides, the effects of a particular environmental factor can be modulated by the presence of others [5,14,18].

The design of adaptive strategies to mitigate the potential negative impact of climate change on grapevine performance is still a matter of discussion. Proposed adaptation strategies to new climatic conditions includes changes in the cultivation areas [19], vineyard practices (i.e. canopy management, modification of cultivation and field architecture, etc.) [19], or the use of genotypes, both for scion and

rootstock, more adapted to the new environmental conditions, as new varieties with late ripening [20] and the use of intra-varietal diversity [19,21]. New varieties are difficult to introduce in viticulture due to the narrow dependency of consumer appreciation, often linked to a certain particular wine taste [22]. Therefore, the selection of clones of reputed cultivars, more adapted to the foreseen climate conditions, can help to maintain wine typicity, associated with the use of varieties very attached to certain regions (as Tempranillo in Spain, Merlot in France, Sangiovese in Italy or Fernao Pires in Portugal) [23]. Red Tempranillo is a wine variety extensively grown in Spain, nowadays spread to many other countries, with a wide number of well characterised clones [24]. This makes it a good candidate to explore its intra-cultivar genetic diversity looking for clones better adapted to future environmental conditions.

The aim of the present work was to determine the phenological, physiological and growth response of five clones of Tempranillo (RJ43, CL306, T3, VN31 and 1084) to changes in air temperature and atmospheric CO<sub>2</sub> concentration (both individually and combined) predicted by 2100. The hypothesis that clones within the same variety that differ in their phenological development may respond in a different manner to the above mentioned environmental factors from a physiological point of view was tested. To this end, fruit-bearing cuttings of grapevine were grown in Temperature Gradient Greenhouses (TGGs), where climate change conditions were simulated under a high level of control [25]. The fruit-bearing cutting system helps to translate this kind of studies (extremely complex to execute in the vineyard due to the difficulty to control environmental variables, heterogeneity or economic cost) to facilities under controlled conditions. In addition, this plant model system has been validated, revealing many similarities with plants grown in the field [26].

## **2. MATERIAL AND METHODS**

### *2.1. Plant material: origin and development*

The plant material consisted of five clones of grapevine (*Vitis vinifera* L.) cv. Tempranillo: three commercial clones (VN31, RJ43 and CL306; the last two being the Tempranillo clones most widely used in Spain); and two non-commercialised accessions (1084 and T3). Material of VN31 was provided by the nursery Vitis Navarra S.A. (Navarra, Spain); RJ43, CL306 and T3 by the Estación de Viticultura y Enología de Navarra (EVENA, Navarra, Spain); and 1084 was selected by the Institute of Sciences of Wine and Vine (La Rioja, Spain). These Tempranillo clones had been previously characterized in the field on the basis of agronomic characters and life cycle duration: RJ43 had been characterised as a clone with an average maturity period [27]; CL306 and T3 as short phenological development clones [28–30]; and VN31 and 1084 had been defined as clones with long phenological development [31,32].



Dormant cuttings (400-500 mm-long) were selected and grown according to a protocol adapted from Mullins and Rajasekaran [33]. Rooting was induced by applying a solution of indole butyric acid (300 mg L<sup>-1</sup>) and maintained in a heat-bed (27 °C) placed within a cold room (5 °C). Once the roots were developed, the cuttings were transplanted to pots of 0.8 L, filled with 2:1 peat:perlite (v/v), and moved to a pre-culture greenhouse. They grew at 25 °C/15 °C (day/night) until fruit set (from March to May 2016). The light consisted on natural daylight supplemented with high-pressure metal halide lamps (OSRAM, Augsburg, Germany), achieving a minimum photosynthetic photon flux density (PPFD) of 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at plant level. The photoperiod had a diary length of 15 hours-light/9 hours-darkness. After bud-break, plant growth was manipulated to obtain a single bunch per plant, and the vegetation was controlled by manual pruning until fruit set. When plants reached this stage, those that presented similar phenological and bunch size characteristics were transferred to 13 L pots using a 2:1 peat:perlite mixture (v/v). One week later, they were moved to Temperature Gradient Greenhouses (TGGs) located in the campus of the University of Navarra (42 °48'N, 1 °40'W; Pamplona, Navarra, Spain) for the application of the CO<sub>2</sub> and temperature treatments. Irrigation (both at the pre-culture greenhouse and at the TGGs) was performed using the nutritive solution described by Ollat et al. [34].

## *2.2. Growth of plants in Temperature Gradient Greenhouses (TGGs)*

TGGs are special infrastructures that allow the growth of plants at different environmental conditions. They contain three temperature modules and a gradient of temperature is created along them, from module 1 of ambient temperature to module 3 of ambient temperature + 4 °C; in addition, CO<sub>2</sub> can be injected inside modifying the air CO<sub>2</sub> concentration [25]. The plants, homogeneously selected to have similar fruit set date and bunch size, were distributed in the most extreme modules (modules 1 and 3) of four TGGs, locating them systematically separated and leaving the central module free of plants. The air CO<sub>2</sub> concentration was modified in two TGGs, upraising it up to 700 ppm, meanwhile the air CO<sub>2</sub> concentration of the other two TGGs was not modified and it corresponded to the current atmospheric CO<sub>2</sub> concentration (ca. 400 ppm). Therefore, the treatments applied to the five Tempranillo clones were the combination of: i) two temperature regimes, ambient (T) and ambient + 4 °C (T+4); and ii) two CO<sub>2</sub> levels, ambient ca. 400 ppm (ACO<sub>2</sub>) and elevated 700 ppm (ECO<sub>2</sub>). That is, four treatments with 6 to 8 plants per clone within each treatment. Treatments were applied from fruit set (May 2016) to maturity, the later considered when the berries in the bunch of each plant reached a total soluble sugar solid content (TSS) of ca. 22 °Brix (September 2016).

### *2.3. Carbon labelling and sampling*

The CO<sub>2</sub> injection (CO<sub>2</sub> provided by Carbueros Metálicos, Spain) was not only used to change the CO<sub>2</sub> concentration of the air but also to modify the isotopic signature in the elevated CO<sub>2</sub> treatment. Therefore, the isotopic labelling period lasted from fruit set to maturity. The air in the ECO<sub>2</sub> treatment had a carbon isotopic composition ( $\delta^{13}\text{C}$ ) of -21.4 ‰, meanwhile the air  $\delta^{13}\text{C}$  in the ACO<sub>2</sub> treatment was -11.4 ‰. The air inside the TGGs was sampled using 50 mL syringes (SGE International Pty Ltd, Ringwood, Vic., Australia) and kept in 10 mL pressurised vacutainers (BD Vacutainers, Plymouth, UK). Air samples were analysed by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS), as described in more detail below.

### *2.4. Phenological development and plant growth*

The phenological development was determined according to the elapsed time between fruit set (beginning of the treatments) and maturity. Fruit set dates were assessed visually, and maturity was established by sampling periodically two berries and measuring the level of TSS in the must using a refractometer (Abbe Digital 315RS, Zuzi, Beriain, Spain). Every plant was assessed individually.

Leaf area was estimated at the onset of veraison (when berries had already softened and just began to colour), veraison (50 % of red berries in the bunch), one and two weeks after veraison, and at maturity, measuring the shoot length and using a regression model adapted from Costanza et al. [35] for Tempranillo: Leaf area (dm<sup>2</sup>) = 13.859x + 200.33; R<sup>2</sup> = 0.9239 (x = shoot length) [32]. The final dry weight of leaves, stem and roots were considered for determining the final vegetative growth of the plants after oven-drying the plant material at 80 °C until constant weight. Bunch production was determined according to the bunch fresh weight, the number of berries per bunch and the fresh weight of individual berries.

### *2.5. Photosynthetic activity*

Photosynthetic activity was measured at the onset of veraison, in young and fully expanded leaves, using a portable photosynthesis system (LCi-SD with the PLUS5 compact light unit, ADC BioScientific, England). The measurements were carried out under the corresponding CO<sub>2</sub> and temperature growth conditions of each plant, using a 1200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  LED light with a wave length emission between 380 and 840 nm. Net photosynthesis, transpiration and stomatal conductance were measured.

## 2.6. Carbon and nitrogen concentration, and carbon isotopic composition ( $\delta^{13}\text{C}$ )

C and N, as well as carbon isotopic analysis, were performed in the 1084 and T3 accessions. These accessions were chosen because, in the present experiment, they were characterised as clones with long and short phenological development, respectively, while showing the most similar bunch sizes. C and N concentration were determined in leaf, shoot, cutting, root and berry samples taken at maturity. Samples were dried, ground to powder and analysed using an elemental analyser (vario MICROCube, Elementar, Hanau, Germany), according to the methodology described by Delgado et al. [36]. To study the plant isotopic signature of  $^{13}\text{C}$ , leaf and grape samples were taken at the onset of veraison, one week after veraison and maturity, whereas root, stem and cutting samples were taken at maturity.

The  $^{13}\text{C}/^{12}\text{C}$  ratio (R) of plant material was determined using an isotope ratio mass spectrometer (IsoPrime 100, Cheadle, UK) as referred in Delgado et al. [36]. The  $^{13}\text{C}/^{12}\text{C}$  (R) ratio of air samples was determined by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) using an Agilent 6890 Gas Chromatograph coupled to an isotope ratio mass spectrometer Delta<sup>plus</sup> via a GC-C Combustion III interphase (ThermoFinnigan), according to Salazar-Parra et al. [17]. The  $^{13}\text{C}/^{12}\text{C}$  (R) ratios of the plant material and air samples were expressed as  $\delta^{13}\text{C}$  using Pee Dee Belemnite (V-PDB) as international standard and calculated according to the formula:  $\delta^{13}\text{C}(\text{‰}) = [((R \text{ sample}) / (R \text{ standard})) - 1] \times 1000$  [37].

## 2.7. Statistical analysis

The statistical analysis was performed with R (3.5.1). Data were analysed using a three-way ANOVA (clone, temperature and  $\text{CO}_2$  concentration). A Fisher's least significant difference (LSD) was carried out as a post-hoc test.

# 3. RESULTS

## 3.1. Temperature

The record of the temperatures confirmed the alteration of 4 °C between the two temperature regimes assayed, ambient (T) and elevated temperature (T+4) throughout the experiment (Figure 1). In addition, the number of extreme temperature events clearly differed between temperature treatments, with 44 and 16 days with maximum temperature above 35 °C and 40 °C, respectively, in T+4; and 21 days above 35 °C in T (plants were not exposed to temperatures higher than 40 °C in the T treatment). Five heat wave events, defined as five consecutive days with maximum temperatures higher than 35 °C or three consecutive days with maximum temperatures higher than 40 °C, according

to Hayman et al. [38], occurred in the T+4 treatments. In contrast, high temperatures lasting several consecutive days rarely occurred in the T treatment.

### *3.2. Phenological development*

The time period between fruit set and maturity was significantly longer in 1084 (up to 15 days) compared with the other clones, which showed a similar behaviour (Figure 2A). Even though the statistical analysis did not show any significant interactions among factors (clone, temperature and CO<sub>2</sub>), when all the treatments were compared simultaneously (Figure 2A), climate change conditions (the combination of ECO<sub>2</sub> and T+4) shortened in 9 days the phenological development of RJ43. In contrast, this trend was not so evident in the rest of clones. Taking into account all the clones, elevated temperature reduced slightly but significantly the total time needed to reach fruit maturity, meanwhile CO<sub>2</sub> did not have any significant effect on plant phenology (Figure 2B).

### *3.3. Plant growth and bunch characteristics*

The clones studied showed different total leaf surface values from 1 week after veraison onwards, the variant 1084 having the largest leaf area (Figure 3A). In general, plants grown under elevated temperature had a higher total leaf area all over the experiment (onset of veraison,  $P_T < 0.001$ ; veraison,  $P_T < 0.001$ ; 1 week after veraison,  $P_T < 0.001$ ; maturity,  $P_T = 0.017$ ), whereas plants grown under ECO<sub>2</sub> showed an increased leaf area only at maturity ( $P_{CO_2} = 0.002$ ) (Figure 3B). At maturity, temperature and CO<sub>2</sub> had additive effects on plant leaf area when applied together (Figure 3B).

When analysed separately, both elevated temperature and elevated CO<sub>2</sub> increased significantly vegetative dry weight ( $P_T = 0.008$ ,  $P_{CO_2} < 0.001$ ) at maturity. Significant differences among clones were observed for leaf, stem and root dry weight, as well as for total vegetative production at maturity (Figure 4A). This was due to a higher vigour of 1084 in comparison to the other clones. In general, plants grown under climate change conditions (T+4/ECO<sub>2</sub>) had a higher vegetative production (Figure 4B), this effect being especially noticeable in VN31 and 1084 (Figure 4A).

Regarding bunch size and berry characteristics, VN31 had the highest bunch weight, number of grapes per bunch and individual berry weight, whereas 1084 was the variant with the lowest bunch size (bunch weight and number of berries per bunch), CL306 having the lowest individual berry weight (Table 1). Taking the five clones into consideration, elevated temperature significantly reduced grape yield and berry weight. In contrast, elevated CO<sub>2</sub> modified neither grape yield nor yield traits (Table 1). Elevated CO<sub>2</sub> combined with high temperature reduced especially the bunch size of the CL306 and RJ43 accessions from  $217.1 \pm 47.7$  g and  $193.3 \pm 39.3$  g, respectively, in the T/ACO<sub>2</sub> treatment, to  $134.9 \pm 32.2$  g and  $133.1 \pm 19.1$  g (CL306 and RJ43, respectively) in the T+4/ECO<sub>2</sub> treatment.

### 3.4. *Photosynthetic activity*

Considering all the clones, net photosynthesis ( $A_N$ ) and leaf transpiration ( $E$ ) did not change under elevated temperature, even though  $A_N$  was affected by T+4 when applied under  $ECO_2$  (Figure 5A). Stomatal conductance values ( $g_s$ ) rose with high temperature, considering all the clones and  $CO_2$  levels ( $P_T = 0.035$ ). Plants grown under  $ECO_2$  showed higher  $A_N$  and reduced  $g_s$  rates compared with  $ACO_2$  ( $A_N$ ,  $P_{CO_2} < 0.001$ ;  $g_s$ ,  $P_{CO_2} < 0.001$ ) (Figure 5A). Clones showed significant differences neither in  $A_N$ , in  $E$ , nor in their  $g_s$  measured at the onset of veraison, although the T3 accession had lower values of  $g_s$  than RJ43 when measured under  $ACO_2$  conditions (Figure 5B). Despite the absence of significant interactions among factors, the clones seemed to have different photosynthetic responses to the combination of  $ECO_2$  and T+4. Whereas CL306, T3, VN31 and 1084 had a significantly higher photosynthetic performance under climate change conditions (T+4/ $ECO_2$ ) compared with current conditions (T/ $ACO_2$ ), RJ43 maintained similar photosynthetic rates in both situations (Figure 5B).

### 3.5. *C and N concentration*

The 1084 and T3 accessions had a similar concentration of C, N and ratio C:N in the organs analysed (Table 2). Considering the two clones as a whole, plants grown under T+4 had a significantly higher concentration of C in the leaves and cutting, whereas plants grown under  $ECO_2$  had higher C in the cutting (Table 2). In general, N concentration in the leaf, berry and stem was reduced significantly in plants grown under  $ECO_2$ , although this effect of  $CO_2$  in the stem was more pronounced in the T3 accession when exposed to T+4.  $ECO_2$  also tended to reduce the N levels in the roots of 1084, but significantly increased those of T3, especially under ambient temperature, resulting in a significant interaction between clone and  $CO_2$  concentration (Table 2, Table S1).

The C:N ratio was significantly higher in the leaves of plants grown under T+4, as well as in the leaves, berries and stem of plants grown under  $ECO_2$  (Table 2). However, the effect of  $CO_2$  on the C:N ratio in the stem depended on the temperature regime and the clone, resulting in a significant interaction between temperature and  $CO_2$  and in another one between clone and  $CO_2$  (Table S1), the effect of  $ECO_2$  being statistically significant only under T+4 in the T3 accession. Additionally, the effect of  $ECO_2$  on the root C:N ratio also depended on the clone, increasing the ratio in 1084 but decreasing in T3 (Table 2). It is worth pointing out that plants of the 1084 accession growing under climate change conditions (T+4/ $ECO_2$ ) showed significantly higher C concentration in leaves and C:N ratio in leaves and berries compared with plants grown under current conditions (T/ $ACO_2$ ). In contrast, climate change conditions did not affect significantly the C:N ratio of leaves and berries of T3 plants (Table 2).

### 3.6. *Isotopic C signature*

Leaves were the most C-labelled organs at maturity (lower  $\delta^{13}\text{C}$  values), followed by berries and stem, whereas the presence of labelled C in roots and cutting was lower (Table 3A). The clones studied (T3 and 1084) had significantly different isotopic C signatures in the cutting, the cutting of 1084 accession being more C-labelled than that of T3 (Table 3A). T+4 reduced significantly  $\delta^{13}\text{C}$  in berries at the onset of veraison and in leaves at maturity compared with T (Table 3A). Compared with  $\text{ACO}_2$ ,  $\text{ECO}_2$  reduced significantly the  $\delta^{13}\text{C}$  in leaves and berries from the onset of veraison onwards, as well as in the rest of the tissues at maturity, as a consequence of the C-labelling (Table 3A, 3B). This reduction produced by  $\text{ECO}_2$  was more pronounced under T+4 in the cutting of the 1084 accession (Table 3B).

## 4. DISCUSSION

### 4.1. *Performance of clones*

Clonal selection has been carried out in viticulture since the late 1950s [39]. Among the reasons favouring the development of clonal selection there is the possibility to search within a variety for the genotypes better adapted to a certain environment, and to produce a certain type of wine. In general, the existing wine protection figures include in their regulation an exhaustive list of varieties authorized. The selection of accessions within the authorized varieties led to a plant material, which is immediately accepted by the protection figure [24]. In the case of Tempranillo, Ibañez et al. [24] reported recently a high number of available certified clones, which may make the selection of the best adapted ones to future environmental conditions a suitable tool to face climate change undesired effects in viticulture.

Regarding the Tempranillo clones studied in the present work, the 1084 accession, stood out from the other clones because of presenting a different phenological behaviour, as well as vegetative and reproductive growth. The long ripening period of 1084 in comparison to the others would explain its higher vegetative production, as a delayed maturity involved a longer period for growing, facilitating to reach a higher vegetative dry mass [32]. We can rule out a lower photosynthetic activity as the main reason to explain the longer ripening period of this clone, since neither the photosynthetic rates measured around veraison nor the leaf isotopic analysis showed differences with the other variants studied. Also, the delayed maturity was not associated with a high bunch production in 1084, as this variant had the lowest bunch weight and the lower number of berries per bunch. However, it had one of the highest weights per berry, which is in line with what other authors have already described: a tendency to compensate the low number of berries with a large berry size [40]. Taking into account the desired bunch characteristics in vitiviculture, the most interesting clone from the ones studied in

this experiment would be CL306 due to its low weight of individual berries [41] and high number of berries. In contrast, VN31 had a too large production and high berry sizes to align with the demands of growers and wine producers.

The C, N and  $\delta^{13}\text{C}$  analysis performed in the present study allowed us to analyse if differences in C allocation were involved in the delayed maturity observed in the 1084 accession. However, no significant differences were observed in the C and N concentration between 1084 and T3, despite having clearly different ripening lengths. Also, both clones showed a similar  $\delta^{13}\text{C}$  signature under current conditions (treatment T/ $\text{ACO}_2$ ), with a larger  $^{12}\text{C}$ -labelling in leaves, berries and stem. The lower presence of labelled C in the cutting and roots could be explained by the fact that, unlike berries and leaves, these organs were formed mainly before the  $^{12}\text{C}$ -labelling period, thus having a low C sink activity from fruit set to maturity. In grapevine, the pool of starch reserves is restored in the wood before veraison, and the new fixed C is then directed to berry maturation [42–44].

#### *4.2. Effects of an increment of temperature in 4 °C*

Plants grown under a 4 °C increased temperature ripened faster than those that grew at ambient temperature, which agrees with previous studies [9,11,45,46] and different predicting models [47,48]. This effect of temperature was also reflected in a higher C-labelling in the berries at the onset of veraison, indicating a higher import of photoassimilates to this organ just before the beginning of the ripening period. Elevated temperature also increased vegetative plant growth (leaf area and dry matter). The literature on the effects of high temperature on grapevine growth shows contradictory results, with reductions in leaf area as a consequence of an increase in temperature from 24 °C /14 °C to 28 °C /18 °C (day/night) [14] or the absence of changes in this parameter [18]. In contrast, temperature-induced stimulations of vegetative growth have been reported for other plant species under similar experimental conditions [49]. The increase in plant dry matter at elevated temperature was a consequence of an enhanced integrated C fixation rate at the whole plant level, rather than an increased photosynthesis per unit of area, which in turn was a result of the increased leaf area observed from veraison to maturity in T+4. However, it should also be considered that the photosynthesis determinations were conducted around veraison and variations in photosynthetic activity during the experiment should not be discarded as possible factors explaining differences in plant growth. Also, the total photosynthesis enhancement may have caused an increment of the photoassimilates intended to the bunch, thus hastening the ripening process [49].

At maturity,  $\delta^{13}\text{C}$  in leaves decreased in the T+4 treatments compared with T, indicating a higher  $\text{CO}_2$  discrimination during the pathway of  $\text{CO}_2$  through stomata and the photosynthetic processes, as well

as an increment of the leaf labelled C stored in this organ. Reductions in the  $\delta^{13}\text{C}$  values with increasing temperatures have been previously reported [50]. Simultaneously, the concentration of C and the ratio between C and N in leaves increased. These results suggest that plants grown under T+4 were not capable of avoiding carbohydrates accumulation in the leaves [17].

T+4 reduced bunch weight, as a consequence of the reduction in the number of berries per bunch, as well as in the weight of individual berries. The five heat-wave events that T+4 plants experienced in this experiment, compared with only one heat wave in T, may explain the reduction in grape yield observed. The frequency of extreme events such as heat waves is expected to increase with global temperature increase [2] and, although this qualitative information is difficult to integrate in an adaptation approach, it is necessary to keep in mind its potential effects [8]. Reductions in berry weight with high temperatures in different grapevine cultivars have been reported in previous studies [9,51].

#### *4.3. Effects of an increment of atmospheric CO<sub>2</sub> concentration up to 700 ppm*

Plants grown under elevated CO<sub>2</sub> showed, in general, a higher photosynthetic activity measured around veraison, which explains the increase in vegetative growth, especially in the final dry weight [13,14,52,53]. Such effect on net photosynthesis, however, did not impact either grape production or phenological development, which agrees with the results of a three year study on red and white Tempranillo under similar growing conditions [18]. The lack in CO<sub>2</sub> effect on bunch weight and on the number of berries per bunch may be related to the fact that inflorescence initiation (in the previous season) occurred under ambient CO<sub>2</sub> conditions, as suggested by Wohlfahrt et al. [54]. In a vineyard FACE-experiment, these authors reported a higher impact of ECO<sub>2</sub> on bunch weight on the second and third year after the commencement of the treatments for Cabernet Sauvignon and Riesling, with no effects on grape yield in the first year.

Although net photosynthesis was not measured in later stages of development, a significant increase in the leaf C:N ratio was observed in ECO<sub>2</sub> plants, which has been reported as an important indicator of photosynthetic acclimation to elevated CO<sub>2</sub> [17,55,56]. In fact, signs of photosynthesis acclimation could already be observed even at the onset of veraison in some of the clones analysed (i.e. RJ43), in which net photosynthesis under ECO<sub>2</sub> were similar to that under ACO<sub>2</sub> at ambient temperature. Although elevated CO<sub>2</sub> enhances photosynthesis rates in leaves of almost all C<sub>3</sub> species, such effect is only temporary and net photosynthesis usually slows or stabilizes at a lower level particularly under relatively long-term elevated CO<sub>2</sub> exposure [57]. In our case, the imbalance in the C:N ratio was due to a reduction in N, suggesting a N limitation to some extent in these plants [58]. Leaf N concentration can be considered a central point to the down-regulation of grapevine photosynthesis in response to



ECO<sub>2</sub>, due to the large fraction of leaf N allocated to the photosynthetic apparatus [16]. Interestingly, the effect of ECO<sub>2</sub> on the leaf C:N ratio was particularly marked under elevated temperature. This result suggests that warmer temperatures exacerbates photosynthetic acclimation in grapevine, phenomenon already observed in other plant species [55]. This probably was due to a higher accumulation of assimilated C in this organ, as indicates the higher C and the lower  $\delta^{13}\text{C}$  values in the treatment T+4/ECO<sub>2</sub>.

Although ECO<sub>2</sub> plants showed a higher leaf dry matter, this treatment did not have such a marked effect on leaf area, thus indicating a significant increase in the specific leaf weight of these plants (P = 0.0197). The results suggest that grapevine had the capacity to use a CO<sub>2</sub>-induced surplus of carbohydrates into structural growth, such as thicker leaves [59], which may also explain the already mentioned N dilution [16].

#### *4.4. Response of clones to combined effects of high temperature and elevated CO<sub>2</sub> concentration*

Plants grown under climate change conditions (T+4/ECO<sub>2</sub> treatment) had, in general, a significantly larger vegetative production and a faster grape development, showing an additive effect of the environmental factors studied. However, some differences could be appreciated among clones. The RJ43 accession was the most affected by climate change in terms of phenology, with an earlier maturity under combined ECO<sub>2</sub> and T+4. In contrast, according to the present results, other clones such as CL306 would be more suitable in a future climate change scenario, maintaining similar developmental time between fruit set and maturity. Regarding vegetative growth and C partitioning, the long-cycle variant 1084 seemed to be one of the most affected by climate change. First, the increased vegetative production and higher presence of labelled C in the cutting in the T+4/ECO<sub>2</sub> treatment, compared with the current conditions (T/ACO<sub>2</sub> treatment), may be interpreted as a consequence of its longer phenology, and consequently a longer exposure time to elevated CO<sub>2</sub> and temperature, in comparison with other variants, thus having more time to increase C reserves in the cutting and to produce more vegetative biomass (shoots, leaves and roots). Second, with regards to the higher levels of total and labelled C in the leaves under T+4/ECO<sub>2</sub>, compared with T/ACO<sub>2</sub>, changes observed were not so marked in T3, which suggests a differential response of these two clones to climate change, in terms of C allocation. Such result may have implication on C reserves for next growing season.

## 5. CONCLUSION

In general, combined elevated temperature and high CO<sub>2</sub> atmospheric concentration hastened grape development and stimulated vegetative growth, showing additive effects on these parameters. In contrast, yield and yield-related parameters were negatively affected, mainly due to the high frequency of heat waves in the high temperature treatments, regardless of CO<sub>2</sub> levels. Despite initial stimulations of net photosynthesis at veraison, signs of photosynthesis acclimation to elevated CO<sub>2</sub> were observed, especially when E-CO<sub>2</sub> was combined with high temperature. N limitations produced by elevated CO<sub>2</sub> and higher leaf C accumulation induced by high temperatures may produce photosynthesis down regulation. Clones showed differences in their phenological development, which conditioned some responses to elevated CO<sub>2</sub> and temperature in terms of vegetative growth and the potential to allocate C into different organs. One of the most widely used clones nowadays, the RJ43 accession, seemed to be the most affected by climate change in terms of phenological development. The present study reveals the importance of testing the performance of the genetic variants available inside a cultivar under the foreseen climate conditions, and adds new knowledge in order to exploit such genetic diversity in the viticulture of the future.

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## TABLES

Table 1. Bunch weight, number of berries per bunch and individual berry weight of five Tempranillo clones (RJ43, CL306, T3, VN31 and 1084) grown at ambient temperature (T) or ambient temperature +4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). Results are shown according to the clone identity (values are means ± SE; n = 28), temperature regime (values are means ± SE; n = 60-80), CO<sub>2</sub> concentration (values are means ± SE; n = 60-80) and the three factors together (values are means ± SE; n = 6-8). Means with letters in common within the same parameter and factor (clone, temperature, CO<sub>2</sub> or their interactions) are not significantly different (P > 0.05) according to LSD test. All probability values for the interactions of factors (P(CL x T), P(CL x CO<sub>2</sub>), P(T x CO<sub>2</sub>) and P(CL x T x CO<sub>2</sub>)) were statistically not significant (P > 0.05).

|                  |     |                  | BUNCH WEIGHT (g FW) | NUMBER OF BERRIES/BUNCH | WEIGHT OF INDIVIDUAL GRAPE (g FW berry <sup>-1</sup> ) |
|------------------|-----|------------------|---------------------|-------------------------|--|
| RJ43             |     |                  | 176.7 ± 14.6 b      | 151.0 ± 12.8 ab         | 1.13 ± 0.06 ab   |
| CL306            |     |                  | 181.0 ± 21.1 b      | 157.0 ± 19.0 a          | 1.11 ± 0.06 b  |
| T3               |     |                  | 147.0 ± 18.5 b      | 115.9 ± 14.3 b          | 1.18 ± 0.06 ab   |
| VN31             |     |                  | 243.9 ± 14.8 a      | 177.6 ± 9.4 a           | 1.29 ± 0.05 a  |
| 1084             |     |                  | 95.7 ± 13.8 c       | 69.9 ± 9.7 c            | 1.28 ± 0.09 ab   |
| T                |     |                  | 192.7 ± 13.3 a      | 149.3 ± 11.2 a          | 1.28 ± 0.05 a  |
| T+4              |     |                  | 151.0 ± 10.6 b      | 123.0 ± 8.2 b           | 1.14 ± 0.04 b  |
| ACO <sub>2</sub> |     |                  | 161.2 ± 11.9 a      | 130.3 ± 9.5 a           | 1.17 ± 0.04 a  |
| ECO <sub>2</sub> |     |                  | 174.6 ± 11.8 a      | 137.3 ± 9.5 a           | 1.22 ± 0.04 a  |
| RJ43             | T   | ACO <sub>2</sub> | 193.3 ± 39.3 abcdef | 165.5 ± 43.1 abc        | 1.23 ± 0.29 abcde                                      |
|                  |     | ECO <sub>2</sub> | 213.8 ± 30.7 abcde  | 177.8 ± 29.7 ab         | 1.20 ± 0.10 abcde                                      |
|                  | T+4 | ACO <sub>2</sub> | 175.1 ± 27.4 bcdef  | 149.1 ± 18.6 abc        | 1.07 ± 0.09 bcde                                       |
|                  |     | ECO <sub>2</sub> | 133.1 ± 19.1 def    | 118.8 ± 18.2 abcde      | 1.07 ± 0.12 bcde                                       |
| CL306            | T   | ACO <sub>2</sub> | 217.1 ± 47.7 abcde  | 203.8 ± 35.8 a          | 0.98 ± 0.08 cde  |
|                  |     | ECO <sub>2</sub> | 221.3 ± 52.7 abc    | 187.3 ± 49.5 a          | 1.21 ± 0.16 abcde                                      |
|                  | T+4 | ACO <sub>2</sub> | 168.6 ± 32.6 bcdef  | 126.6 ± 22.1 abcde      | 1.23 ± 0.10 abcde                                      |
|                  |     | ECO <sub>2</sub> | 134.9 ± 32.2 cdef   | 133.9 ± 33.9 abcd       | 0.95 ± 0.03 e  |
| T3               | T   | ACO <sub>2</sub> | 126.8 ± 30.3 def    | 108.8 ± 34.4 abcde      | 1.16 ± 0.11 bcde                                       |
|                  |     | ECO <sub>2</sub> | 186.2 ± 29.0 abcdef | 119.5 ± 21.8 abcde      | 1.52 ± 0.11 a  |
|                  | T+4 | ACO <sub>2</sub> | 103.2 ± 33.0 f      | 94.6 ± 29.8 bcde        | 0.98 ± 0.08 de   |
|                  |     | ECO <sub>2</sub> | 161.6 ± 44.0 bcdef  | 137.3 ± 31.7 abcd       | 1.05 ± 0.08 cde  |
| VN31             | T   | ACO <sub>2</sub> | 266.6 ± 15.7 ab     | 180.3 ± 17.6 ab         | 1.42 ± 0.12 abc  |
|                  |     | ECO <sub>2</sub> | 271.3 ± 29.3 a      | 191.1 ± 14.8 a          | 1.31 ± 0.09 abc  |
|                  | T+4 | ACO <sub>2</sub> | 228.1 ± 29.2 ab     | 179.6 ± 19.0 ab         | 1.19 ± 0.10 bcde                                       |
|                  |     | ECO <sub>2</sub> | 221.1 ± 30.8 abcd   | 160.6 ± 22.0 abc        | 1.30 ± 0.08 abcd                                       |
| 1084             | T   | ACO <sub>2</sub> | 72.3 ± 19.8 f       | 50.8 ± 14.2 e           | 1.46 ± 0.30 ab   |
|                  |     | ECO <sub>2</sub> | 114.3 ± 29.1 ef     | 89.6 ± 22.1 cde         | 1.21 ± 0.15 abcde                                      |
|                  | T+4 | ACO <sub>2</sub> | 96.3 ± 25.0 f       | 72.5 ± 19.0 de          | 1.18 ± 0.10 bcde                                       |
|                  |     | ECO <sub>2</sub> | 88.3 ± 30.2 f       | 57.3 ± 16.7 e           | 1.36 ± 0.21 abc  |



Table 2. Carbon concentration (C, %), nitrogen concentration (N, %) and ratio between carbon and nitrogen (C:N) at maturity in different tissues (leaf, berry, stem, cutting and root) of plants of two Tempranillo clones (1084 and T3) grown at ambient (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm; ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm; ECO<sub>2</sub>). Results are shown according to the clone identity (values are means ± SE; n = 15-16), temperature regime (values are means ± SE; n = 15-16), CO<sub>2</sub> concentration (values are means ± SE; n = 15-16), and the three factors together (values are means ± SE; n = 3-4). Means with letters in common within the same parameter and factor (clone, temperature, CO<sub>2</sub> or their interactions) are not significantly different (P > 0.05) according to LSD test. Probability values for the interactions of factors are shown in Table S1.

|      |                  | LEAF                 | BERRY                | STEM            | CUTTING         | ROOT            |                |                |
|------|------------------|----------------------|----------------------|-----------------|-----------------|-----------------|----------------|----------------|
| C    | 1084             | 43.1 ± 0.5 a         | 43.6 ± 0.2 a         | 46.61 ± 0.4 a   | 45.0 ± 0.7 a    | 42.7 ± 0.5 a    |                |                |
|      | T3               | 42.9 ± 0.3 a         | 43.0 ± 0.2 a         | 46.53 ± 0.2 a   | 45.0 ± 0.7 a    | 41.4 ± 0.8 a    |                |                |
| N    | 1084             | 1.7 ± 0.2 a          | 0.61 ± 0.04 a        | 0.61 ± 0.03 a   | 0.42 ± 0.03 a   | 1.27 ± 0.07 a   |                |                |
|      | T3               | 1.8 ± 0.1 a          | 0.65 ± 0.04 a        | 0.60 ± 0.05 a   | 0.40 ± 0.02 a   | 1.37 ± 0.13 a   |                |                |
| C:N  | 1084             | 28.3 ± 2.6 a         | 75.5 ± 5.6 a         | 78.93 ± 4.0 a   | 113.0 ± 6.1 a   | 35.2 ± 2.2 a    |                |                |
|      | T3               | 24.9 ± 1.1 a         | 68.6 ± 3.2 a         | 86.81 ± 8.7 a   | 115.9 ± 5.3 a   | 33.5 ± 2.4 a    |                |                |
| C    | T                | 42.3 ± 0.3 b         | 43.1 ± 0.2 a         | 46.38 ± 0.2 a   | 44.0 ± 0.8 b    | 42.0 ± 0.5 a    |                |                |
|      | T+4              | 43.7 ± 0.4 a         | 43.5 ± 0.2 a         | 46.77 ± 0.4 a   | 45.9 ± 0.6 a    | 42.1 ± 0.8 a    |                |                |
| N    | T                | 1.90 ± 0.14 a        | 0.65 ± 0.03 a        | 0.60 ± 0.02 a   | 0.43 ± 0.03 a   | 1.38 ± 0.14 a   |                |                |
|      | T+4              | 1.59 ± 0.09 a        | 0.62 ± 0.04 a        | 0.62 ± 0.06 a   | 0.39 ± 0.02 a   | 1.27 ± 0.07 a   |                |                |
| C:N  | T                | 23.7 ± 1.3 b         | 67.8 ± 2.7 a         | 78.98 ± 2.7 a   | 108.9 ± 5.9 a   | 34.0 ± 2.6 a    |                |                |
|      | T+4              | 29.4 ± 2.4 a         | 75.8 ± 5.5 a         | 86.76 ± 9.3 a   | 121.3 ± 4.8 a   | 34.7 ± 1.9 a    |                |                |
| C    | ACO <sub>2</sub> | 42.6 ± 0.3 a         | 43.3 ± 0.2 a         | 46.90 ± 0.4 a   | 43.5 ± 0.8 b    | 41.9 ± 0.5 a    |                |                |
|      | ECO <sub>2</sub> | 43.4 ± 0.5 a         | 43.3 ± 0.2 a         | 46.26 ± 0.3 a   | 46.5 ± 0.4 a    | 42.1 ± 0.8 a    |                |                |
| N    | ACO <sub>2</sub> | 1.98 ± 0.13 a        | 0.70 ± 0.03 a        | 0.69 ± 0.04 a   | 0.38 ± 0.03 a   | 1.28 ± 0.07 a   |                |                |
|      | ECO <sub>2</sub> | 1.50 ± 0.08 b        | 0.57 ± 0.03 b        | 0.53 ± 0.03 b   | 0.43 ± 0.02 a   | 1.36 ± 0.14 a   |                |                |
| C:N  | ACO <sub>2</sub> | 22.5 ± 1.0 b         | 63.7 ± 2.8 b         | 71.75 ± 4.2 b   | 119.6 ± 7.0 a   | 34.2 ± 1.9 a    |                |                |
|      | ECO <sub>2</sub> | 30.6 ± 2.3 a         | 79.3 ± 4.9 a         | 93.05 ± 7.4 a   | 109.6 ± 3.9 a   | 34.5 ± 2.6 a    |                |                |
| C    | 1084 T           | ACO <sub>2</sub>     | 41.2 ± 0.5 c         | 44.0 ± 0.5 ab   | 46.96 ± 0.1 a   | 41.0 ± 0.7 b    | 41.5 ± 1.4 a   |                |
|      |                  | ECO <sub>2</sub>     | 43.2 ± 0.4 abc       | 43.2 ± 0.4 ab   | 45.55 ± 0.4 a   | 46.6 ± 0.1 a    | 43.2 ± 1.1 a   |                |
|      |                  | T+4 ACO <sub>2</sub> | 43.2 ± 0.3 abc       | 43.2 ± 0.5 ab   | 47.18 ± 1.5 a   | 45.4 ± 1.8 a    | 42.6 ± 0.5 a   |                |
|      |                  | ECO <sub>2</sub>     | 44.7 ± 1.4 a         | 44.1 ± 0.4 a    | 46.75 ± 0.6 a   | 46.8 ± 0.1 a    | 43.2 ± 0.7 a   |                |
|      | T3 T             | ACO <sub>2</sub>     | 42.5 ± 0.4 abc       | 42.7 ± 0.0 b    | 46.68 ± 0.2 a   | 41.6 ± 1.2 b    | 41.0 ± 0.7 a   |                |
|      |                  | ECO <sub>2</sub>     | 42.4 ± 0.9 bc        | 42.8 ± 0.3 b    | 46.33 ± 0.5 a   | 46.8 ± 0.1 a    | 42.3 ± 0.7 a   |                |
|      |                  | T+4 ACO <sub>2</sub> | 43.5 ± 0.3 ab        | 43.5 ± 0.4 ab   | 46.75 ± 0.5 a   | 45.9 ± 1.1 a    | 42.5 ± 1.4 a   |                |
|      |                  | ECO <sub>2</sub>     | 43.3 ± 1.1 abc       | 43.0 ± 0.5 ab   | 46.42 ± 0.4 a   | 45.6 ± 1.5 a    | 39.8 ± 2.7 a   |                |
|      | N                | 1084 T               | ACO <sub>2</sub>     | 2.18 ± 0.54 a   | 0.70 ± 0.06 ab  | 0.64 ± 0.05 b   | 0.43 ± 0.09 a  | 1.42 ± 0.21 ab |
|      |                  |                      | ECO <sub>2</sub>     | 1.73 ± 0.16 abc | 0.62 ± 0.05 ab  | 0.64 ± 0.04 b   | 0.42 ± 0.03 a  | 1.17 ± 0.15 b  |
|      |                  |                      | T+4 ACO <sub>2</sub> | 1.74 ± 0.09 abc | 0.66 ± 0.07 ab  | 0.63 ± 0.08 b   | 0.35 ± 0.03 a  | 1.40 ± 0.03 ab |
|      |                  |                      | ECO <sub>2</sub>     | 1.16 ± 0.14 c   | 0.51 ± 0.10 b   | 0.53 ± 0.06 bc  | 0.46 ± 0.03 a  | 1.13 ± 0.15 b  |
| T3 T |                  | ACO <sub>2</sub>     | 2.01 ± 0.09 ab       | 0.73 ± 0.00 a   | 0.59 ± 0.02 bc  | 0.40 ± 0.04 a   | 0.99 ± 0.08 b  |                |
|      |                  | ECO <sub>2</sub>     | 1.66 ± 0.17 abc      | 0.59 ± 0.05 ab  | 0.52 ± 0.03 bc  | 0.47 ± 0.04 a   | 1.93 ± 0.35 a  |                |
|      |                  | T+4 ACO <sub>2</sub> | 1.99 ± 0.09 ab       | 0.73 ± 0.11 a   | 0.94 ± 0.01 a   | 0.34 ± 0.02 a   | 1.33 ± 0.12 b  |                |
|      |                  | ECO <sub>2</sub>     | 1.46 ± 0.04 bc       | 0.57 ± 0.04 ab  | 0.44 ± 0.08 c   | 0.39 ± 0.03 a   | 1.22 ± 0.24 b  |                |
| C:N  | 1084 T           | ACO <sub>2</sub>     | 21.7 ± 3.9 b         | 64.1 ± 4.4 b    | 74.30 ± 5.4 bc  | 109.5 ± 20.0 ab | 30.4 ± 4.5 bc  |                |
|      |                  | ECO <sub>2</sub>     | 25.5 ± 2.2 b         | 70.9 ± 5.3 b    | 71.75 ± 3.8 bc  | 113.8 ± 7.9 ab  | 38.6 ± 4.6 ab  |                |
|      |                  | T+4 ACO <sub>2</sub> | 25.1 ± 1.4 b         | 67.6 ± 6.4 b    | 78.29 ± 10.9 bc | 128.5 ± 9.8 ab  | 30.5 ± 0.4 bc  |                |
|      |                  | ECO <sub>2</sub>     | 40.8 ± 6.2 a         | 96.5 ± 16.3 a   | 91.39 ± 9.3 ab  | 104.0 ± 7.2 ab  | 40.2 ± 5.0 ab  |                |
|      | T3 T             | ACO <sub>2</sub>     | 21.2 ± 0.8 b         | 58.8 ± 0.0 b    | 79.11 ± 2.0 bc  | 107.2 ± 11.5 ab | 42.0 ± 2.4 a   |                |
|      |                  | ECO <sub>2</sub>     | 26.4 ± 2.6 b         | 74.1 ± 5.6 ab   | 90.78 ± 5.0 ab  | 101.9 ± 8.7 b   | 24.1 ± 4.2 c   |                |
|      |                  | T+4 ACO <sub>2</sub> | 22.0 ± 0.9 b         | 63.3 ± 7.8 b    | 49.80 ± 0.2 c   | 135.5 ± 9.0 a   | 32.9 ± 4.1 abc |                |
|      |                  | ECO <sub>2</sub>     | 29.8 ± 1.4 b         | 75.8 ± 5.3 ab   | 118.30 ± 24.4 a | 119.0 ± 6.7 ab  | 35.1 ± 4.1 abc |                |

Table 3. Isotopic C signature ( $\delta^{13}\text{C}$  values) in different tissues (leaf, berry, stem, cutting and root) at different ripening moments of two Tempranillo clones (1084 and T3) grown at ambient (T) or ambient temperature + 4 °C (T+4), combined with ambient  $\text{CO}_2$  (ca. 400 ppm;  $\text{ACO}_2$ ) or elevated  $\text{CO}_2$  (700 ppm;  $\text{ECO}_2$ ). Data are expressed in ‰ and shown according to: (A) the clone identity (values are means  $\pm$  SE; n = 15-16), temperature regime (values are means  $\pm$  SE; n = 15-16) and  $\text{CO}_2$  concentration (values are means  $\pm$  SE; n = 15-16); and (B) the three factors together (clone, temperature and  $\text{CO}_2$  concentration) (values are means  $\pm$  SE; n = 3-4). Means with letters in common within the same tissue (leaf, berry, stem, cutting or root), ripening status (onset of veraison, 1 week after veraison or maturity) and factor (clone, temperature,  $\text{CO}_2$  or their interactions) are not significantly different ( $P > 0.05$ ) according to LSD test. All probability values for the interactions of factors ( $P(\text{CL} \times \text{T})$ ,  $P(\text{CL} \times \text{CO}_2)$ ,  $P(\text{T} \times \text{CO}_2)$  and  $P(\text{CL} \times \text{T} \times \text{CO}_2)$ ) were statistically not significant ( $P > 0.05$ ).

| A       |                   | CLONES |       |           | TEMPERATURE |     |       | $\text{CO}_2$ |   |                |       |           |   |
|---------|-------------------|--------|-------|-----------|-------------|-----|-------|---------------|---|----------------|-------|-----------|---|
| LEAF    | Onset of veraison | 1084   | -36.4 | $\pm 1.7$ | a           | T   | -35.9 | $\pm 1.6$     | a | $\text{ACO}_2$ | -29.6 | $\pm 0.2$ | a |
|         |                   | T3     | -35.7 | $\pm 1.6$ | a           | T+4 | -36.2 | $\pm 1.7$     | a | $\text{ECO}_2$ | -41.7 | $\pm 0.4$ | b |
|         | Veraison+1week    | 1084   | -35.7 | $\pm 1.6$ | a           | T   | -35.9 | $\pm 1.6$     | a | $\text{ACO}_2$ | -29.4 | $\pm 0.2$ | a |
|         |                   | T3     | -36.2 | $\pm 1.8$ | a           | T+4 | -36.0 | $\pm 1.8$     | a | $\text{ECO}_2$ | -42.0 | $\pm 0.5$ | b |
|         | Maturity          | 1084   | -35.9 | $\pm 1.7$ | a           | T   | -34.5 | $\pm 1.5$     | a | $\text{ACO}_2$ | -30.5 | $\pm 0.9$ | a |
|         |                   | T3     | -35.8 | $\pm 1.6$ | a           | T+4 | -37.2 | $\pm 1.7$     | b | $\text{ECO}_2$ | -41.2 | $\pm 0.9$ | b |
| BERRY   | Onset of veraison | 1084   | -33.3 | $\pm 1.3$ | a           | T   | -32.9 | $\pm 1.3$     | a | $\text{ACO}_2$ | -28.3 | $\pm 0.2$ | a |
|         |                   | T3     | -33.0 | $\pm 1.2$ | a           | T+4 | -33.4 | $\pm 1.3$     | b | $\text{ECO}_2$ | -38.0 | $\pm 0.2$ | b |
|         | Veraison+1week    | 1084   | -35.1 | $\pm 1.6$ | a           | T   | -34.9 | $\pm 1.7$     | a | $\text{ACO}_2$ | -28.1 | $\pm 0.2$ | a |
|         |                   | T3     | -34.4 | $\pm 1.7$ | a           | T+4 | -34.6 | $\pm 1.6$     | a | $\text{ECO}_2$ | -40.1 | $\pm 0.3$ | b |
|         | Maturity          | 1084   | -34.7 | $\pm 1.7$ | a           | T   | -34.3 | $\pm 1.6$     | a | $\text{ACO}_2$ | -27.9 | $\pm 0.2$ | a |
|         |                   | T3     | -34.3 | $\pm 1.7$ | a           | T+4 | -34.7 | $\pm 1.7$     | a | $\text{ECO}_2$ | -40.2 | $\pm 0.4$ | b |
| STEM    | Maturity          | 1084   | -34.5 | $\pm 1.8$ | a           | T   | -34.7 | $\pm 1.8$     | a | $\text{ACO}_2$ | -28.0 | $\pm 0.2$ | a |
|         |                   | T3     | -35.7 | $\pm 1.9$ | a           | T+4 | -35.5 | $\pm 1.9$     | a | $\text{ECO}_2$ | -41.7 | $\pm 0.6$ | b |
| CUTTING | Maturity          | 1084   | -31.0 | $\pm 1.0$ | b           | T   | -29.9 | $\pm 0.9$     | a | $\text{ACO}_2$ | -27.0 | $\pm 0.1$ | a |
|         |                   | T3     | -29.7 | $\pm 0.8$ | a           | T+4 | -30.8 | $\pm 1.0$     | a | $\text{ECO}_2$ | -33.7 | $\pm 0.5$ | b |
| ROOT    | Maturity          | 1084   | -32.6 | $\pm 1.7$ | a           | T   | -32.2 | $\pm 1.5$     | a | $\text{ACO}_2$ | -26.6 | $\pm 0.3$ | a |
|         |                   | T3     | -31.5 | $\pm 1.3$ | a           | T+4 | -31.9 | $\pm 1.5$     | a | $\text{ECO}_2$ | -37.1 | $\pm 0.8$ | b |

| B    |     | LEAF              |                   |                   | BERRY              |                    |                   | STEM               | CUTTING           | ROOT               |                   |
|------|-----|-------------------|-------------------|-------------------|--------------------|--------------------|-------------------|--------------------|-------------------|--------------------|-------------------|
|      |     | Onset of veraison | Veraison+1week    | Maturity          | Onset of veraison  | Veraison+1week     | Maturity          | Maturity           | Maturity          | Maturity           |                   |
| 1084 | T   | $\text{ACO}_2$    | -29.7 $\pm 0.5$ a | -29.9 $\pm 0.5$ a | -29.3 $\pm 0.9$ a  | -27.5 $\pm 0.4$ a  | -28.2 $\pm 0.1$ a | -27.4 $\pm 0.5$ a  | -27.9 $\pm 0.6$ a | -26.9 $\pm 0.2$ a  | -26.1 $\pm 1$ a   |
|      |     | $\text{ECO}_2$    | -41.7 $\pm 0.6$ b | -41.1 $\pm 0.6$ b | -37.8 $\pm 3.1$ bc | -38.1 $\pm 0.5$ cd | -40.3 $\pm 0.4$ b | -39.8 $\pm 0.9$ bc | -40 $\pm 2$ b     | -33.9 $\pm 1.2$ bc | -37.8 $\pm 1.7$ b |
|      | T+4 | $\text{ACO}_2$    | -29.9 $\pm 0.3$ a | -29.5 $\pm 0.2$ a | -33.3 $\pm 3.4$ ab | -28.8 $\pm 0.2$ b  | -28.8 $\pm 0.7$ a | -28.5 $\pm 0.3$ a  | -27.9 $\pm 0.2$ a | -27.4 $\pm 0.3$ a  | -26.3 $\pm 0.6$ a |
|      |     | $\text{ECO}_2$    | -42.7 $\pm 1$ b   | -42.3 $\pm 0.7$ b | -43.3 $\pm 0.6$ d  | -38.5 $\pm 0.4$ d  | -39.8 $\pm 0.3$ b | -41.2 $\pm 0.3$ c  | -42 $\pm 0.6$ b   | -35.7 $\pm 0.5$ c  | -38.6 $\pm 0.9$ b |
| T3   | T   | $\text{ACO}_2$    | -29.7 $\pm 0.6$ a | -29.1 $\pm 0.5$ a | -29.8 $\pm 0.6$ a  | -28.4 $\pm 0.2$ ab | -27.6 $\pm 0.3$ a | -27.9 $\pm 0$ a    | -28.2 $\pm 0.4$ a | -26.7 $\pm 0.1$ a  | -27 $\pm 0.1$ a   |
|      |     | $\text{ECO}_2$    | -40.9 $\pm 1.1$ b | -41.7 $\pm 1.5$ b | -41 $\pm 1.2$ cd   | -37.4 $\pm 0.2$ c  | -39.9 $\pm 1$ b   | -38.8 $\pm 0.9$ b  | -42.6 $\pm 0.4$ b | -32.3 $\pm 0.9$ b  | -36.5 $\pm 1.4$ b |
|      | T+4 | $\text{ACO}_2$    | -29.1 $\pm 0.6$ a | -29.1 $\pm 0.5$ a | -29.5 $\pm 0.7$ a  | -28.3 $\pm 0.3$ ab | -27.9 $\pm 0.5$ a | -27.9 $\pm 0.4$ a  | -28.3 $\pm 0.2$ a | -27 $\pm 0.5$ a    | -26.9 $\pm 0.3$ a |
|      |     | $\text{ECO}_2$    | -41.5 $\pm 0.2$ b | -43 $\pm 1.1$ b   | -42.7 $\pm 0.4$ cd | -37.9 $\pm 0.4$ cd | -40.4 $\pm 0.7$ b | -41.1 $\pm 0.5$ c  | -42.1 $\pm 1.3$ b | -33 $\pm 1$ b      | -35.7 $\pm 2.5$ b |

**FIGURES**

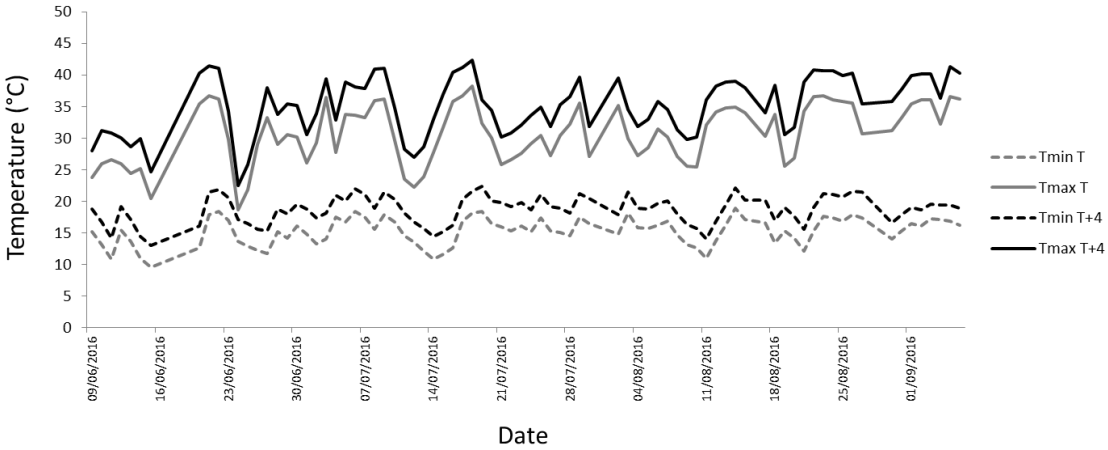


Figure 1. Evolution of the daily minimum and maximum temperatures registered in the modules of ambient temperature (T) and ambient temperature + 4 °C (T+4) of the TGGs. The values correspond to the mean of the four modules at ambient temperature (T) and of the four modules at ambient temperature + 4 °C (T+4).

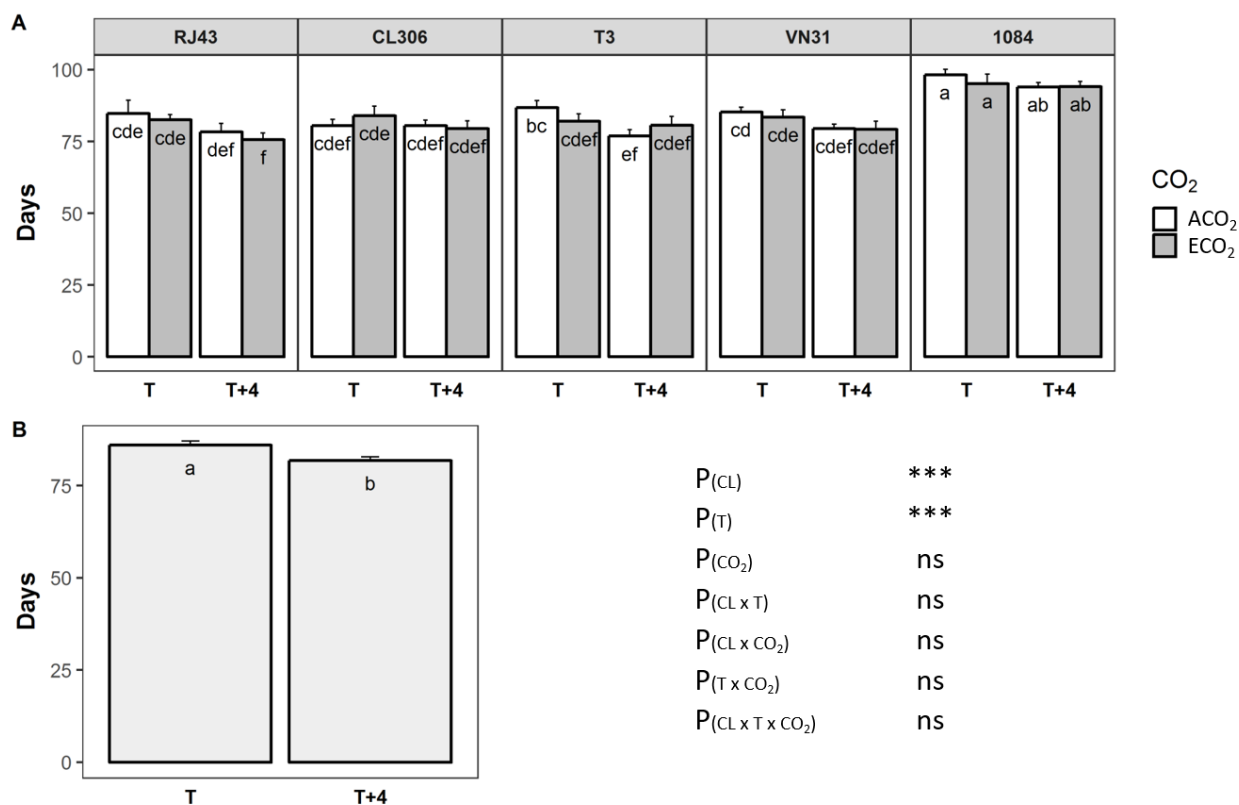


Figure 2. Elapsed time between fruit set and maturity of the five Tempranillo clones (RJ43, CL306, T3, VN31 and 1084) grown at ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>) (A) presented individually for each clone, temperature and CO<sub>2</sub> regime (values are means ± SE, n = 4-8) and (B) presented according to the temperature (T or T+4) considering all the clones as a whole (values are means ± SE, n = 40-80). Means with letters in common are not significantly different ( $P > 0.05$ ) according to LSD test. Probability values (P) for the main effects of clone,  $P_{(CL)}$ ; temperature  $P_{(T)}$ ; CO<sub>2</sub>,  $P_{(CO_2)}$ ; and their interactions,  $P_{(CL \times T)}$ ,  $P_{(CL \times CO_2)}$ ,  $P_{(T \times CO_2)}$  and  $P_{(CL \times T \times CO_2)}$ . \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant.

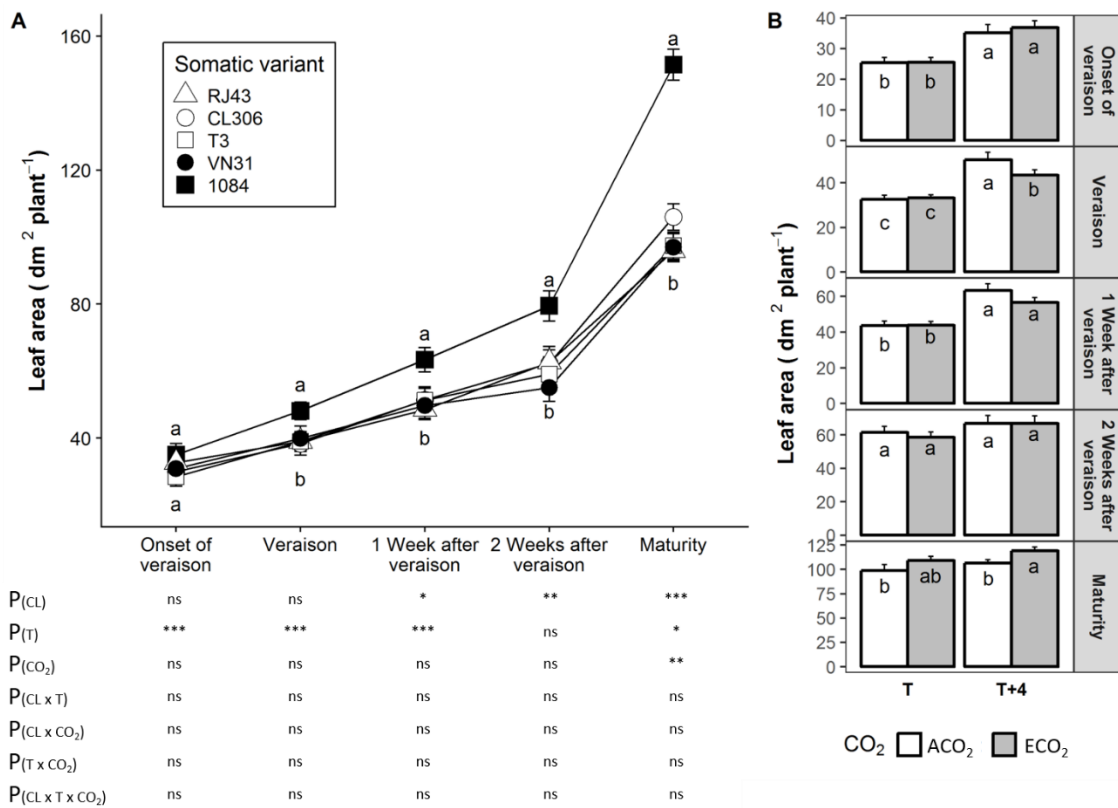


Figure 3. Leaf area of the five Tempranillo clones (RJ43, CL306, T3, VN31 and 1084) grown at ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>) measured at the onset of veraison, veraison, one week after veraison, two weeks after veraison and maturity, (A) presented according to the clone identity (values are means ± SE, n = 28) and (B) presented according to the temperature (T or T+4) and CO<sub>2</sub> regime (ACO<sub>2</sub> or ECO<sub>2</sub>), considering all the clones as a whole (values are means ± SE, n = 20-40). Means with letters in common are not significantly different (P > 0.05) according to LSD test. Probability values (P) for the main effects of clone, P(CL); temperature P(T); CO<sub>2</sub>, P(CO<sub>2</sub>); and their interactions, P(CL x T), P(CL x CO<sub>2</sub>), P(T x CO<sub>2</sub>) and P(CL x T x CO<sub>2</sub>). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant.

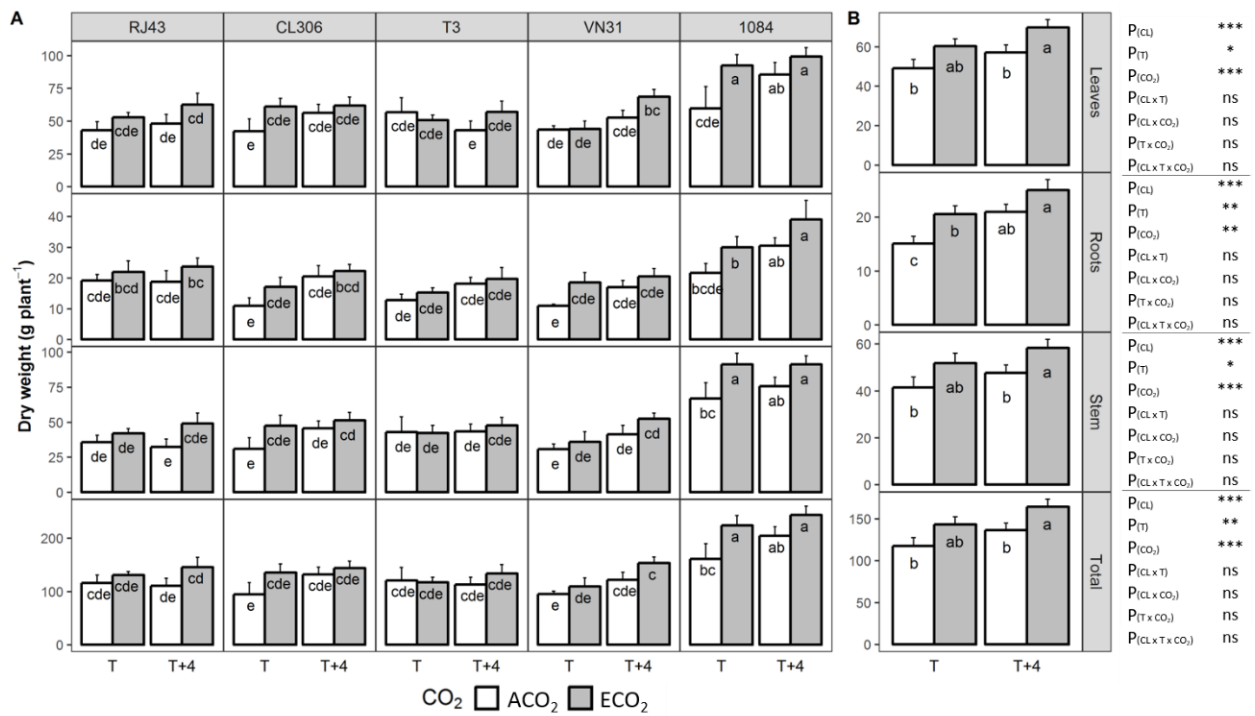


Figure 4. Leaves, stem, roots and total dry weight of the five Tempranillo clones (RJ43, CL306, T3, VN31 and 1084) grown at ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (ca. 700 ppm, ECO<sub>2</sub>) at maturity according to the temperature regime (T or T+4) and CO<sub>2</sub> concentration (ACO<sub>2</sub> or ECO<sub>2</sub>), (A) presented individually for each clone (values are means ± SE, n = 4-8) and (B) considering all the clones as a whole (values are means ± SE, n = 20-40). Means with letters in common within the same chart (A or B) and organ are not significantly different (P > 0.05) according to LSD test. Probability values (P) for the main effects of clone, P(CL); temperature P(T); CO<sub>2</sub>, P(CO<sub>2</sub>); and their interactions, P(CL x T), P(CL x CO<sub>2</sub>), P(T x CO<sub>2</sub>) and P(CL x T x CO<sub>2</sub>). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant.

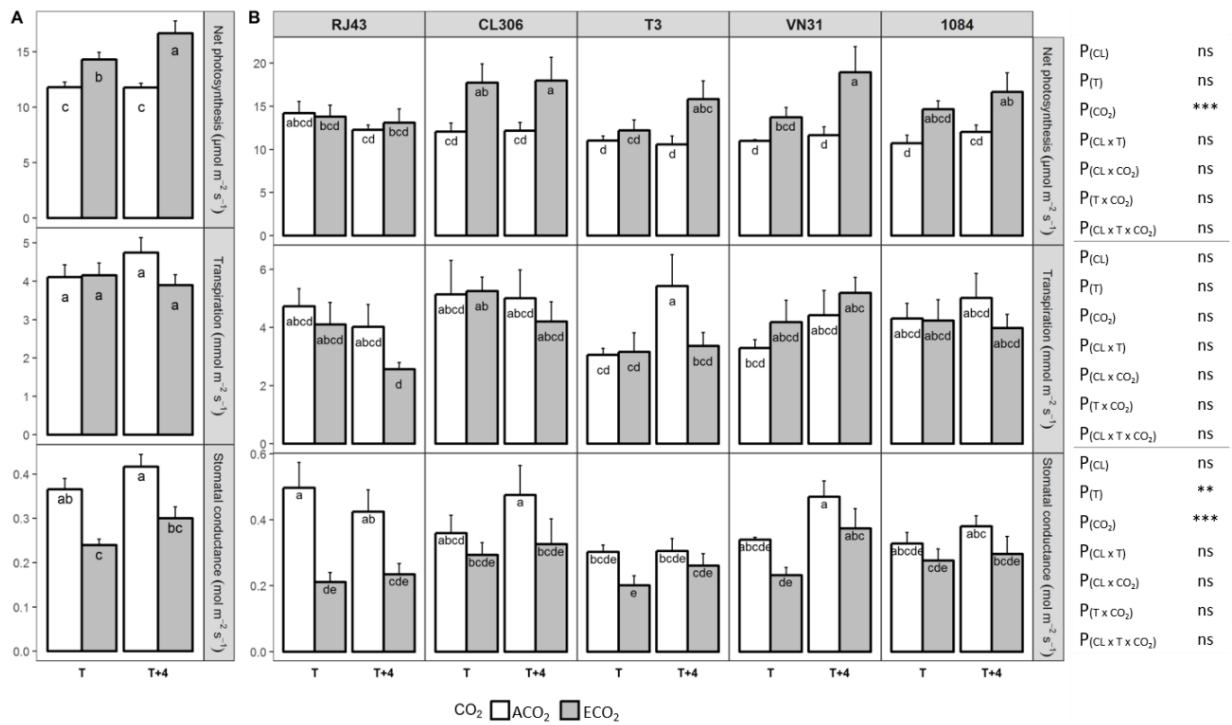


Figure 5. Net photosynthesis, transpiration and stomatal conductance of the five Tempranillo clones (RJ43, CL306, T3, VN31 and 1084) grown at ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>) measured at the onset of veraison and presented according to the temperature regime (T or T+4) and CO<sub>2</sub> concentration (ACO<sub>2</sub> or ECO<sub>2</sub>), (A) considering all the clones as a whole (values are means ± SE, n = 20-40) and (B) presented individually for each clone (values are means ± SE, n = 4-8). Means with letters in common within the same chart (A or B) and parameter are not significantly different (P > 0.05) according to LSD test. Probability values (P) for the main effects of clone, P(Cl); temperature P(T); CO<sub>2</sub>, P(CO<sub>2</sub>); and their interactions, P(Cl × T), P(Cl × CO<sub>2</sub>), P(T × CO<sub>2</sub>) and P(Cl × T × CO<sub>2</sub>). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant.

## SUPPLEMENTARY DATA

Table S1. Result of the ANOVA analysis of carbon (C) and nitrogen (N) concentration, as well as the ratio between carbon and nitrogen (C:N) in different tissues (leaf, berry, stem, root and cutting) of plants of two clones of *Vitis vinifera* cv. Tempranillo (1084 and T3) at maturity. Probability values (P) for the main effects of clone, P(CL); temperature P(T); CO<sub>2</sub>, P(CO<sub>2</sub>); and their interactions, P(CL x T), P(CL x CO<sub>2</sub>), P(T x CO<sub>2</sub>) and P(CL x T x CO<sub>2</sub>). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant.

|     |                              | Leaf | Berry | Stem | Root | Cutting |
|-----|------------------------------|------|-------|------|------|---------|
| C   | P(CL)                        | ns   | ns    | ns   | ns   | ns      |
|     | P(T)                         | *    | ns    | ns   | ns   | *       |
|     | P(CO <sub>2</sub> )          | ns   | ns    | ns   | ns   | ***     |
|     | P(CL x T)                    | ns   | ns    | ns   | ns   | ns      |
|     | P(CL x CO <sub>2</sub> )     | ns   | ns    | ns   | ns   | ns      |
|     | P(T x CO <sub>2</sub> )      | ns   | ns    | ns   | ns   | **      |
|     | P(CL x T x CO <sub>2</sub> ) | ns   | *     | ns   | ns   | ns      |
| N   | P(CL)                        | ns   | ns    | ns   | ns   | ns      |
|     | P(T)                         | ns   | ns    | ns   | ns   | ns      |
|     | P(CO <sub>2</sub> )          | **   | *     | ***  | ns   | ns      |
|     | P(CL x T)                    | ns   | ns    | *    | ns   | ns      |
|     | P(CL x CO <sub>2</sub> )     | ns   | ns    | **   | *    | ns      |
|     | P(T x CO <sub>2</sub> )      | ns   | ns    | **   | ns   | ns      |
|     | P(CL x T x CO <sub>2</sub> ) | ns   | ns    | ns   | ns   | ns      |
| C:N | P(CL)                        | ns   | ns    | ns   | ns   | ns      |
|     | P(T)                         | *    | ns    | ns   | ns   | ns      |
|     | P(CO <sub>2</sub> )          | ***  | *     | **   | ns   | ns      |
|     | P(CL x T)                    | ns   | ns    | ns   | ns   | ns      |
|     | P(CL x CO <sub>2</sub> )     | ns   | ns    | *    | **   | ns      |
|     | P(T x CO <sub>2</sub> )      | ns   | ns    | *    | ns   | ns      |
|     | P(CL x T x CO <sub>2</sub> ) | ns   | ns    | ns   | ns   | ns      |





### **CHAPTER 3**

Irrigation regime and increase of temperature and CO<sub>2</sub> levels influence the growth and physiology of different *Vitis vinifera* L. cv. Tempranillo clones



# Irrigation regime and increase of temperature and CO<sub>2</sub> levels influence the growth and physiology of different *Vitis vinifera* L. cv. Tempranillo clones

## ABSTRACT

In recent decades, atmospheric CO<sub>2</sub> levels and air temperature have increased accompanied by altered precipitation patterns and reduced water availability in most grape growing regions. Global atmospheric changes present new challenges for viticulture, which will require the adaptation of varieties in their traditional growing regions. The intra-varietal genetic diversity can provide the basis for new clones better suited to the projected climate conditions. In this study, we tested the hypothesis of Tempranillo clones that differ in their phenological development having a differential physiological and growing response when exposed to simulated 2100 environmental conditions. This way, we explored the potential interactions between climate change and water deficit, as well as their impact on four clones of *Vitis vinifera* cv. Tempranillo. Two T/CO<sub>2</sub>/Relative Humidity (RH) regimes: climate change (CC; 28 °C/18 °C, 700 ppm CO<sub>2</sub> and 33 %/53 % RH, day/night) vs. current situation (CS; 24 °C/14 °C, 400 ppm CO<sub>2</sub> and 45 %/65 % RH), and two water conditions: well-watered (WW) vs. water deficit (WD), were applied to grapevine fruit-bearing cuttings, from fruit set to maturity, in greenhouses. Under CS and WW conditions, the clones studied exhibited some differences in their photosynthetic rates ( $A_N$ ) after mid-veraison, which may partially explain the differences in their phenological development and vegetative biomass production at maturity. Water deficit strongly limited leaf C assimilation, through stomatal closure, and consequently slowed down grape ripening and grapevine growth. The increased  $A_N$  under CC conditions seemed to ameliorate the reduced C fixation rates under drought at mid-veraison, but not two weeks later. CC increased intrinsic water use efficiency ( $WUE_i$ ,  $A_N/g_s$ ), especially when combined with WD. However, due to the higher vapour pressure deficit in CC, no differences in the instantaneous WUE ( $WUE_{int}$ ,  $A_N/E$ ) were observed. Clones responded, in general, in a similar manner to the simulated year 2100 expected environmental conditions, but some degree of variability in the response of  $A_N$  to changes in the T/CO<sub>2</sub>/RH conditions, as well as in the response of  $A_N$ , plant phenology and vegetative growth to water deficit, were observed.

**Keywords:** Climate change; Tempranillo clones; Grapevine (*Vitis vinifera*); Elevated temperature; Elevated CO<sub>2</sub>; Water deficit; Phenology; Photosynthesis; Vegetative and reproductive growth.

## 1. INTRODUCTION

The impact of climate change on crop production holds the attention of researchers worldwide. According to the Intergovernmental Panel on Climate Change (IPCC), the worst scenarios proposed foresee an increase in the atmospheric CO<sub>2</sub> concentration up to between 670 ppm and 936 ppm (RCP 6.0 and RCP 8.5, respectively) for 2100, contributing to expected increases in global mean temperature (T) of  $2.2 \pm 0.5$  °C and  $3.7 \pm 0.7$  °C [1]. Besides, recent models also report a decrease in near-surface land relative humidity (RH) with global warming [2]. At the same time, precipitation patterns are also expected to suffer modifications, with more frequent drought events predicted for regions that are already arid [1]. In addition, even if locally rainfall does not decrease, water deficit experienced by crops will increase, because of the impact of temperature on reference evapotranspiration, especially in summer [3].

Grapevine (*Vitis vinifera* L.) is one of the most widespread crops worldwide (7.5 Mha in 2016) [4]. Europe represents the largest vineyard area in the world (39 % of the world's total area), the main part being located in the Mediterranean region [4]. This region is considered as one of the most vulnerable to the impacts of global warming, especially as concerns water availability (IPCC) [1]. Grapevine development and grape ripening are sensitive to environmental factors. Therefore, given the high cultural and economic value of grapevine, and in the context of climate change, it is adequate to anticipate and predict the response of this crop to changes in environmental conditions. At a physiological level, combined high temperatures and elevated CO<sub>2</sub> concentrations have been reported to hasten phenological development [5,6], and to increase plant vegetative biomass. Elevated CO<sub>2</sub> has been reported to increase grape production in some cases [7,8], but this effect was not so marked in others [9–11]. Also, photosynthetic activity is stimulated during the first days of exposure to elevated CO<sub>2</sub> then experiencing in some cases an acclimation process resulting in down-regulated photosynthesis rates [6,12]. Under water deficit conditions, stomata closure is one of the early grapevine responses in order to prevent hydraulic failure [13], thus restricting water loss but also C assimilation [14]. Also, drought has been reported to decrease plant growth [9,12,15] and final fruit production [15], with variable effects on plant phenology depending on its intensity [16].

In order to mitigate the negative impact of climate change on grape growth and quality, some adaptation of future viticulture is needed [17]. Within this context, the choice of adequate plant material by identifying genotypes more adapted to the new environmental conditions is certainly one of the most powerful tools to adapt the vineyards to future climate conditions. Grapevine plants are reproduced by vegetative propagation and new features can appear spontaneously in a bud after accidental modifications in the DNA, including point mutations, large deletions, illegitimate

recombinations or variable number of repeats in microsatellite sequences [18]. This emergence of genetic variability leads to clonal variation within a variety and slightly different plants can be identified and their characteristics transmitted by vegetative propagation. The existing clone collections can be explored to detect any phenotypic variation that could be useful in the adaptation of varieties to climate change in their traditional growing region [19,20], without changing wine typicity, and giving rise to plant material immediately accepted by the corresponding protection figure [21]. In this sense, the selection of late-ripening clones has been proposed as a strategy to mitigate the grape quality alterations caused by high temperatures during fruit ripening [3]. Despite clonal selection is a promising tool to adapt vineyards to future environmental conditions, few studies have assessed the performance of different clones of the same cultivar to climate conditions foreseen by 2100, combining different factors such as temperature, CO<sub>2</sub>, relative humidity and water availability. Additionally, it is not possible to extrapolate plant responses to combined environmental conditions from the response derived from a single condition [22].

Among the cultivars grown in Spain (an area expected to suffer a decline of precipitations as consequence of climate change), Tempranillo is one of the most internationally recognized, with the highest number of certified clones [21]. Variability among Tempranillo clones for phenological development, water use efficiency, leaf antioxidant compounds, berry sugar and anthocyanin accumulation and profile has been previously reported [23–26]. The purpose of our study was to evaluate the response of four Tempranillo clones, differing in their maturing times, to the projected conditions for the year 2100 (combined effects of elevated CO<sub>2</sub>, elevated temperature and reduced RH) in plants exposed to two irrigation regimes. The hypothesis behind this experiment was the higher suitability of long phenological development clones in comparison to short phenological development ones to grow under environmental conditions similar to the ones expected in 2100, especially in regards to plant phenology, photosynthesis performance and plant growth. One of the strengths of the present paper lies in the assessment of three-way interactions among clones, T/CO<sub>2</sub>/RH regimes, and water availability, since the information obtained of multi-stress approaches is crucial to predict the impact of the projected environmental conditions and to design mitigation and adaptation strategies allowing viticulture to cope with climate change.

## 2. MATERIAL AND METHODS

### 2.1. *Plant material: origin and development*

In this experiment, dormant cuttings of four clones of grapevine (*Vitis vinifera* L.) cv. Tempranillo were used: three certified clones (CL306, RJ43 and VN31) and a non-commercialised one (1084). CL306 and RJ43 are two Spanish clones widely distributed during the previous decade [21] and considered as short and intermediate cycle variants respectively [27–29]. The plant material of these two clones was provided by Estación de Viticultura y Enología de Navarra (EVENA, Olite, Spain). VN31 is a certified clone provided by Vitis Navarra nursery (Larraga, Spain) and described as a long-phenological development clone according to previous field characterization [30,31]. The non-commercialized accession 1084 was provided by the Instituto de Ciencias de la Vid y del Vino (ICVV, Logroño, Spain) and has been classified as a long-phenological development clone according to previous field characterization (ICVV, unpublished data) and on the basis of our previous findings [24].

An adapted protocol from Mullins and Rajasekaran [32] was used to produce fruit-bearing cuttings as described in Arrizabalaga et al. [24] and Morales et al. [33]. Cuttings were treated with a solution of indole butyric acid ( $300 \text{ mg L}^{-1}$ ) and kept for several weeks in a hot-bed at  $27 \text{ }^{\circ}\text{C}$ , placed in a cold room at  $5 \text{ }^{\circ}\text{C}$ , until they developed roots. Then, cuttings were set in pots of 0.8 L with a mixture of 2:1 peat:sand (v/v) and transferred to growth chamber greenhouses (GCGs) [34]. They grew up to fruit set at  $25 \text{ }^{\circ}\text{C}/15 \text{ }^{\circ}\text{C}$  (day/night), air relative humidity (RH) of 50 % and natural light supplemented with high-pressure metal halide lamps (POWERSTAR® HQI®-TS 400W/D PRO, OSRAM, Augsburg, Germany) with a photosynthetic photon flux density of  $500 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$  at plant level for 15 hours a day. Vegetative growth was controlled by manual pruning, and a single flowering stem was allowed to develop on each plant in order to obtain a single berry bunch per plant. After fruit set, and for homogenisation purposes, plants of each clone showing similar phenological development were transferred to 7 L pots with a mixture of 2:1 peat:sand (v/v). Plants were irrigated with the nutritive solution described in Ollat et al. [35].

### 2.2. *Experimental design*

At fruit set, plants of the four clones were distributed in two growth chamber greenhouses (GCGs) and subjected to two temperature,  $\text{CO}_2$  concentration and RH (T/ $\text{CO}_2$ /RH) regimes according to Leibar et al. [36]: climate change conditions (CC), which consisted in simulated year 2100 expected environmental conditions ( $28 \text{ }^{\circ}\text{C}/18 \text{ }^{\circ}\text{C}$ , day/night,  $700 \text{ } \mu\text{mol mol}^{-1}$  (ppm)  $\text{CO}_2$  and 35 %/53 % RH, day/night) vs. current situation (CS;  $24 \text{ }^{\circ}\text{C}/14 \text{ }^{\circ}\text{C}$ , 400 ppm  $\text{CO}_2$  and 45 %/63 % RH). Moreover, plants

within each greenhouse were subjected to two water regimes: well-watered (WW) vs. water deficit (WD; 60 % of the irrigation received by the WW plants).

The CO<sub>2</sub> and temperature conditions in the CC treatment were set according to the IPCC [1]. Regarding RH, ENSEMBLES models (based on IPCC data), according to the Max Planck Institute model (MPI-ECHAM5, [37]), state that the RH for the summer period will be 12 % lower at the end of the present century in the area of study [36]. Irrigation regimes were designed following our previous experiences with grapevine fruit-bearing cuttings [9,36,38]. The water deficit level was chosen to match conditions predicted for the end of the present century in the grid of Navarra and La Rioja (region of interest) by the model of the Max Planck Institute, i.e. 40 % lower precipitation in the summer [36]. Soil water sensors (EC-5 Soil Moisture Sensors, Decagon Devices Inc., Pullman, WA, USA) were placed in the substrate to monitor soil water content. Well-watered plants were maintained at ca. 90 % of the substrate field capacity (sensor value between 40-50 %, m<sup>3</sup> H<sub>2</sub>O x 100 m<sup>-3</sup> substrate), equivalent to 400-500 g H<sub>2</sub>O L<sup>-1</sup> substrate. In the water deficit treatment, plants were subjected to a drought that consisted in withholding irrigation until the soil moisture sensors reached a value of ca. 10 % (m<sup>3</sup> H<sub>2</sub>O x 100 m<sup>-3</sup>), equivalent to 100 g H<sub>2</sub>O L<sup>-1</sup> substrate. Then, plants were irrigated with 60 % of the volume received by the WW plants during the corresponding drought period. In order to provide the same amount of nutrients to all the treatments, irrigation of WW plants was performed using nutrient solution alternated with water, whereas WD plants were irrigated only with nutrient solution.

### *2.3. Pre-dawn water potential*

Pre-dawn leaf water potential ( $\Psi_{\text{leaf}}$ ) was measured at mid-veraison (half of the berries in the bunch had started to change colour) and two weeks after mid-veraison, in young fully expanded leaves, using a pressure chamber (SKYE SKPM 1400, Skye Instruments Ltd, Llandrindod, Wales, UK) and according to the methodology described by Scholander et al. [39].

### *2.4. Phenological development*

The number of days between fruit set and maturity (corresponding to a total soluble solid, TSS, content of ca. 22 °Brix) was determined. Fruit set was assessed visually, whereas maturity was established by sampling periodically two berries and measuring the level of TSS in the must using a refractometer (Abbe Digital 315RS, Zuzi, Beriain, Spain). Every plant was assessed individually.



## *2.5. Leaf gas exchange and chlorophyll content*

Net photosynthesis ( $A_N$ ), transpiration ( $E$ ) and stomatal conductance ( $g_s$ ) measurements were conducted at mid-veraison and 2 weeks after mid-veraison in young fully expanded leaves using a portable photosynthesis system (LCi-SD with the PLUS5 compact light unit, ADC BioScientific, England). Measurements started three hours after the sunrise and extended over a time window of about 3 hours. Temperature,  $CO_2$  and RH conditions used in the measurement chamber corresponded to the respective growth conditions (400 ppm  $CO_2$ , 24 °C and 45 % RH for CS condition, and 700 ppm  $CO_2$ , 28 °C and 35 % RH for CC condition). Measurements were performed under a photosynthetic photon flux density of  $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

Chlorophyll levels were assessed by non-destructive fluorescence measurements using a multiparametric portable optical sensor (Multiplex\_Research, FORCE-A, Orsay, France) [40]. Chlorophyll concentration is correlated to a parameter resulting from the ratio of far-red to red fluorescence (SFR\_R index) [41].

## *2.6. Plant growth and fruit production*

Leaf area was determined at mid-veraison, 2 weeks after mid-veraison and maturity. It was estimated by measuring the shoot length and using a regression model adapted for Tempranillo from Costanza et al. [42], by relating leaf area measured using a leaf area meter (LI-300 model; Li-Cor Biosciences, Lincoln, USA) ( $y$ ) and total shoot length ( $x$ ): Leaf area ( $\text{dm}^2$ ) =  $13.859x + 200.33$ ;  $R^2 = 0.9239$  ( $x$  = shoot length). The vegetative production, expressed as dry matter weight, was measured at maturity by weighing the oven-dried leaves, stem and roots. The drying was done introducing the plant material in an oven at 80 °C for until constant weight.

At maturity, fresh bunch weight and the number of berries per bunch were determined. The fresh weight of individual berries was measured throughout the ripening process (mid-veraison, 1 week after mid-veraison, 2 weeks after mid-veraison and maturity).

## *2.7. Statistical analysis*

The software used for the statistical analysis was R (3.5.1). The tests used were a three-way ANOVA (clone, T/ $CO_2$ /RH regime and water availability). A Fisher's least significant difference (LSD) test was used as a post-hoc ( $p < 0.05$ ). Results were also analysed using a principal component analysis (PCA).

### 3. RESULTS

#### 3.1. Pre-dawn water potential

Clones had similar pre-dawn leaf water potential ( $\Psi_{\text{leaf}}$ ) values ( $P_{\text{CL}} = 0.243$  at mid-veraison and  $P_{\text{CL}} = 0.765$  two weeks after mid-veraison, Figure 1). WD significantly reduced  $\Psi_{\text{leaf}}$  compared with plants grown under WW conditions. A significant interaction between the T/CO<sub>2</sub>/RH and irrigation regimes was observed at mid-veraison, when, under WD conditions, CS plants had lower  $\Psi_{\text{leaf}}$  than the CC ones.

#### 3.2. Leaf gas exchange parameters and photosynthetic pigments

The clones showed similar photosynthetic rates ( $A_N$ ), leaf transpiration (E) and stomatal conductance ( $g_s$ ) values at mid-veraison, considering all the T/CO<sub>2</sub>/RH and water regime situations ( $P_{\text{CL}}$  values of 0.295, 0.222 and 0.160 for  $A_N$ , E, and  $g_s$ , respectively; Figure 2A). Nevertheless, under WW conditions, 1084 tended to have lower  $A_N$ , E and, especially,  $g_s$  values than the other clones. Two weeks later, a significant interaction between clone and irrigation was observed for the above mentioned gas exchange parameters (Figure 2A). At this time, comparing the four clones under WW conditions, the 1084 accession showed significantly lower  $A_N$  and E compared with RJ43 and VN31 (CS conditions) and with VN31 (CC conditions), as well as lower  $g_s$  compared with all the clones (CS conditions). These differences, however, disappeared under WD conditions, which drastically reduced the values of the  $A_N$ , E and  $g_s$ . Considering the clones altogether, CC conditions enhanced significantly  $A_N$  at mid-veraison, regardless of the water regime applied ( $P_{\text{T/CO}_2/\text{RH}} = 0.001$ , Figure 2B). Two weeks after mid-veraison, differences in the  $A_N$  between CS and CC plants were maintained in WW plants, but they disappeared under WD (significant interaction between T/CO<sub>2</sub>/RH and water availability). This effect was especially seen in VN31 and 1084 (Figure 2A). Stomatal conductance was reduced by CC conditions in WW plants, both at mid-veraison and 2 weeks later, but these differences disappeared under WD due to a significant interaction between T/CO<sub>2</sub>/RH and water availability. The clones studied showed some variability in their gas-exchange response to climate change both at mid-veraison and 2 weeks later, the 1084 accession showing the highest increase in  $A_N$  and minor changes in  $g_s$  between CC and CS compared with the other clones (Figure 2A).

Clones did not differ systematically either in the intrinsic ( $WUE_i$ ) or the instantaneous ( $WUE_{\text{inst}}$ ) water use efficiency, but under WD, CL306 and VN31 showed higher  $WUE_{\text{inst}}$  than the other accessions 2 weeks after mid-veraison (CS and CC, respectively) (Table 1). CC conditions significantly increased  $WUE_i$ , especially under WD (significant interaction observed 2 weeks after mid-veraison), but they did not impact  $WUE_{\text{inst}}$ . WD significantly increased both  $WUE_i$  and  $WUE_{\text{inst}}$  at mid-veraison and 2 weeks after mid-veraison.

The SFR\_R index, parameter correlated with the leaf chlorophyll concentration, did not show significant differences among clones either at mid-veraison ( $P_{CL} = 0.334$ ) or 2 weeks after mid-veraison ( $P_{CL} = 0.253$ ) (data not shown). WD significantly increased the chlorophyll index ( $P_I = 0.001$ ) from  $2.05 \pm 0.05$  to  $2.25 \pm 0.03$ , WW and WD, respectively. Two weeks after mid-veraison, CC significantly increased the SFR\_R index compared with CS ( $2.21 \pm 0.03$  and  $2.09 \pm 0.05$  relative units, CC and CS, respectively,  $P_{T/CO_2/RH} = 0.042$ ), regardless of the clone and the irrigation regime.

### *3.3. Phenological development*

Considering the clones altogether, CC conditions significantly shortened the elapsed time between fruit set and maturity, especially under WD conditions, while WD delayed it (Figure 3A). A significant interaction was observed between clones and water availability, where WD significantly delayed maturity in RJ43, CL306 and VN31, but not in the 1084 accession (Figure 3B). The elapsed time between fruit set and maturity was significantly different among clones, the 1084 accession showing a longer grape developmental period.

### *3.4. Total leaf area and vegetative growth*

A significant interaction between clone and irrigation level was observed for total leaf area measured at mid-veraison, 2 weeks after mid-veraison and maturity, as leaf area was significantly reduced by WD throughout all the experiment, this reduction being more noticeable in the 1084 accession (Figure 4A). Clones showed significant differences in total leaf area at maturity in WW plants (higher in 1084), but these differences disappeared under WD conditions. The T/CO<sub>2</sub>/RH regime also interacted with the irrigation level, and CC conditions significantly reduced leaf area at mid-veraison and 2 weeks after mid-veraison, compared with CS, only in WW plants (Figure 4B). No difference between T/CO<sub>2</sub>/RH regimes were observed at maturity (Figure 4B).

CC conditions raised total leaf dry weight at maturity, when considering all the clones as a whole, and especially in VN31, but it did not affect either stem or root growth (Figures 5A, 5B). Clones showed significant differences in the final dry matter production of the different organs analysed, the 1084 accession having the highest dry weight under WW conditions (Figure 5B). WD reduced the final dry weight of all the organs, regardless of the T/CO<sub>2</sub>/RH regime applied, the 1084 being the most affected clone (significant interaction between clone and irrigation regime for leaves, stem and total dry weight) (Figure 5B).

### 3.5. *Bunch characteristics*

No significant interactions among factors were observed for yield and grape characteristics (Table 2). The RJ43 accession had the highest bunch weight and number of berries per bunch, whereas 1084 showed higher individual berry weight all over the ripening period (significant differences with VN31, which had the lowest values). CC did not affect either grape yield or yield components at maturity, although this treatment had the lowest grape weight values at mid-veraison. The bunch weight, number of berries per bunch and individual berry weight were significantly reduced by WD. Even though there were not significant interactions between factors, CL306 was the clone that experienced the strongest reduction in bunch weight as a consequence of WD, especially under CC conditions (reduction of 63 %), compared with the rest of the clones (decreases of 24 %, 14 % and 25 % in RJ43, VN31 and 1084, respectively).

### 3.6. *Principal component analysis*

Phenology, gas exchange as well as vegetative and reproductive growth parameters were analysed by principal component analysis (PCA). The first two principal components explained more than 75 % of the total variability (Figure 6). Differences between water availability regimes were clearly observed along PC1, and they were mainly associated with reduced vegetative growth (dry weight and total leaf area), leaf water potential,  $A_N$ , and E in the WD treatment. Under WW conditions, the 1084 accession was separated from the other clones along PC2, regardless of the T/CO<sub>2</sub>/RH regime, due to a longer fruit set to maturity period and a lower bunch size (fresh weight and number of berries). In contrast, these differences among clones disappeared under water deficit conditions.

## 4. DISCUSSION

In the present study, the impact of simulated year-2100 expected climatic conditions (elevated temperature, high CO<sub>2</sub> and reduced RH acting simultaneously) combined with water deficit on phenology, leaf gas exchange and vegetative and reproductive growth parameters were evaluated in four Tempranillo clones previously selected due to their differences in the timing of grape development.

#### *4.1. Response of leaf water potential and leaf gas exchange traits of Tempranillo clones to the year-2100-expected climate conditions*

All the clones studied were affected in a similar manner by WD both at mid-veraison and maturity, reaching lower  $\Psi_{\text{leaf}}$  values (more negative) than their respective WW treatments, regardless of the T/CO<sub>2</sub>/RH regime. The low  $\Psi_{\text{leaf}}$  values reached in WD plants (close to -1.5 MPa) compared with those of WW plants (around -0.6 MPa) indicate that, with a 40 % reduction in the water availability, plants were exposed to a severe water deficit, also reflected in large differences in the  $g_s$  between WW and WD plants. CC conditions did not affect, in general,  $\Psi_{\text{leaf}}$  of WW plants, although, in WD plants, those under CS conditions showed lower  $\Psi_{\text{leaf}}$  compared with CC. The analysis of each clone individually reveals that these differences were more pronounced in the VN31 accession, but this result was not likely associated with either higher transpiration rates or lower root development of this clone in the treatments CC/WD compared with CS/WD. These differences disappeared 2 weeks after mid-veraison.

$A_N$  values of the 1084 variant were the lowest among the four clones studied, both at mid-veraison and, especially, 2 weeks later. Thus, the 1084 plants grown under CS /WW conditions exhibited a significantly lower  $A_N$  compared with other clones under the same T/CO<sub>2</sub>/RH regime and water availability conditions 2 weeks after mid-veraison. Differences in the levels of leaf chlorophyll do not explain these differences in  $A_N$ , which in part could be partially attributed to a lower stomatal conductance in 1084. Variability for  $A_N$  among grapevine cultivars has been previously reported by Bota et al., Tomás et al., and more recently by Greer [43–45]. Greer et al. attributed, in part, such variability to differences in stomatal conductance, rather than to biochemical factors such as RUBP carboxylation and regeneration [45]. Potential mesophyll diffusion limitations may also explain these differences, although they remain largely unexplored in Tempranillo clones [12].

The impact of water deficit on grapevine photosynthesis performance has been extensively studied [14] and references therein [46,47]. In the present study, water availability was the factor that most affected gas exchange parameters, reducing  $A_N$  values in all the clones studied, and overshadowed the differences among clones observed in WW plants. Such reduction in C assimilation was presumably related to a decrease in CO<sub>2</sub> availability when plants closed stomata to prevent water loss [48], as suggested by the reduction in  $g_s$ . Under mild to moderate water deficits, stomata closure is among the earliest plant responses, restricting water loss and C assimilation [49].

Considering all the clones, CC conditions increased  $A_N$  at mid-veraison, regardless of the water availability, thus compensating partially the impact of WD on C assimilation. Unfortunately, we cannot attribute either to the CO<sub>2</sub> or temperature factor these effects on  $A_N$ , but it is very likely that CO<sub>2</sub>,

rather than temperature, was the main factor affecting photosynthetic rates under the present experimental conditions. Grapevine photosynthesis, as in other  $C_3$  species, is limited by  $CO_2$ , and therefore high  $CO_2$  has been reported to increase  $A_N$  [11,50]. In addition, previous studies in different grapevine cultivars, grown in both natural and controlled environments show that changes in temperature in the range of the present work (24 °C to 28 °C) had not significant effects on grapevine photosynthesis [45,51]. Two weeks after mid-veraison, a significant interaction between T/ $CO_2$ /RH regime and irrigation regime was observed, and the positive effect of CC in WW plants was completely abolished by drought. The results agree with those reported by Leibar et al. under similar experimental conditions [36]. Despite the absence of significant interaction between clone identity and T/ $CO_2$ /RH regime, it is worth to mention that, under WW conditions, the 1084 accession exhibited a more pronounced increase in  $A_N$  in response to CC compared with the rest of clones studied, both at mid-veraison and 2 weeks later. These results may reflect some differences among clones in their photosynthetic response to the projected environmental conditions.

Within the frame of the IPCC predictions for a decrease in water availability, the improvement of the crops water use efficiency has become a priority in basic and applied research in the later years [23]. Clonal variability of the  $WUE_i$  has been reported for the Tempranillo cultivar [23,25]. However, considering all the T/ $CO_2$ /RH and irrigation regimes, we did not observe significant differences in  $WUE_i$  and  $WUE_{inst}$  among the clones considered in the present work. It must be noted that Tortosa et al. studied 30 different genotypes, among them the RJ43, VN31 and 1084 accessions, and the differences among these three clones were not as remarkable as among other clones included in their study [25]. More recently, Tortosa et al. reported no statistical differences between RJ43 and 1084 accessions either under field or in pot conditions [23].

Despite the absence of systematic differences in both  $WUE_i$  and  $WUE_{inst}$  among clones, under WD, CL306 and VN31 showed higher values of  $WUE_{inst}$  than the other accessions (CS and CC, respectively) [23]. The result suggests that these Tempranillo accessions may perform better WUE than others depending on the T/ $CO_2$ /RH and/or the water availability regime.

Considering all the clones, both WD and CC significantly increased  $WUE_i$ , which was associated with a drop in  $g_s$  values in the first case, and with a higher photosynthetic capacity and lower  $g_s$ , in the second case. Such improvement in  $WUE_i$  was especially remarkable when these two environmental conditions were combined (CC/WD), thus suggesting that, in a future environment with high  $CO_2$  and elevated temperature,  $WUE_i$  of grapevine may be improved under drought conditions [36]. However, when leaf transpiration was considered to calculate  $WUE_{inst}$  ( $A_N/E$ ), the gain in water use efficiency of plants under CC conditions disappeared. That is because the reduced  $g_s$  observed under CC were not

accompanied by low transpiration rates, probably related to the higher vapour pressure deficit in these conditions. Therefore, the increase in water vapour concentration difference between leaf and air, as a consequence of the projected air temperature and RH conditions, would largely offset the potential gain in WUE produced by elevated CO<sub>2</sub> [52]. Such results highlight the importance of studying the combined effect of CO<sub>2</sub> with other climate change factors also involved in climate change, which may modulate the photosynthetic and water use efficiency response of plants to CO<sub>2</sub>. In addition, the WUE<sub>inst</sub> seems to be a more suitable parameter to estimate the photosynthetic water use efficiency under environmental conditions that modify vapour pressure deficit as in this case.

#### *4.2. Phenological response of Tempranillo clones to the year-2100-expected climate conditions*

Differences in the timing of maturity among Tempranillo clones have been reported in previous studies of our group [24], the 1084 accession being characterised as having a long phenological development clone. Such behaviour, however, could not be completely explained in the present study by a lower photosynthetic activity in this clone, although the differences observed may have partially contributed to the delayed maturity in the 1084 accession. Grapevine phenology is greatly influenced by temperature [19] (and references therein), especially the early phenological events. However, for later phenological events, the level of complexity increases and other environmental factors may also influence the timing of phenophases [5]. In the present study, water availability was the factor that most strongly affected grape development. The severe reduction in the A<sub>N</sub> and total leaf area in WD plants probably limited C availability, thus slowing down grape ripening in these treatments. Whereas mild water deficit has been proven to enhance ripening, severe water deficit has been reported to induce stomatal closure, greatly reducing C fixation, and consequently, impairing berry ripening [14,53]. In addition, the statistical interaction between clone and irrigation revealed a differential response of the studied genotypes to WD, the 1084 accession being the least responsive. This result may be explained with a lower difference in the A<sub>N</sub> between WW and WD conditions observed in this accession. Contrary to WD, CC advanced grape maturity when all the clones were taken into consideration and compensated the delaying effect produced by drought. This is in accordance with previous studies under controlled and semi-controlled conditions [5,36,53]. The advancement of ripeness in CC indicates a faster sugar accumulation, which was concomitant with higher A<sub>N</sub> in CC (both in WW and WD at mid-veraison, and in WW 2 weeks later), compared with CS, as from mid-veraison onwards the main part of the photoassimilates is directed to berry maturation [54] (and references therein).

#### *4.3. Vegetative and reproductive response of Tempranillo clones to the year-2100-expected climate conditions*

Water deficit was the most limiting factor in the present study, and its impact on C assimilation was clearly reflected at a plant level reducing leaf area and dry matter production of all the vegetative organs analysed, regardless of the clone and the T/CO<sub>2</sub>/RH regime. However, roots were less affected than the above-ground part, as was also reported by Kizildeniz et al. [9,55]. The significant interaction between clone and irrigation regime reveals a certain degree of intra-varietal diversity in the growth response of Tempranillo to WD, the 1084 being one of the accessions more negatively affected. Probably, the long life cycle of this variant, and consequently, the longer exposure to WD contributed to exacerbate the differences between WW and WD plants at maturity.

WD also reduced the reproductive growth. Medrano et al., in a ten-year study on the effect of water availability on two Spanish grapevine cultivars, concluded that there is a close link between water availability and grape yield, through water stress effects on photosynthesis [47]. The reduction in bunch weight in the present study was concomitant with a lower berry size and a lower number of berries per bunch, the later suggesting a loss of berries produced by a severe water deficit. The reduction in average berry weight for WD plants was already evident at mid-veraison, and differences between the WW and WD treatments were maintained constant thereafter until maturity, thus reflecting a high sensitivity of berry growth to water limitations imposed before mid-veraison. McCarthy and Roby and Matthews also showed that berry size is more sensitive to water deficit before mid-veraison, whereas water deficit after mid-veraison had only minor effects on berry weight at maturity [56,57].

The impact of CC conditions on the vegetative and reproductive growth was similar in all the clones studied. The higher photosynthetic rates of plants grown under CC conditions were associated with an increased leaf dry mass production under WW conditions, with a minor impact on reproductive growth. These results are in agreement with those observed by Kizildeniz et al. who reported that elevated CO<sub>2</sub> stimulated more vegetative than reproductive growth both in red and white Tempranillo [55]. In contrast, Bindi et al. and Moutinho-Pereira et al. observed a positive impact of elevated CO<sub>2</sub> on grape yield, although the later only found significant differences in one of the three seasons studied [7,11].



## 5. CONCLUSION

Simulated year-2100-expected temperature, CO<sub>2</sub>, and RH conditions (CC) hastened grape phenological development and increased leaf biomass of the clones of Tempranillo studied, which was associated with increased photosynthetic rates. Such effect was abolished by WD, which was the factor that most strongly affected gas exchange, vegetative and reproductive growth of grapevine fruit-bearing cuttings. CC increased the WUE<sub>i</sub>, especially when combined with WD in all the clones, but it did not modify the WUE<sub>int</sub>, probably due to the higher temperature and lower RH in CC, which increased the vapour pressure deficit in this treatment. Although the studied clones showed, in general, a similar behaviour under the simulated CC conditions, some degree of variability in their response to changes in the T/CO<sub>2</sub>/RH regime was observed for A<sub>N</sub> and g<sub>s</sub>, as well as in the response to WD for gas exchange parameters, phenology and vegetative and reproductive growth. The differences among clones observed in terms of phenological development seemed to condition the impact of the environmental conditions assayed on the vegetative growth.

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## TABLES

Table 1. Intrinsic Water Use Efficiency (WUE<sub>i</sub>) and Instantaneous Water Use Efficiency (WUE<sub>inst</sub>) of the *V. vinifera* cv. Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation (“CS”: 24 °C/14°C, 400 ppm and 45%/65 % RH) and climate change (“CC”: 28 °C/18 °C, 700 ppm and 33%/53 % RH), combined with two irrigation regimes: well-watered (WW) and water deficit (WD), at mid-veraison and 2 weeks after mid-veraison. Results (values are means ± SE) are shown according to the clone identity (n = 21-32), T/CO<sub>2</sub>/RH condition (n = 52-60), irrigation regime (n = 39-61) and the three factors together (n = 3-8).

|       |                                   | WUE <sub>i</sub> (μmol CO <sub>2</sub> mol <sup>-1</sup> H <sub>2</sub> O) |      |                            |    | WUE <sub>inst</sub> (μmol CO <sub>2</sub> mmol <sup>-1</sup> H <sub>2</sub> O) |       |                            |     |
|-------|-----------------------------------|--|------|----------------------------|----|--|-------|----------------------------|-----|
|       |                                   | Mid-veraison   |      | 2 weeks after mid-veraison |    | Mid-veraison   |       | 2 weeks after mid-veraison |     |
|       | RJ43                              | 104.0 ± 12.2   | a    | 130.2 ± 14.4               | a  | 4.93 ± 0.40  | a     | 5.16 ± 0.24                | a   |
|       | CL306                             | 119.5 ± 14.6   | a    | 123.2 ± 16.8               | a  | 4.85 ± 0.28  | a     | 4.93 ± 0.39                | a   |
|       | VN31                              | 104.5 ± 9.9  | a    | 109.7 ± 10.3               | a  | 4.50 ± 0.38  | a     | 5.42 ± 0.37                | a   |
|       | 1084                              | 115.2 ± 11.7   | a    | 123.4 ± 13.3               | a  | 4.50 ± 0.25  | a     | 4.63 ± 0.24                | a   |
|       | CS                                | 78.5 ± 5.8   | b    | 83.5 ± 5.9                 | b  | 4.52 ± 0.24  | a     | 4.81 ± 0.20                | a   |
|       | CC                                | 140.6 ± 8.4  | a    | 165.5 ± 9.7                | a  | 4.86 ± 0.23  | a     | 5.27 ± 0.24                | a   |
|       | WW                                | 87.5 ± 5.2   | b    | 94.0 ± 5.8                 | b  | 4.15 ± 0.11  | b     | 4.60 ± 0.13                | b   |
|       | WD                                | 147.2 ± 10.9   | a    | 157.2 ± 11.7               | a  | 5.45 ± 0.34  | a     | 5.49 ± 0.28                | a   |
| RJ43  | CS Water                          | 62.9 ± 4.4   | fg   | 64.0 ± 8.5                 | d  | 4.21 ± 0.17  | bcde  | 4.73 ± 0.34                | bcd |
|       | Drought                           | 107.7 ± 18.4   | bcde | 96.8 ± 18.7                | c  | 6.25 ± 1.21  | a     | 5.64 ± 0.56                | bc  |
|       | CC Water                          | 132.9 ± 12.4   | cde  | 130.4 ± 11.1               | bc | 4.35 ± 0.44  | bcde  | 4.78 ± 0.23                | bcd |
|       | Drought                           | 158.1 ± 30.6   | abc  | 224.2 ± 28.2               | a  | 5.58 ± 1.35  | abc   | 5.54 ± 0.70                | bc  |
| CL306 | CS Water                          | 50.7 ± 2.9   | fg   | 63.0 ± 11.1                | d  | 3.91 ± 0.26  | cde   | 4.47 ± 0.28                | bcd |
|       | Drought                           | 115.8 ± 15.8   | bcde | 99.3 ± 17.0                | cd | 4.90 ± 0.87  | abcde | 6.23 ± 1.15                | ab  |
|       | CC Water                          | 133.2 ± 9.1  | bcd  | 134.7 ± 21.0               | bc | 4.92 ± 0.13  | abcde | 4.87 ± 0.65                | bcd |
|       | Drought                           | 200.4 ± 45.9   | a    | 235.0 ± 39.0               | a  | 5.84 ± 0.71  | ab    | 4.51 ± 0.97                | bcd |
| VN31  | CS Water                          | 55.6 ± 5.2   | g    | 58.9 ± 2.7                 | d  | 3.55 ± 0.23  | e     | 4.12 ± 0.30                | cd  |
|       | Drought                           | 118.1 ± 10.8   | bcd  | 126.8 ± 25.2               | c  | 5.37 ± 1.07  | abcd  | 5.25 ± 0.53                | bcd |
|       | CC Water                          | 111.2 ± 9.4  | def  | 141.6 ± 9.2                | c  | 3.83 ± 0.30  | de    | 5.16 ± 0.37                | bcd |
|       | Drought                           | 161.4 ± 44.3   | ab   | 231.3 ± 42.1               | ab | 5.98 ± 1.09  | ab    | 7.42 ± 1.20                | a   |
| 1084  | CS Water                          | 43.4 ± 2.9   | efg  | 50.8 ± 5.0                 | d  | 3.75 ± 0.31  | de    | 3.85 ± 0.48                | d   |
|       | Drought                           | 129.2 ± 17.1   | cdef | 115.7 ± 8.4                | cd | 5.37 ± 0.60  | abcde | 4.66 ± 0.57                | bcd |
|       | CC Water                          | 105.9 ± 7.5  | bcd  | 114.6 ± 9.4                | c  | 4.65 ± 0.35  | abcde | 4.88 ± 0.20                | bcd |
|       | Drought                           | 165.8 ± 14.5   | abc  | 186.4 ± 30.1               | a  | 4.68 ± 0.71  | abcde | 5.11 ± 0.50                | bcd |
|       | P(CL)                             | ns   |      | ns                         |    | ns   |       | ns                         |     |
|       | P(T/CO <sub>2</sub> /RH)          | ***  |      | ***                        |    | ns   |       | ns                         |     |
|       | P(I)                              | ***  |      | ***                        |    | ***  |       | **                         |     |
|       | P(CL x T/CO <sub>2</sub> /RH)     | ns   |      | ns                         |    | ns   |       | ns                         |     |
|       | P(CL x I)                         | ns   |      | ns                         |    | ns   |       | ns                         |     |
|       | P(T/CO <sub>2</sub> /RH x I)      | ns   |      | *                          |    | ns   |       | ns                         |     |
|       | P(CL x T/CO <sub>2</sub> /RH x I) | ns   |      | ns                         |    | ns   |       | ns                         |     |

Means with letters in common within the same parameter, stage and factor (clone, T/CO<sub>2</sub>/RH, irrigation regime, or their interaction) are not significantly different according to LSD test ( $P > 0.05$ ). Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I), P(T/CO<sub>2</sub>/RH x I) and P(CL x T/CO<sub>2</sub>/RH x I). \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant.

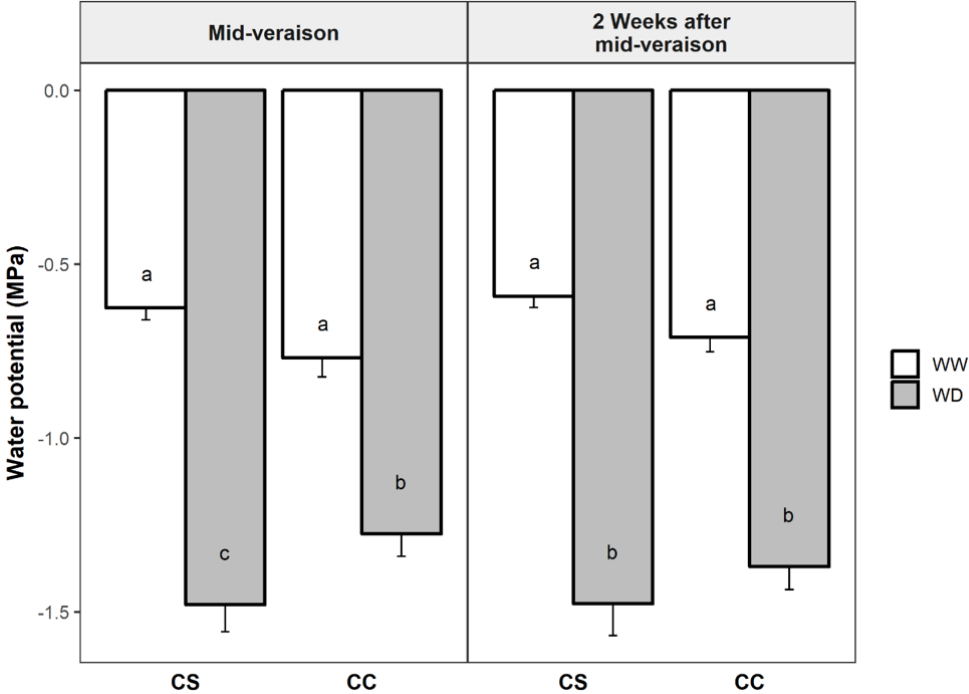
Table 2. Bunch weight, number of berries per bunch and individual berry weight (at mid-veraison, 1 weeks after mid-veraison, 2 weeks after mid-veraison and at maturity) of the *V. vinifera* cv. Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation ("CS": 24 °C/14 °C, 400 ppm and 45%/65% RH) and climate change ("CC": 28 °C/18 °C, 700 ppm and 33%/53% RH), combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Results (values are means ± SE) are shown according to the clone identity (n = 26-32), T/CO<sub>2</sub>/RH condition (n = 59-62), irrigation regime (n = 58-62) and the three factors together (n = 5-8).

|       | BUNCH WEIGHT (g FW) | NUMBER OF BERRIES/BUNCH | WEIGHT OF IND. BERRY (g FW berry <sup>-1</sup> ) |                           |                                 |                            | Maturity |
|-------|---------------------|-------------------------|--|---------------------------|---------------------------------|----------------------------|----------|
|       |                     |                         | Mid-veraison                                     | 1 week after mid-veraison | mid- 2 weeks after mid-veraison | 2 weeks after mid-veraison |          |
| RJ43  | 154,47 ±12,10 a     | 152,91 ±9,90 a          | 0,85 ±0,04 b                                     | 1,02 ±0,05 ab             | 1,16 ±0,04 a                    | 0,95 ±0,04 ab              |          |
| CL306 | 98,01 ±11,29 b      | 103,31 ±10,58 b         | 0,76 ±0,04 bc                                    | 0,97 ±0,04 b              | 1,11 ±0,04 ab                   | 0,99 ±0,05 a               |          |
| VN31  | 98,31 ±8,94 b       | 114,23 ±8,98 b          | 0,74 ±0,04 c                                     | 0,92 ±0,04 b              | 1,02 ±0,04 b                    | 0,85 ±0,04 b               |          |
| 1084  | 97,00 ±8,89 b       | 91,23 ±6,40 b           | 1,02 ±0,05 a                                     | 1,10 ±0,05 a              | 1,19 ±0,05 a                    | 0,99 ±0,06 a               |          |
| CS    | 114,53 ±8,30 a      | 116,80 ±7,01 a          | 0,93 ±0,03 a                                     | 1,04 ±0,03 a              | 1,15 ±0,03 a                    | 0,93 ±0,03 a               |          |
| CC    | 110,27 ±7,53 a      | 115,64 ±7,05 a          | 0,76 ±0,03 b                                     | 0,97 ±0,03 a              | 1,09 ±0,03 a                    | 0,96 ±0,03 a               |          |
| WW    | 133,75 ±8,36 a      | 125,76 ±6,85 a          | 0,94 ±0,03 a                                     | 1,14 ±0,03 a              | 1,24 ±0,03 a                    | 1,01 ±0,04 a               |          |
| WD    | 91,05 ±6,40 b       | 106,05 ±6,98 b          | 0,74 ±0,03 b                                     | 0,86 ±0,03 b              | 1,00 ±0,03 b                    | 0,87 ±0,03 b               |          |
| RJ43  | 184,9 ±39,5 a       | 159,8 ±28,6 ab          | 0,99 ±0,10 bc                                    | 1,21 ±0,11 ab             | 1,28 ±0,09 abc                  | 1,03 ±0,11 ab              |          |
| WD    | 126,2 ±17,8 bcd     | 130,1 ±21,0 abc         | 0,90 ±0,05 bcd                                   | 0,94 ±0,04 cdef           | 1,13 ±0,03 abcde                | 0,93 ±0,05 abc             |          |
| CC    | 174,6 ±12,6 ab      | 158,5 ±11,6 ab          | 0,88 ±0,03 bcde                                  | 1,14 ±0,06 abc            | 1,30 ±0,06 ab                   | 1,06 ±0,07 ab              |          |
| WD    | 132,2 ±13,6 abc     | 163,3 ±15,5 a           | 0,63 ±0,07 fg                                    | 0,76 ±0,06 f              | 0,93 ±0,06 efg                  | 0,77 ±0,07 c               |          |
| CL306 | 129,2 ±17,4 abcd    | 112,0 ±15,5 bcd         | 1,00 ±0,04 bc                                    | 1,14 ±0,03 abc            | 1,29 ±0,06 abc                  | 1,17 ±0,13 a               |          |
| WD    | 95,0 ±16,8 cdef     | 106,1 ±23,4 cd          | 0,73 ±0,07 defg                                  | 0,89 ±0,05 def            | 1,08 ±0,07 cdefg                | 0,87 ±0,07 bc              |          |
| CC    | 122,5 ±26,4 bcde    | 113,1 ±24,1 bcd         | 0,77 ±0,06 cdef                                  | 1,07 ±0,08 abcd           | 1,19 ±0,07 abcd                 | 1,01 ±0,08 abc             |          |
| WD    | 45,3 ±16,9 f        | 73,4 ±21,9 d            | 0,53 ±0,05 g                                     | 0,78 ±0,08 ef             | 0,89 ±0,09 fg                   | 0,85 ±0,09 bc              |          |
| VN31  | 117,3 ±17,5 cde     | 134,4 ±17,7 abc         | 0,92 ±0,04 bcd                                   | 1,06 ±0,07 abcd           | 1,13 ±0,03 abcde                | 0,87 ±0,05 bc              |          |
| WD    | 74,8 ±9,4 def       | 92,9 ±12,7 cd           | 0,73 ±0,07 defg                                  | 0,80 ±0,07 ef             | 0,94 ±0,07 efg                  | 0,75 ±0,04 c               |          |
| CC    | 108,3 ±20,7 cde     | 114,9 ±21,6 abcd        | 0,68 ±0,10 efg                                   | 1,02 ±0,10 bcd            | 1,14 ±0,10 abcde                | 0,93 ±0,12 abc             |          |
| WD    | 92,8 ±21,1 cdef     | 114,9 ±19,2 abcd        | 0,62 ±0,07 fg                                    | 0,79 ±0,05 ef             | 0,87 ±0,06 g                    | 0,86 ±0,04 bc              |          |
| 1084  | 116,8 ±18,8 cde     | 113,8 ±12,9 bcd         | 1,23 ±0,10 a                                     | 1,27 ±0,10 a              | 1,33 ±0,10 a                    | 0,92 ±0,12 bc              |          |
| WD    | 71,4 ±17,3 ef       | 78,7 ±11,8 d            | 0,90 ±0,09 bcde                                  | 0,93 ±0,13 cdef           | 1,00 ±0,10 defg                 | 0,89 ±0,10 bc              |          |
| CC    | 114,3 ±17,6 cde     | 96,4 ±11,1 cd           | 1,06 ±0,12 ab                                    | 1,22 ±0,08 ab             | 1,29 ±0,07 abc                  | 1,10 ±0,12 ab              |          |
| WD    | 85,5 ±15,1 cdef     | 74,5 ±12,3 d            | 0,88 ±0,07 bcde                                  | 0,98 ±0,07 cde            | 1,11 ±0,11 bcdef                | 1,04 ±0,11 ab              |          |

Means with letters in common within the same parameter, stage and factor (clone, temperature and CO<sub>2</sub> irrigation regime, or their interaction) are not significantly different according to LSD test (P > 0.05). All probability values for the interactions of factors (P(CL x T/CO<sub>2</sub>/RH), P(CL x I), P(T/CO<sub>2</sub>/RH x I) and P(CL x T/CO<sub>2</sub>/RH x I)) were statistically not significant (P > 0.05).



**FIGURES**



|                               |     |     |
|-------------------------------|-----|-----|
| P(CL)                         | ns  | ns  |
| P(T/CO <sub>2</sub> /RH)      | ns  | ns  |
| P(I)                          | *** | *** |
| P(CL x T/CO <sub>2</sub> /RH) | ns  | ns  |
| P(CL x I)                     | ns  | ns  |
| P(T/CO <sub>2</sub> /RH x I)  | **  | ns  |

Figure 1. Pre-dawn leaf water potential at mid-veraison and 2 weeks after mid-veraison of the *V. vinifera* cv. Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation (“CS”: 24 °C/14 °C, 400 ppm and 45 %/65 % RH) and climate change (“CC”: 28 °C/18 °C, 700 ppm and 33 %/53 % RH), combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Results (values are means ± SE) are represented according to the T/CO<sub>2</sub>/RH and irrigation regimes (n = 14-19). Means with letters in common within the same stage are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I) and P(T/CO<sub>2</sub>/RH x I).\*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. Interaction of all factors P(CL x T/CO<sub>2</sub>/RH x I) was statistically not significant (P > 0.05).

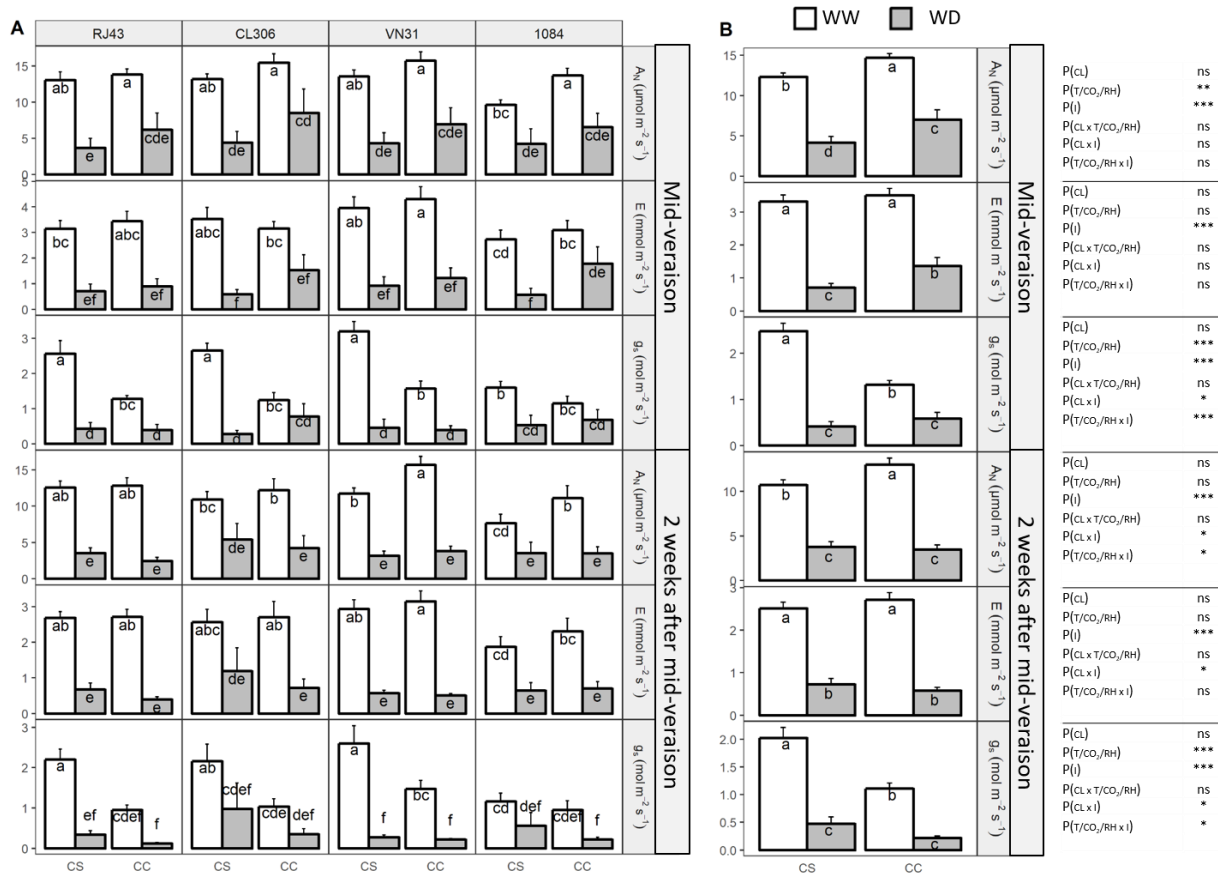


Figure 2. Net photosynthesis ( $A_N$ ), transpiration ( $E$ ) and stomatal conductance ( $g_s$ ) of the *V. vinifera* cv. Tempranillo clones grown under two  $T/CO_2/RH$  conditions: current situation (“CS”: 24 °C/14 °C, 400 ppm, and 45%/65% RH) and climate change (“CC”: 28 °C/18 °C, 700 ppm and 33%/53% RH), combined with two irrigation regimes: well-watered (WW) and water deficit (WD), at mid-veraison and 2 weeks after mid-veraison. Results (values are means  $\pm$  SE) are represented according to (A) the clones,  $T/CO_2/RH$  and irrigation regimes ( $n = 5-8$ ) and (B) to  $T/CO_2/RH$  and irrigation regimes, considering the clones altogether ( $n = 18-31$ ). Means with letters in common within the same parameter and stage are not significantly different according to LSD test ( $P > 0.05$ ). Probability values ( $P$ ) for the main effects of clone,  $P(CL)$ ;  $T/CO_2/RH$  regime,  $P(T/CO_2/RH)$ ; irrigation regime,  $P(I)$ ; and their interactions,  $P(CL \times T/CO_2/RH)$ ,  $P(CL \times I)$  and  $P(T/CO_2/RH \times I)$ . \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant. Interaction of all factors  $P(CL \times T/CO_2/RH \times I)$  was statistically not significant ( $P > 0.05$ ).

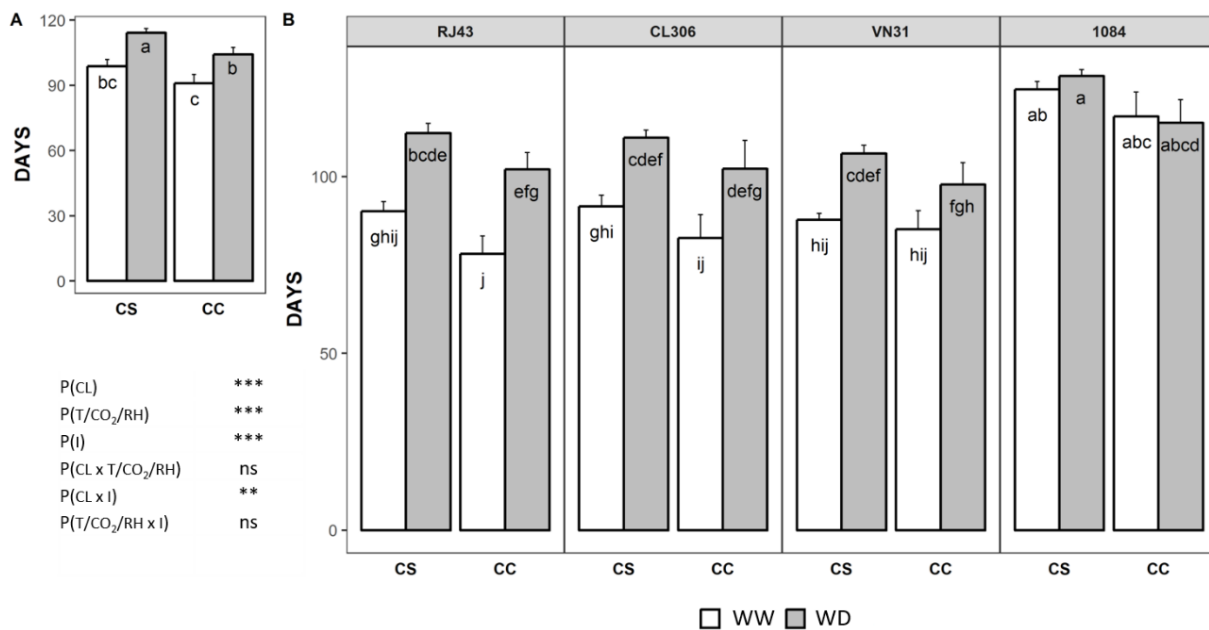


Figure 3. Number of days between fruit set and maturity (ca. 22°Brix) of the *V. vinifera* cv. Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation (“CS”: 24 °C/14 °C, 400 ppm and 45 %/65 % RH) and climate change (“CC”: 28 °C/18°C, 700 ppm and 33 %/53 % RH), combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Data (values are means ± SE) are presented according to: (A) the T/CO<sub>2</sub>/RH and irrigation regimes considering the clones altogether (n = 28-31) and (B) the clones, T/CO<sub>2</sub>/RH and irrigation regimes (n = 6-8). Means with letters in common within the same chart (A or B) are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I) and P(T/CO<sub>2</sub>/RH x I).\*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. Interaction of all factors P(CL x T/CO<sub>2</sub>/RH x I) was statistically not significant (P > 0.05).

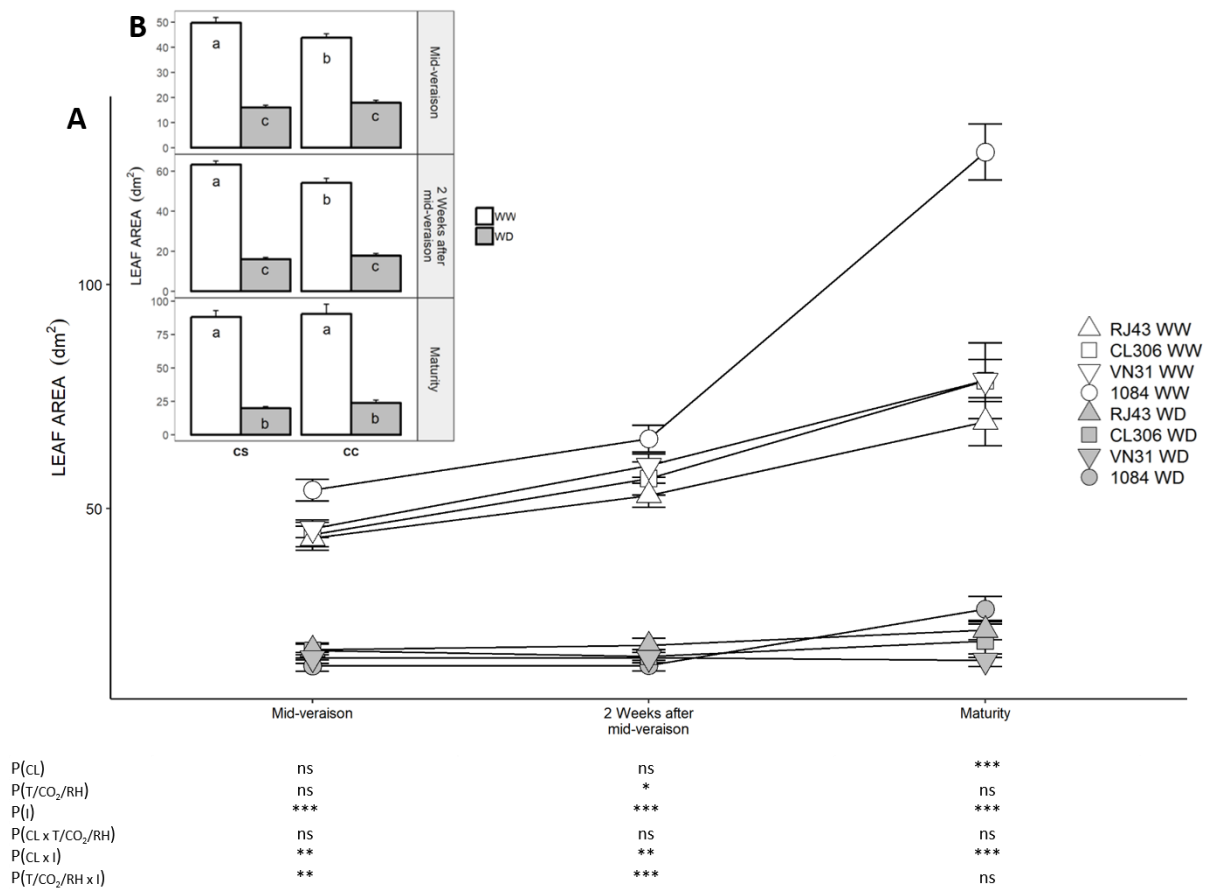


Figure 4. Total leaf area at mid-veraison, 2 weeks after mid-veraison and maturity of the *V. vinifera* cv. Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation ("CS": 24 °C/14 °C, 400 ppm and 45 %/65 % RH) and climate change ("CC": 28 °C/18 °C, 700 ppm and 33 %/53 % RH), combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Data (values are means ± SE) are represented according to (A) the clones, and irrigation regimes (n = 14-16) and to (B) the T/CO<sub>2</sub>/RH and irrigation regimes (n = 31). Means with letters in common within the same stage in chart B are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I) and P(T/CO<sub>2</sub>/RH x I).\*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. Interaction of all factors P(CL x T/CO<sub>2</sub>/RH x I) was statistically not significant (P > 0.05).

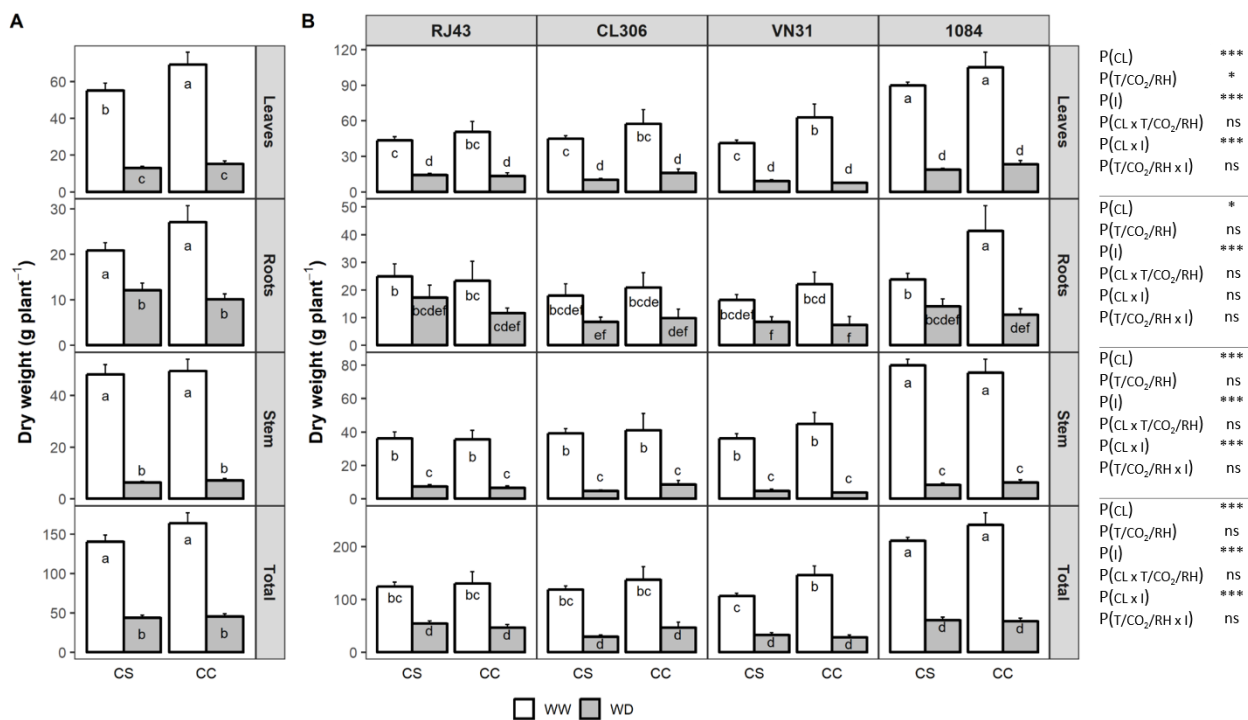


Figure 5. Leaves, roots, stem and total dry weight of the *V. vinifera* cv. Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation (“CS”: 24 °C/14 °C, 400 ppm and 45 %/65 % RH) and climate change (“CC”: 28 °C/18 °C, 700 ppm and 33 %/53 % RH), combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Data (values are means ± SE) are represented according to (A) the T/CO<sub>2</sub>/RH and irrigation regimes (n = 28-31) and (B) the clones, T/CO<sub>2</sub>/RH and irrigation regimes (n = 5-8). Means with letters in common within the same chart (A or B) and organ are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I) and P(T/CO<sub>2</sub>/RH x I).\*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. Interaction of all factors P(CL x T/CO<sub>2</sub>/RH x I) was statistically not significant (P > 0.05).

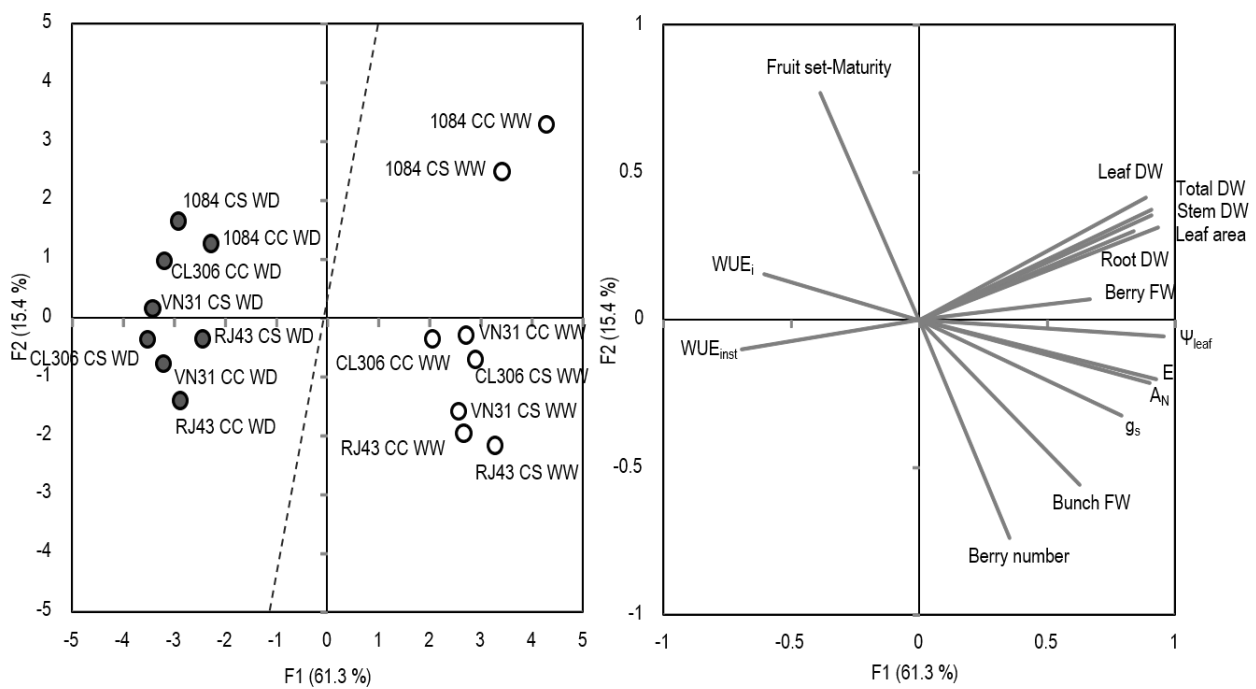


Figure 6. Principal component analysis of pre-dawn water potential, phenology, gas exchange and dry mass production parameters: score (A) and loading plot (B). CS, current conditions (24 °C/14 °C, 400 ppm and 45 %/65 % RH); CC, climate change conditions (28 °C/18 °C, 700 ppm and 33 %/53 % RH); WW, well-watered; WD, water deficit. FW, fresh weight; DW, dry weight;  $A_N$ , net photosynthesis; E, leaf transpiration;  $g_s$ , stomatal conductance;  $WUE_i$ , intrinsic water use efficiency;  $WUE_{inst}$ , instantaneous water use efficiency;  $\Psi_{leaf}$ , pre-dawn leaf water potential; fruit set-maturity, number of days between fruit set and maturity.



## **CHAPTER 4**

High temperature and elevated CO<sub>2</sub> modify berry composition of different clones of grapevine (*Vitis vinifera* L.) cv. Tempranillo





# High temperature and elevated CO<sub>2</sub> modify berry composition of different clones of grapevine (*Vitis vinifera* L.) cv. Tempranillo

## ABSTRACT

Tempranillo is a grapevine (*Vitis vinifera* L.) variety extensively used for world wine production which is expected to be affected by the environmental parameters modified by on-going global climate changes: increases in average air temperature and rise of atmospheric CO<sub>2</sub> levels. Apart from determining their effects on grape development and biochemical characteristics, this paper considers the intra-varietal diversity of the cultivar Tempranillo as a tool to develop future adaptive strategies to face the impact of climate change on grapevine. Fruit-bearing cuttings of five clones (RJ43, CL306, T3, VN31 and 1084) were grown in temperature gradient greenhouses (TGGs), from fruit set to maturity, under two temperature regimes (ambient temperature vs ambient temperature plus 4 °C) and two CO<sub>2</sub> levels (ambient, ca. 400 ppm, vs elevated, 700 ppm). Treatments were applied separately or in combination. The analyses carried out included berry phenological development, the evolution in the concentration of must compounds (organic acids, sugars and amino acids) and total skin anthocyanins. Elevated temperature hastened berry ripening as well as malic acid breakdown, especially when combined with high CO<sub>2</sub> concentration, as the latter seemed to induce the anaplerotic flux through the tricarboxylic acid cycle. Elevated CO<sub>2</sub> reduced the decoupling effect of temperature on the anthocyanin to sugar ratio. The impact of these factors, taken individually or combined, was dependent on the clone analysed, thus indicating certain intra-varietal variability in the response of Tempranillo to these climate change-related factors.

**Keywords:** Climate change; Grapevine (*Vitis vinifera*); Genetic variability; Sugars; Malic acid; Amino acids; Anthocyanins.

## 1. INTRODUCTION

Grapevine is one of the most prominent crops in agriculture given the cultural and economic importance of grape and wine production. Among the grape varieties cultivated worldwide, Tempranillo ranked #3 in 2017 with 231,000 ha, behind Cabernet-Sauvignon and Kyoho [1] and is one of the most important red grape varieties grown in Spain. This cultivar is characterized by subtle aroma, producing wines with fruity and spicy flavours, low acidity and low tannins. However, wine organoleptic characteristics are highly determined by the characteristics of the grapes used for its production. Therefore, changes in grapevine growing conditions that affect berry composition are also likely to affect the wine produced. Grape quality is a complex trait that mainly refers to berry composition, including sugars, organic acids (malic and tartaric acid), amino acids and a wide range of secondary metabolites such as phenolic compounds, aromas and aroma precursors [2]. Among the factors that affect berry content at harvest, climate parameters, and notably temperature, play a prominent role.

The Intergovernmental Panel on Climate Change (IPCC) has pointed out the ineluctable increase in the temperature worldwide and has identified climate change as an important threat to global food supply. Indeed, greenhouse gas (GHG) emissions have increased greatly during the last decades, affecting the equilibrium of biogeochemical cycles and, hence, the composition of the atmosphere [3]. Some of the consequences of the rise in the atmospheric concentration of GHG are at global climate level, as high levels of GHG block heat loss of the planet, thus contributing to the so-called “greenhouse effect” and to global warming. Another effect of the anthropogenic GHG emissions is the increase in the concentration of CO<sub>2</sub> in the atmosphere [4]. The IPCC has carried out different researches to determine the magnitude of these changes according to different scenarios. Some of the predictions for global mean temperature in 2100 show an increment between 2.2 ± 0.5 °C and 3.7 ± 0.7 °C and the estimations for future atmospheric CO<sub>2</sub> concentration are between 669.7 and 935.9 ppm (scenarios RCP 6.0 and RCP 8.5 of the IPCC) [3].

Research on the response of grapevine to the above mentioned environmental factors has concluded that high temperature affects the phenology of grapevine, as well as grape berry development and ripening, hastening the latter [5–9] and affecting both primary and secondary metabolisms. Berry sugar accumulation is altered under climate change conditions [7,10], meanwhile malic acid degradation is enhanced by high temperatures [11,12] and by its combination with elevated CO<sub>2</sub> [13,14]. Secondary metabolism is also sensitive to high temperatures, particularly the flavonoid and aroma precursor pathways, as evidenced by transcriptomic and metabolomic approaches [8,15–20], thus affecting the balance of berry quality-related compounds at ripeness [10,21]. In addition, whereas

increased temperature has been consistently shown to reduce anthocyanin levels [18–20,22], the effect of CO<sub>2</sub> on these compounds is more controversial with some authors reporting no effect [23], whereas others reported an increase in anthocyanin levels [24]. Finally, the decoupling in the accumulation of anthocyanins and sugars, thus leading to an imbalance between these two compounds at maturity, has been also described as a consequence of elevated temperature [10,21].

Therefore, in order to avoid detrimental wine traits, it is important to determine how grape characteristics will be affected by the above mentioned climate change-related factors, acting not only individually but also combined, and to investigate approaches to mitigate the undesired effects, ensuring the sustainability of this crop. Among the strategies proposed to adapt viticulture to a changing environment, the genetic improvement and adaptation of elite and autochthonous varieties are very relevant to keep their intrinsic varietal values and typicality [9,25,26]. Accordingly, the selection of grapevine varieties and clones within the same variety with longer ripening periods has been suggested as a valuable tool to exploit in order to find accessions keeping current traits under future climate conditions [27].

Certain varieties used for wine production are tightly linked to specific production areas. This is the case of Tempranillo in Spain, Merlot in France, Sangiovese in Italy or Fernao Pires in Portugal [28]. Tempranillo requires warm, sunny days to reach full maturity but also cool nights to keep its natural acidity. Maturity occurs fairly early in comparison with Grenache, the variety commonly blended with Tempranillo. Nevertheless, the constant increase in temperature and CO<sub>2</sub> levels in the future could significantly shift the optimal maturation conditions in these areas, which would have a significant effect on berry quality. For these reasons, successfully exploiting the intra-varietal diversity has potential to face with the putative negative impacts of climate change, as it would allow to keep the culture of traditional varieties. The research done so far in Tempranillo includes the identification and characterisation of a large number of clones (49 certified clones), which differ either in reproductive or vegetative traits [29], making possible the research of clones well adapted to future climate conditions.

In this context, the objective of this work was to study the effects of increased temperature and rise in atmospheric CO<sub>2</sub>, alone or in combination, on berry development and composition of five different clones of *Vitis vinifera* cv. Tempranillo exhibiting different length of their reproductive cycle. The study was focused on the evolution of must composition (malic acid, sugars and amino acids) and skin total anthocyanins throughout the ripening period, aiming to assess whether the impact of the above mentioned factors differs among different clones of Tempranillo.

## 2. MATERIAL AND METHODS

### 2.1. *Plant material: origin and development*

Dormant cuttings of five clones of grapevine (*Vitis vinifera* L.) cv. Tempranillo were obtained from the germplasm bank of three institutions: RJ43, CL306 (both clones widely cultivated in Spain) and T3 were obtained from Estación de Viticultura y Enología de Navarra (EVENA), located in Olite, Navarra (Spain); 1084 was obtained from the Institute of Sciences of Vine and Wine (Rioja Government, Spain), located in “La Grajera”, La Rioja (Spain); and VN31 was facilitated by Vitis Navarra, located in Larraga, Navarra (Spain). The reproductive cycle length of these clones had been characterised previously, presenting differences among them: VN31 and 1084 have been described as long-reproductive cycle accessions [15,30], CL306 and T3 have been defined as short cycle accessions [31–33], and RJ43 is considered to have an intermediate reproductive cycle length [34].

Fruit-bearing cuttings were obtained as described in Arrizabalaga et al. [15] with minor modifications. They were manipulated to develop a single berry bunch and they were grown at the same conditions as described in Arrizabalaga et al. [15] from March to May 2016, when the fruit set took place. Then, plants were transferred to 13 L pots with 2:1 peat:perlite mixture (v/v) and moved afterwards to Temperature Gradient Greenhouses (TGGs) located in the campus of the University of Navarra (42°48'N, 1°40'W; Pamplona, Navarra, Spain). The irrigation, both before fruit set and at the TGGs, was carried out using the nutritive solution described by Ollat et al. [35].

### 2.2. *Treatments and plant growth at Temperature Gradient Greenhouses (TGGs)*

Treatment application was conducted in TGGs, a structure for plant growth with semi-controlled conditions, taking into consideration natural environmental conditions. Each TGG is divided into three temperature modules which create a gradient of temperature (from module 1 of ambient temperature to module 3 of ambient temperature + 4 °C), as the air heats up when passing through them [36]. The temperature records are included in Figure S1. In addition, CO<sub>2</sub> can be injected inside the TGGs, modifying the air CO<sub>2</sub> concentration.

An equal number of plants of each clone was placed in the first and the third module of four TGGs, leaving the central module free of plants. Half of the plants (those located in the modules 1) grew at ambient temperature (T), corresponding to the ambient temperature outdoors, meanwhile the other half of the plants (those located in the modules 3) grew under ambient plus 4 °C warmer temperature (T+4). Besides, the air CO<sub>2</sub> concentration was modified in two out of the four TGGs, resulting in half of the plants growing at current atmospheric CO<sub>2</sub> concentration (ca. 400 ppm; ACO<sub>2</sub>) and the other half

under increased air CO<sub>2</sub> concentration (700 ppm; ECO<sub>2</sub>). Therefore, plants grew under four different CO<sub>2</sub> and temperature conditions: i) ambient temperature and ambient CO<sub>2</sub> (T/ACO<sub>2</sub>), ii) ambient temperature and elevated CO<sub>2</sub> (T/ECO<sub>2</sub>), iii) elevated temperature and ambient CO<sub>2</sub> (T+4/ACO<sub>2</sub>), iv) elevated temperature and elevated CO<sub>2</sub> (T+4/ECO<sub>2</sub>). The treatments were applied from fruit set (2016, May) to berry maturity (2016, September), which was considered to occur when the total soluble solid (TSS) content of the berries was ca. 22 °Brix, each plant being measured individually.

### *2.3. Phenological development and berry size*

The length of the phenological development of grapes was determined for each plant by individually recording the dates corresponding to fruit set, mid-veraison (half of the berries in the bunch had started to change colour) and maturity. The time intervals between fruit set and mid-veraison and between mid-veraison and maturity were calculated. Fruit set and mid-veraison were determined visually and maturity was considered to be reached when the levels of TSS of two berries of each bunch was, at least, 22 °Brix. This analysis was done periodically for each bunch every 2-3 days during the last weeks of development of the berries.

In order to carry out the different analyses of berries, 5 sampling points were determined: i) onset of veraison, when berries had already softened and just began to colour; ii) mid-veraison (determined as described previously in this section), at this stage berries with the same proportion of coloured skin surface were sampled (ca. 50 %); iii) 1 week after mid-veraison; iv) 2 weeks after mid-veraison; and v) maturity, determined as described previously in this section. At the onset of veraison, 3-4 berries were sampled from each bunch, 3 berries at mid-veraison, 1 week after mid-veraison and 2 weeks after mid-veraison, and 10 berries at maturity. The diameter was measured with a caliper and berries were frozen in liquid nitrogen and stored at -80 °C until analyses.

### *2.4. Berry analyses preparation*

Analyses were carried out by doing pools of berries (two or three samples of 3 berries -10 berries at maturity- from different plants per pool). Berries were manually peeled and separated into skin, pulp and seeds. Fresh skins, pulps and seeds were weighed and the data obtained were used to determine the relative skin mass (relation between skin fresh weight and total berry fresh weight, expressed as a percentage). The pulp was crushed to obtain the must, which was centrifuged and used for sugar, malic acid and amino acid analyses. The skin was freeze-dried in an Alph1-4, lyophilizer (CHRIST, Osterode, Germany), weighed to calculate the water content and grinded into powder using an MM200 ball grinder (Retsch, Haan, Germany) for carrying out the anthocyanin analysis.

## 2.5. *Sugar, malic acid and amino acid profile analyses*

Sugar (glucose and fructose) concentration was determined enzymatically using an automated absorbance microplate reader (Elx800UV, Biotek Instruments Inc., Winooski, VT, USA) using the Glucose/Fructose kit from BioSenTec (Toulouse, France) according to the manufacturer. Malic acid was determined with a Bran and Luebbe TRAACS 800 autoanalyser (Bran & Luebbe, Plaisir, France) as described in detail in Bobeica et al. [37].

For the amino acid analysis, samples were derivatized with the AccQ•Fluor Reagent (6-aminoquinolyl-N-hydroxy-succinimidyl-carbamate, Waters, Milford, MA, USA) [38] as described by Hilbert et al. [39]. The products of this reaction were analysed with an UltiMate 3000 UHPLC system (Thermo Electron SAS, Waltham, MA, USA) equipped with an FLD-3000 Fluorescence Detector (Thermo Electron SAS, Waltham, MA, USA). Amino acids were separated using as eluents sodium acetate buffer (eluent A, 140 mM at pH 5.7), acetonitrile (eluent B) and water (eluent C) at 37 °C and 0.5 ml min<sup>-1</sup> through an AccQ•Tag Ultra column, 2.1 × 100 mm, 1.7 µm (Waters, Milford, MA, USA) according to Habran et al. [40]. The concentration and identification of each compound was determined through a chromatographic analysis as described in Pereira et al. [41], using an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Samples were loaded alternated with control samples as in Torres et al. [42].

## 2.6. *Total skin anthocyanin analysis*

Anthocyanin analyses were carried out according to Torres et al. and described in detail by Acevedo De la Cruz et al. and Hilbert et al. [42–44] with minor changes. In brief, ground dried skins were treated with methanol containing 0.1 % HCl (v/v), in order to extract the pigments, and filtered using a polypropylene syringe filter of 0.45 µm (Pall Gelman Corp., Ann Arbor, USA). The obtained extracts were separated using a Synchronis C18, 2.1 × 100 mm, 1.7 µm Column (Thermo Fisher Scientific, Waltham, MA, USA) and analysed with an UltiMate 3000 UHPLC system (Thermo Electron SAS, Waltham, MA, USA) equipped with DAD-3000 diode array detector (Thermo Electron SAS, Waltham, MA, USA). The wavelength used for recording the chromatographic profiles was 520 nm and the standard was malvidin-3-*O*-glucoside (Extrasynthese, Genay, France). The peak areas of chromatograms were calculated using the Chromeleon software (version 7.1) (Thermo Electron SAS, Waltham, MA, USA). Concentration of total anthocyanins was calculated as the sum of the concentration of individual anthocyanins.

## 2.7. Statistical analysis

The data were statistically analysed using R (3.5.1), carrying out a three-way ANOVA (clone, temperature and CO<sub>2</sub> concentration) and a Fisher's least significant difference (LSD) was carried out as a post-hoc test when statistically significant differences were found ( $P < 0.05$ ).

## 3. RESULTS

### 3.1. Phenological development

In general, elevated CO<sub>2</sub> had a higher impact on grape phenology in the period comprised between fruit set to mid-veraison, whereas ripening (mid-veraison to maturity) was more affected by elevated temperature (Figure 1A). The number of days elapsed between fruit set and mid-veraison was slightly, but significantly, shortened by ECO<sub>2</sub>, and especially when it was combined with T+4 (Figure 1A). However, this hastening effect of CO<sub>2</sub> was nullified between mid-veraison and maturity, whereas the increase of temperature reduced significantly this period in up to 5 days. Also, the duration from mid-veraison to maturity was affected significantly by the clone identity, mainly because 1084 needed more time to reach maturity, regardless of the growing condition (Figure 1B). Although significant interactions among factors were not detected, it is worth pointing out the significant difference in the elapsed days between mid-veraison and maturity of T3 plants grown under T+4/ACO<sub>2</sub> compared to T/ACO<sub>2</sub>, as maturity was reached 12 days earlier when T+4 was applied.

### 3.2. Berry characteristics

Berry diameter differed significantly among clones at the onset of veraison, mid-veraison and maturity (Table 1). It was also modified by temperature, decreasing under T+4 2 weeks after mid-veraison and at maturity. By contrast, the CO<sub>2</sub> level did not markedly affect this berry characteristic. The only noteworthy interaction among the parameters was at mid-veraison, when the effect of ECO<sub>2</sub> was different depending on the temperature regime and the clone (triple interaction).

In general, berries from all the studied clones presented similar relative skin mass throughout the experiment except at maturity, when 1084 showed significantly lower values than CL306, T3 and VN31 (Table 2). Relative skin masses were higher 1 and 2 weeks after mid-veraison in grapes developed under T+4 compared with those grown at T. Grapes under ECO<sub>2</sub> had a lower relative skin mass than ACO<sub>2</sub> at the onset of veraison, but higher 1 and 2 weeks after mid-veraison. At maturity, the T3 clone was the most affected one by the increase in temperature of T+4, especially when combined with ACO<sub>2</sub>.



### 3.3. Malic acid

The evolution of malic acid concentration throughout the ripening process was not affected by the clone identity, decreasing in a similar manner in all of them until maturity (Figure 2A). However, at maturity, the 1084 accession had the lowest malic acid levels and CL306 the highest (Figure 2B). Considering all the clones as a whole, T+4 decreased significantly malic acid from mid-veraison onwards with respect to T, while ECO<sub>2</sub> raised acid malic significantly at the onset of veraison and reduced it at maturity compared to ACO<sub>2</sub> (Figure 2A). For all the clones studied, grapes developed under current situation (T/ACO<sub>2</sub>) presented significantly higher levels of malic acid at maturity than those developed under climate change conditions (T+4/ECO<sub>2</sub>) (Figure 2B). In the case of 1084, this difference was observed with plants grown at T+4, regardless of the CO<sub>2</sub> regime. Globally, there were no significant interactions among factors.

### 3.4. Sugars

In general, the level of sugars (glucose and fructose) depended significantly on the clone from mid-veraison onwards, being strongly affected by this factor 2 weeks after mid-veraison and at maturity (Figure 3A). Notably, 2 weeks after mid-veraison, the most contrasted clones were 1084 and CL306, with the lowest and highest sugar levels, respectively, (Figure 3B). The T+4 treatment increased significantly the sugar concentration 2 weeks after mid-veraison compared with T, whereas the atmospheric CO<sub>2</sub> level did not have any effect on this parameter. Nonetheless, there were no significant interactions between the factors considered.

### 3.5. Amino Acids

The concentration of total amino acids was similar among clones throughout berry development, except 2 weeks after mid-veraison, when 1084 had lower levels in comparison to the other clones (Figure 4A; Tables S1A to S1E). At this stage, and considering all the clones, the T+4 treatment reduced total amino acid concentration compared to the T treatment (from  $15.9 \pm 1.33 \mu\text{mol ml}^{-1}$  to  $12.3 \pm 1.18 \mu\text{mol ml}^{-1}$ , respectively, Table S1D). Also ECO<sub>2</sub> diminished the amino acid levels with respect to ACO<sub>2</sub> (from  $17.1 \pm 1.48 \mu\text{mol ml}^{-1}$  to  $11.2 \pm 0.82 \mu\text{mol ml}^{-1}$ , respectively) 2 weeks after mid-veraison (Table S1D). At maturity, there were no significant effects of temperature and CO<sub>2</sub> (neither individually nor combined). However, T+4 tended to reduce the concentration of total amino acids (especially under ACO<sub>2</sub>) in all the clones and ECO<sub>2</sub> tended to reduce the amino acid levels of CL306, T3 and VN31 at ambient temperature (Figure 4B, Table S1E).

The relative abundance of the different amino acids varied among clones. Specifically, 2 weeks after mid-veraison and at maturity the pyruvate and aspartate derivatives were more abundant in 1084 at the expense of  $\alpha$ -ketoglutarate derivatives (except GABA and arginine, which increased) (Figure 4D, Tables S1D and S1E). Considering all the clones as a whole,  $ECO_2$  significantly reduced the proportion of  $\alpha$ -ketoglutarate derivatives (although arginine and GABA tended to increase in the later stages of the ripening period in  $ECO_2$ ), increasing that of those originated from aspartate and pyruvate (2 weeks after mid-veraison and at maturity, respectively). Even though there were no significant interactions, there were two exceptions to this effect at maturity, as  $ECO_2$  increased  $\alpha$ -ketoglutarate derivatives in the grapes of the clones RJ43 and 1084 exposed to T and T+4, respectively. In addition, the rise in the relative abundance of pyruvate derivatives observed in the  $ECO_2$  treatment at maturity was globally stronger at T+4 (Figure 4C, Table S1E).

### 3.6. Total skin anthocyanins

Anthocyanin levels did not differ significantly in the early ripening period among clones, but 2 weeks after mid-veraison and at maturity the concentration of total anthocyanins was lower in 1084, whereas RJ43 showed the highest values (Figures 5A and 5B). T+4 had a significant enhancing effect on anthocyanins at the onset of veraison and 2 weeks after mid-veraison, regardless of the clone and  $CO_2$  level.  $ECO_2$  increased anthocyanin concentration at the onset of veraison and mid-veraison but reduced it 2 weeks after mid-veraison (Figure 5A). At maturity, although there were no significant interactions between factors, the T+4/ $ECO_2$  treatment seemed to have different effects depending on the clone: whereas in RJ43 the grapes exposed to T+4/ $ECO_2$  (climate change conditions) had significantly lower anthocyanin levels than those exposed to T/ $ACO_2$  (current conditions), in CL306 the concentration of skin anthocyanins increased with climate change conditions (T+4/ $ECO_2$ ) (Figure 5B).

### 3.7. Total anthocyanins to TSS ratio

Clones showed different anthocyanin to TSS ratios at maturity regardless of the temperature and  $CO_2$  regime, RJ43 and T3 having the highest values (Figure 6A). Considering the clones altogether, a significant interaction between temperature and  $CO_2$  was observed (Figure 6B). Thus, the significant decrease of the ratio between anthocyanins and TSS under  $ACO_2$  induced by T+4, with respect to T, disappeared under  $ECO_2$ . When the temperature and  $CO_2$  effects were analysed for each clone independently, the growing conditions showed slightly different effects on the anthocyanin to TSS ratio (Figure 6C). In RJ43 and VN31, the impact of T+4 on the ratio was more evident under  $ACO_2$  conditions.  $ECO_2$  strongly increased the ratio in CL306 plants at T+4, meanwhile it did not have any effect at T. Finally, neither temperature nor  $CO_2$  had a marked impact on the relationship between anthocyanins and TSS in T3 and 1084.

## 4. DISCUSSION

### 4.1. Performance of clones

Clones showed different characteristics in all the studied parameters. The accession that differed the most from the others studied was 1084. It had an extremely long berry ripening period associated to a lower sugar accumulation rate, not even reaching the optimum sugar levels for wine production. The 1084 accession also had berries with low relative skin mass and presented the lowest values of malic acid at maturity. Despite T3, VN31 and RJ43 having similar berry diameter to 1084 (indicating similar size), their malic acid values were higher than in 1084. This result suggests that the low concentration of malic acid in 1084 was not associated with a dilution effect due to high berry size. After veraison, malate is released from the vacuole and becomes available for catabolism through involvement in gluconeogenesis, respiration through the tricarboxylic acid (TCA) cycle, amino acid inter-conversions and the production of secondary compounds such as anthocyanins and flavonols [45–50]. Probably, the longer ripening period of the 1084 accession, already seen in previous experiments [15], contributed to higher malate breakdown, thus reaching a lower malic acid concentration at maturity. In addition, the higher proportion of some amino acids observed at maturity in 1084 (i.e. GABA, arginine, as well as pyruvate and aspartate derivatives) in comparison with other clones, not accompanied by higher sugar level in this accession, may indicate that, in this clone, the use of malate in supplementing TCA cycle may be favoured over its use in gluconeogenesis.

The 1084 accession also presented lower concentration of skin anthocyanins, compared with the other clones. The results are in agreement with our previous work [15] and may be related to the slow sugar accumulation rate observed in 1084. Dai et al., using an experimental system allowing the long-term *in vitro* culture of grape berries, reported an induction in total anthocyanins by rising sugar concentrations in the culture medium, as well as a negative correlation between phenylalanine and total anthocyanin levels [51]. In the present study, phenylalanine was similar and even higher in 1084 (2 weeks after mid-veraison) compared with the other clones. Therefore, the lower anthocyanin accumulation may be a consequence of a limitation in the biosynthetic enzymatic activity rather than to a limitation of its precursor, as suggested by Dai et al. [51]. In opposition to the results obtained by Roby et al. [52], the anthocyanin content in berries was not systematically positively correlated to the relative skin mass, except for 1084, although in our case anthocyanins were measured in dry skin and Roby et al. did it in the whole berry. In addition, both 1084 and T3 had some of the largest diameters. However, the relative skin mass of this two clones differed significantly, T3 having a high value and 1084 the lowest. These results indicate a lack of effect of berry size on the relative skin mass already observed by Barbagallo et al. [53]. These berry parameters are important as they are considered to determine the solutes extraction during the maceration process [52–55].

#### *4.2. Global response of Tempranillo clones to elevated temperature*

The increment of temperature shortened the elapsed time between mid-veraison and maturity and reduced the size of berries at the end of the ripening process. These results agree with previous studies, in which berries stopped increasing their volume after being heat-treated at mid-veraison and mid-ripening [9,11,52,56] and high temperature accelerated the ripening process [5,7,9,15]. Given that maturity was determined by the level of TSS (ca. 22 °Brix), the reduction of the time to reach this stage in the T+4 treatment indicates a faster and more efficient accumulation of sugars. In the present study, the lower berry size in the T+4 may have contributed to the higher concentration of sugars observed. However, the effect of high temperatures on sugar accumulation varies among studies and conditions. Whereas elevated temperature has been reported to enhance sugar accumulation [57,58], in other cases the berry sugar content at harvest was not affected [17], or sugar accumulation was stopped [52,59], or even decreased [10,12,60]. These apparently contradictory results may be due to differences in the experimental procedures, which included more extreme temperatures than in the present one, thus reducing photosynthesis and limiting the supply of sugar to the berries [60].

It is well established that warm temperatures promote the decrease of organic acid levels in grape berries after mid-veraison, by accelerating malate degradation [11,12,61,62]. Malic acid respiration is favoured by heat, and genes involved in its transmembrane transport display a marked regulation by temperature [63]. In addition, the enhancement of the anaplerotic capacity of the TCA cycle for amino acid biosynthesis by elevated temperatures has also been suggested [17,61]. In our work, although malic acid degradation was promoted by T+4 from mid-veraison onwards, the concentration of total amino acids tended to decrease in this treatment compared with T, and changes in the proportion  $\alpha$ -ketoglutarate, pyruvate or aspartate amino acid derivatives were not so obvious. These results may indicate that under the temperature conditions assayed (4 °C of difference between T and T+4 vs increases of 8 °C [17] and 10 °C [61] in the mentioned studies) the anaplerotic capacity of the TCA cycle for amino acid biosynthesis may not markedly be increased. Other pathways such as gluconeogenesis may have played a more important role in malate degradation, thus contributing to the differences in sugar accumulation observed between temperature regimes. Despite the limited changes in the amino acid profile, an increased proportion of proline and arginine was observed under high temperature, as previously reported by other authors [17,42]. Proline has a protective role in plants against abiotic stresses, including elevated temperature, whereas arginine is an important source of nitrogen during winemaking [64], despite being a precursor of putrescine, a compound with negative effects on human's health [65].

Considering the clones altogether, high temperature significantly increased the concentration of anthocyanins 2 weeks after mid-veraison, but it did not affect the final anthocyanin levels at maturity. These results do not agree with previous studies that demonstrate that high temperature during ripening had a negative impact on anthocyanin biosynthesis in berries by acting on the correspondent enzymes and transcription factors [16,17,22,63,66]. In those experiments the expression of genes related to flavonoid biosynthesis in grape skins were found to be repressed by high temperatures, specially genes coding for the key enzyme of the phenylpropanoid pathway, the phenylalanine-ammonia-lyase (PAL) or *MYB* transcription factors, which control anthocyanin biosynthesis. However, the repression of *VvMYBA1* by high temperature described by Yamane et al. [66] was not confirmed by other authors [67]. Besides, in addition to a lower anthocyanin biosynthesis, some authors have reported increases in anthocyanin degradation due to high temperature [22]. Accordingly, the increase in anthocyanin concentration induced by T+4 observed 2 weeks after mid-veraison in our study was not detected at maturity ( $P_{(T)} > 0.05$ ), which might be caused by an earlier degradation of anthocyanins under T+4.

Anthocyanin levels have also been reported to be differentially affected by temperature in different cultivars [68]. Total anthocyanins decreased with high temperature in Merlot [19,20], Malbec [69], Pione [18], Cabernet Sauvignon [17,22,70] and Muscat Hamburg [12], while final concentration of anthocyanins increased in Merlot by reducing the day temperature oscillations [71]. In the case of Tempranillo, Kizildeniz et al., in a three year-experiment under similar conditions than this study, did not observe significant effects of high temperature on final anthocyanin levels [72]. Also, differences in the response of anthocyanins to temperature were detected among different clones of Tempranillo, not all the clones being equally affected [15,42]. Furthermore, some authors also reported that the stage of berry growth at which the heat treatment was applied could modulate the plant response in terms of anthocyanin content [17,73,74].

#### *4.3. Global response of Tempranillo clones to elevated CO<sub>2</sub>*

High atmospheric CO<sub>2</sub> concentration slightly hastened grape phenology, but only from fruit set to mid-veraison, as reported by Martínez-Lüscher et al. for red and white Tempranillo [8]. Regarding fruit composition, the increase in malic acid concentration of grapes exposed to ECO<sub>2</sub> at the onset of veraison may indicate an enhancement of the organic acids biosynthesis at early berry development stages. These differences disappeared in later stages, when the degradation of malic acid took place, the grapes ripened under ECO<sub>2</sub> conditions reaching even lower malic acid levels at maturity than those grown at ACO<sub>2</sub>, possibly because of an accelerated breakdown. Similarly, Bindi et al., in a field experiment using a free air CO<sub>2</sub> enrichment (FACE) facility, observed that organic acid components

were positively affected by increases in CO<sub>2</sub> concentration at the middle of the ripening season, an effect that almost completely vanished at maturity [75]. These results could point towards a regulatory effect of ECO<sub>2</sub> on malic acid degradation, favouring its catabolic flux through the TCA cycle over its use in gluconeogenesis, as suggests the increase of the relative abundance of GABA as well as pyruvate and aspartate derivatives [61] and the absence of effects on sugar levels.

Taking into consideration the clones altogether, plants grown at ECO<sub>2</sub> presented an increased concentration of total grape skin anthocyanins at the beginning of the ripening period, which may indicate a slight hastening in the synthesis of these compounds in this treatment. In contrast, 2 weeks after mid-veraison, ECO<sub>2</sub> plants had lower anthocyanin concentration than ACO<sub>2</sub>, these differences disappearing at maturity. These results do not agree with the observations of other authors, who reported no effect [23] or a rising effect of CO<sub>2</sub> on anthocyanins at maturity [24]. In the same way, studies on the effects of elevated CO<sub>2</sub> on the anthocyanin biosynthetic pathway in different plant species report contradictory results. For example, in strawberry and table grapes, elevated CO<sub>2</sub> levels decreased anthocyanin content by decreasing the expression of genes involved in the phenylpropanoid metabolism, especially the one coding PAL enzyme [76,77]. In contrast, increased anthocyanin content and stimulated enzyme activity have been observed in ginger and *Labisia pumila* under elevated CO<sub>2</sub> [78,79].

#### 4.4. Response of clones to combined elevated temperature and CO<sub>2</sub>

The analysis of the combined effects of high temperature and elevated CO<sub>2</sub> on the parameters analysed indicates low interactive effects of these environmental factors on sugar accumulation and, thus, in the length of the ripening period, as ECO<sub>2</sub> did not modify the hastening effect induced by T+4. Conversely, increase in temperature and CO<sub>2</sub> showed additive effects enhancing malic acid degradation, a phenomenon especially marked in the 1084 accession (probably due to its longer exposure to these conditions) but less evident in RJ43 and CL306. Such reduction of malic acid content and its impact on must acidity should certainly affect wine production, not only for its contribution to sourness and organoleptic proprieties, but also because of its influence on wine microbiological stability. In addition, it might make the winemaking process more expensive due to the need of acidifying the must for achieving a proper fermentation [9].

Interestingly, a significant interaction between temperature and CO<sub>2</sub> was observed for the relationship between anthocyanin and sugar levels: the temperature-induced decrease of this ratio under ACO<sub>2</sub> was not observed under ECO<sub>2</sub>. This result suggests that in a future climate change scenario, elevated CO<sub>2</sub> may, at least partially, mitigate the negative impact of high temperature on the imbalance

between sugars and anthocyanins in ripe berries. In addition, there was a wide range of responses of the different clones to climate change conditions. While in RJ43 and VN31 climate change conditions (T+4/ECO<sub>2</sub>) markedly reduced the anthocyanin to sugar ratio with respect to current conditions (T/ACO<sub>2</sub>) (differences statistically significant in RJ43), the balance between these two compounds was less affected in CL306, T3 and 1084.

## 5. CONCLUSIONS

The projected increases in average air temperature and in atmospheric CO<sub>2</sub> concentration advanced grape maturity, reducing the elapsed time between fruit set and mid-veraison and between mid-veraison and maturity, respectively. High temperature hastened berry ripening, sugar accumulation, as well as malic acid breakdown, especially when combined with elevated CO<sub>2</sub>, which seemed to increase the anaplerotic flux through the TCA cycle. Even though the increase of temperature and high CO<sub>2</sub> concentration (both individually and combined) did not affect anthocyanin concentration, the clones studied showed different values for this parameter. The reduction of the ratio between anthocyanins and TSS under T+4 conditions was partially mitigated by ECO<sub>2</sub>. Additionally, the study reveals the existence of a differential response of Tempranillo clones to the projected future temperature and CO<sub>2</sub> levels in terms of grape composition.

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## TABLES

Table 1. Grape berry diameter of the five Tempranillo clones (RJ43, CL306, T3, VN31 and 1084) grown under four temperature/CO<sub>2</sub> regimes: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). Results (values are means ± SE) are shown according to the clone identity (n = 16), temperature regime (n = 40), CO<sub>2</sub> concentration (n = 40), and the combined factors (n = 4). Means with letters in common within the same stage and factor (clone, temperature, CO<sub>2</sub> or their interactions) are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone P(CL); temperature, P(T); CO<sub>2</sub>, P(CO<sub>2</sub>); and their interactions, P(CL x T), P(CL x CO<sub>2</sub>), P(T x CO<sub>2</sub>) and P(CL x T x CO<sub>2</sub>). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant.

|                              |     | Berry diameter (mm) |                  |                           |                            |                  |                    |
|------------------------------|-----|---------------------|------------------|---------------------------|----------------------------|------------------|--------------------|
|                              |     | Onset of veraison   | Mid-veraison     | 1 week after mid-veraison | 2 weeks after mid-veraison | Maturity         |                    |
| RJ43                         |     | 10.51 ± 0.18 ab     | 10.78 ± 0.23 bc  | 11.36 ± 0.21 b            | 13.19 ± 1.09 a             | 12.71 ± 0.17 ab  |                    |
| CL306                        |     | 10.11 ± 0.16 b      | 10.57 ± 0.13 c   | 11.36 ± 0.19 b            | 12.07 ± 0.19 a             | 12.33 ± 0.20 b   |                    |
| T3                           |     | 10.86 ± 0.20 a      | 11.11 ± 0.19 b   | 11.63 ± 0.23 ab           | 12.40 ± 0.22 a             | 13.11 ± 0.17 a   |                    |
| VN31                         |     | 10.77 ± 0.15 a      | 11.19 ± 0.13 ab  | 13.00 ± 1.25 a            | 13.81 ± 1.07 a             | 13.07 ± 0.16 a   |                    |
| 1084                         |     | 10.86 ± 0.23 a      | 11.60 ± 0.19 a   | 12.10 ± 0.20 ab           | 12.16 ± 0.30 a             | 12.88 ± 0.25 a   |                    |
| T                            |     | 10.75 ± 0.12 a      | 11.18 ± 0.11 a   | 11.85 ± 0.13 a            | 13.39 ± 0.60 a             | 13.14 ± 0.13 a   |                    |
| T+4                          |     | 10.49 ± 0.13 a      | 10.92 ± 0.13 a   | 11.93 ± 0.52 a            | 12.07 ± 0.15 b             | 12.50 ± 0.10 b   |                    |
| ACO <sub>2</sub>             |     | 10.60 ± 0.10 a      | 11.07 ± 0.12 a   | 12.16 ± 0.51 a            | 12.42 ± 0.11 a             | 12.68 ± 0.13 a   |                    |
| ECO <sub>2</sub>             |     | 10.64 ± 0.14 a      | 11.03 ± 0.13 a   | 11.62 ± 0.15 a            | 13.03 ± 0.63 a             | 12.96 ± 0.13 a   |                    |
| RJ43                         | T   | ACO <sub>2</sub>    | 10.93 ± 0.49 abc | 11.18 ± 0.47 abcd         | 11.55 ± 0.24 b             | 12.70 ± 0.15 bc  | 13.01 ± 0.52 bcde  |
|                              |     | ECO <sub>2</sub>    | 10.39 ± 0.36 bcd | 10.44 ± 0.43 def          | 11.45 ± 0.53 b             | 16.43 ± 4.35 ab  | 12.82 ± 0.13 cdef  |
|                              | T+4 | ACO <sub>2</sub>    | 10.50 ± 0.24 bc  | 10.18 ± 0.21 ef           | 10.73 ± 0.44 b             | 11.63 ± 0.18 c   | 12.56 ± 0.42 defg  |
|                              |     | ECO <sub>2</sub>    | 10.21 ± 0.33 cd  | 11.32 ± 0.53 abcd         | 11.70 ± 0.37 b             | 12.01 ± 0.11 c   | 12.46 ± 0.24 defg  |
| CL306                        | T   | ACO <sub>2</sub>    | 10.03 ± 0.18 cd  | 10.52 ± 0.20 def          | 11.35 ± 0.17 b             | 12.11 ± 0.25 c   | 11.84 ± 0.56 g     |
|                              |     | ECO <sub>2</sub>    | 10.32 ± 0.34 cd  | 11.04 ± 0.07 abcde        | 11.93 ± 0.22 b             | 12.31 ± 0.37 c   | 12.95 ± 0.27 bcdef |
|                              | T+4 | ACO <sub>2</sub>    | 10.68 ± 0.18 abc | 10.71 ± 0.26 cdef         | 11.44 ± 0.54 b             | 12.69 ± 0.16 bc  | 12.52 ± 0.34 defg  |
|                              |     | ECO <sub>2</sub>    | 9.42 ± 0.20 d    | 10.02 ± 0.22 f            | 10.71 ± 0.31 b             | 11.18 ± 0.26 c   | 12.03 ± 0.19 fg    |
| T3                           | T   | ACO <sub>2</sub>    | 10.72 ± 0.33 abc | 11.16 ± 0.04 abcd         | 12.09 ± 0.09 b             | 12.45 ± 0.19 bc  | 13.22 ± 0.14 abcd  |
|                              |     | ECO <sub>2</sub>    | 11.59 ± 0.24 a   | 11.87 ± 0.09 a            | 12.46 ± 0.28 b             | 13.30 ± 0.23 abc | 13.97 ± 0.23 a     |
|                              | T+4 | ACO <sub>2</sub>    | 10.29 ± 0.34 cd  | 10.74 ± 0.56 cdef         | 11.17 ± 0.47 b             | 11.95 ± 0.58 c   | 12.43 ± 0.14 defg  |
|                              |     | ECO <sub>2</sub>    | 10.83 ± 0.45 abc | 10.68 ± 0.35 cdef         | 10.82 ± 0.44 b             | 11.91 ± 0.34 c   | 12.81 ± 0.20 cdef  |
| VN31                         | T   | ACO <sub>2</sub>    | 10.94 ± 0.31 abc | 11.25 ± 0.19 abcd         | 11.97 ± 0.57 b             | 12.67 ± 0.08 bc  | 13.57 ± 0.13 abc   |
|                              |     | ECO <sub>2</sub>    | 10.90 ± 0.35 abc | 11.57 ± 0.23 abc          | 11.77 ± 0.75 b             | 16.79 ± 4.33 a   | 13.21 ± 0.25 abcd  |
|                              | T+4 | ACO <sub>2</sub>    | 10.68 ± 0.29 abc | 11.16 ± 0.30 abcd         | 16.81 ± 4.86 b             | 13.04 ± 0.42 abc | 12.54 ± 0.25 defg  |
|                              |     | ECO <sub>2</sub>    | 10.55 ± 0.32 bc  | 10.79 ± 0.21 cdef         | 11.47 ± 0.39 b             | 12.76 ± 0.29 bc  | 12.97 ± 0.44 bcdef |
| 1084                         | T   | ACO <sub>2</sub>    | 10.87 ± 0.24 abc | 11.93 ± 0.33 a            | 12.32 ± 0.54 b             | 12.63 ± 0.60 bc  | 13.02 ± 0.38 bcde  |
|                              |     | ECO <sub>2</sub>    | 10.80 ± 0.53 abc | 10.87 ± 0.41 bcdef        | 11.58 ± 0.50 b             | 12.47 ± 0.22 bc  | 13.84 ± 0.45 ab    |
|                              | T+4 | ACO <sub>2</sub>    | 10.38 ± 0.52 bcd | 11.86 ± 0.18 a            | 12.15 ± 0.35 a             | 12.36 ± 0.15 c   | 12.12 ± 0.33 efg   |
|                              |     | ECO <sub>2</sub>    | 11.38 ± 0.54 ab  | 11.76 ± 0.41 ab           | 12.35 ± 0.18 b             | 11.19 ± 0.95 c   | 12.57 ± 0.52 defg  |
| P(CL)                        |     | *                   | ***              | ns                        | ns                         | *                |                    |
| P(T)                         |     | ns                  | ns               | ns                        | *                          | ***              |                    |
| P(CO <sub>2</sub> )          |     | ns                  | ns               | ns                        | ns                         | ns               |                    |
| P(CL x T)                    |     | ns                  | ns               | ns                        | ns                         | ns               |                    |
| P(CL x CO <sub>2</sub> )     |     | ns                  | ns               | ns                        | ns                         | ns               |                    |
| P(T x CO <sub>2</sub> )      |     | ns                  | ns               | ns                        | ns                         | ns               |                    |
| P(CL x T x CO <sub>2</sub> ) |     | ns                  | **               | ns                        | ns                         | ns               |                    |

Table 2. Relative skin mass (%) in grape berries of the five Tempranillo clones (RJ43, CL306, T3, VN31 and 1084) grown under four temperature/CO<sub>2</sub> regimes: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). Results (values are means ± SE) are shown according to the clone (n = 16), temperature (n = 40), CO<sub>2</sub> concentration (n = 40), and the combined factors (n = 4). Means with letters in common within the same stage and factor (clone, temperature, CO<sub>2</sub> or their interactions) are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone, P(CL); temperature, P(T); and CO<sub>2</sub>, P(CO<sub>2</sub>). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. All probability values for the interactions of factors (P(CL x T), P(CL x CO<sub>2</sub>), P(T x CO<sub>2</sub>) and P(CL x T x CO<sub>2</sub>)) were statistically not significant (P > 0.05).

|                     |     | Relative skin mass (%) |                  |                           |                            |                  |                     |
|---------------------|-----|------------------------|------------------|---------------------------|----------------------------|------------------|---------------------|
|                     |     | Onset of veraison      | Mid-veraison     | 1 week after mid-veraison | 2 weeks after mid-veraison | Maturity         |                     |
| RJ43                |     | 16.76 ± 1.30 a         | 15.60 ± 0.89 a   | 14.02 ± 0.57 a            | 15.02 ± 0.76 a             | 16.50 ± 0.68 ab  |                     |
| CL306               |     | 15.24 ± 0.66 a         | 14.27 ± 0.40 ab  | 14.73 ± 0.88 a            | 17.02 ± 1.57 a             | 17.51 ± 0.76 a   |                     |
| T3                  |     | 14.68 ± 0.64 a         | 13.76 ± 0.70 ab  | 14.20 ± 0.63 a            | 14.83 ± 1.03 a             | 16.88 ± 0.84 a   |                     |
| VN31                |     | 15.43 ± 0.53 a         | 13.90 ± 0.75 ab  | 14.79 ± 0.55 a            | 15.62 ± 0.90 a             | 17.72 ± 0.87 a   |                     |
| 1084                |     | 14.57 ± 0.58 a         | 13.24 ± 0.44 b   | 14.35 ± 0.60 a            | 14.81 ± 1.61 a             | 14.41 ± 0.78 b   |                     |
| T                   |     | 15.48 ± 0.40 a         | 14.36 ± 0.37 a   | 13.85 ± 0.31 b            | 13.72 ± 0.32 b             | 16.03 ± 0.52 a   |                     |
| T+4                 |     | 15.19 ± 0.59 a         | 13.94 ± 0.48 a   | 14.98 ± 0.47 a            | 17.31 ± 1.01 a             | 17.22 ± 0.51 a   |                     |
| ACO <sub>2</sub>    |     | 16.56 ± 0.57 a         | 13.74 ± 0.46 a   | 13.67 ± 0.42 b            | 14.35 ± 0.77 b             | 16.81 ± 0.55 a   |                     |
| ECO <sub>2</sub>    |     | 14.11 ± 0.33 b         | 14.57 ± 0.39 a   | 15.17 ± 0.37 a            | 16.56 ± 0.75 a             | 16.48 ± 0.5 a    |                     |
| RJ43                | T   | ACO <sub>2</sub>       | 17.79 ± 0.71 ab  | 15.33 ± 0.91 abc          | 12.24 ± 0.35 c             | 12.33 ± 0.57 c   | 15.80 ± 1.19 bcdef  |
|                     |     | ECO <sub>2</sub>       | 13.75 ± 1.16 bcd | 15.51 ± 2.80 abc          | 14.52 ± 0.79 abc           | 14.63 ± 0.86 bc  | 16.04 ± 1.72 abcdef |
|                     | T+4 | ACO <sub>2</sub>       | 20.68 ± 4.63 a   | 16.15 ± 2.06 a            | 12.66 ± 0.18 bc            | 15.87 ± 1.39 bc  | 17.12 ± 1.93 abcdef |
|                     |     | ECO <sub>2</sub>       | 14.81 ± 0.89 bcd | 15.41 ± 1.64 abc          | 16.66 ± 1.27 a             | 17.26 ± 2.08 abc | 17.02 ± 0.80 abcdef |
| CL306               | T   | ACO <sub>2</sub>       | 16.64 ± 1.35 abc | 12.91 ± 0.41 abc          | 13.90 ± 0.29 abc           | 14.07 ± 1.08 bc  | 18.16 ± 2.76 abcd   |
|                     |     | ECO <sub>2</sub>       | 14.48 ± 0.44 bcd | 15.75 ± 0.86 ab           | 12.55 ± 0.74 bc            | 15.70 ± 0.98 bc  | 17.84 ± 1.15 abcd   |
|                     | T+4 | ACO <sub>2</sub>       | 15.04 ± 1.59 bcd | 13.94 ± 0.74 abc          | 16.64 ± 3.13 a             | 15.30 ± 1.59 bc  | 18.60 ± 1.64 abc    |
|                     |     | ECO <sub>2</sub>       | 14.79 ± 1.80 bcd | 14.46 ± 0.63 abc          | 15.84 ± 1.35 ab            | 22.58 ± 5.00 a   | 15.60 ± 0.71 bcdef  |
| T3                  | T   | ACO <sub>2</sub>       | 17.56 ± 1.15 abc | 15.18 ± 1.05 abc          | 14.48 ± 1.75 abc           | 13.10 ± 0.82 bc  | 14.21 ± 1.50 def    |
|                     |     | ECO <sub>2</sub>       | 12.05 ± 0.81 d   | 13.18 ± 0.74 abc          | 13.26 ± 0.65 abc           | 13.29 ± 1.53 bc  | 15.10 ± 0.29 cdef   |
|                     | T+4 | ACO <sub>2</sub>       | 15.17 ± 0.80 bcd | 12.13 ± 2.36 bc           | 12.47 ± 0.60 bc            | 15.82 ± 3.01 bc  | 20.05 ± 0.91 a      |
|                     |     | ECO <sub>2</sub>       | 13.94 ± 0.52 bcd | 14.54 ± 0.83 abc          | 16.58 ± 0.90 a             | 17.88 ± 2.06 abc | 18.16 ± 1.95 abcd   |
| VN31                | T   | ACO <sub>2</sub>       | 17.32 ± 1.59 abc | 14.92 ± 1.19 abc          | 14.36 ± 0.85 abc           | 13.66 ± 0.93 bc  | 15.99 ± 1.79 abcdef |
|                     |     | ECO <sub>2</sub>       | 14.85 ± 0.75 bcd | 13.44 ± 0.50 abc          | 14.78 ± 0.53 abc           | 15.82 ± 0.84 bc  | 19.61 ± 1.34 ab     |
|                     | T+4 | ACO <sub>2</sub>       | 15.43 ± 0.41 bcd | 11.81 ± 2.22 c            | 13.36 ± 1.07 abc           | 14.09 ± 2.50 bc  | 17.34 ± 2.17 abcde  |
|                     |     | ECO <sub>2</sub>       | 14.10 ± 0.65 bcd | 15.44 ± 1.42 abc          | 16.65 ± 1.42 a             | 19.57 ± 0.95 ab  | 17.94 ± 1.80 abcd   |
| 1084                | T   | ACO <sub>2</sub>       | 15.59 ± 0.67 bcd | 13.07 ± 0.38 abc          | 12.93 ± 1.14 bc            | 12.34 ± 0.35 c   | 14.10 ± 1.16 def    |
|                     |     | ECO <sub>2</sub>       | 14.78 ± 1.10 bcd | 14.35 ± 1.08 abc          | 15.50 ± 1.45 abc           | 12.75 ± 0.50 bc  | 12.03 ± 1.08 f      |
|                     | T+4 | ACO <sub>2</sub>       | 14.38 ± 0.92 bcd | 11.97 ± 0.91 bc           | 13.66 ± 0.95 abc           | 17.12 ± 6.61 abc | 17.07 ± 1.73 abcdef |
|                     |     | ECO <sub>2</sub>       | 13.55 ± 1.85 cd  | 13.57 ± 0.82 abc          | 15.31 ± 1.14 abc           | 17.02 ± 0.96 abc | 13.24 ± 0.94 ef     |
| P(CL)               |     | ns                     | ns               | ns                        | ns                         | *                |                     |
| P(T)                |     | ns                     | ns               | *                         | **                         | ns               |                     |
| P(CO <sub>2</sub> ) |     | ***                    | ns               | **                        | ns                         | ns               |                     |

**FIGURES**

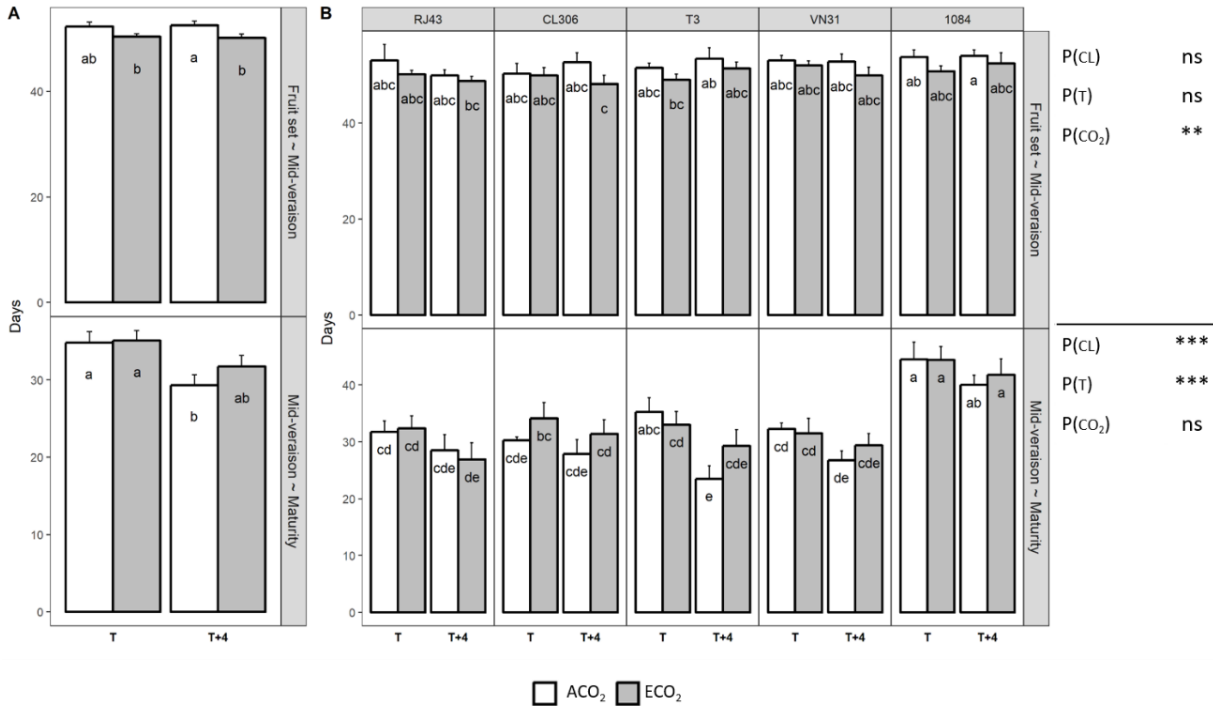


Figure 1. Elapsed time between fruit set and mid-veraison, and between mid-veraison and maturity of the five Tempranillo clones grown under four temperature/CO<sub>2</sub> regimes: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). Results (values are means ± SE) are presented according to the temperature (T or T+4) and CO<sub>2</sub> regime (ACO<sub>2</sub> or ECO<sub>2</sub>), (A) considering all the clones as altogether (n = 20-40) and (B) considering each clone individually (n = 4-8). Means with letters in common within each chart (A and B) and period are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone P(CL); temperature, P(T); and CO<sub>2</sub>, P(CO<sub>2</sub>). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. All probability values for the interactions of factors (P(CL x T), P(CL x CO<sub>2</sub>), P(T x CO<sub>2</sub>) and P(CL x T x CO<sub>2</sub>)) were statistically not significant (P > 0.05).



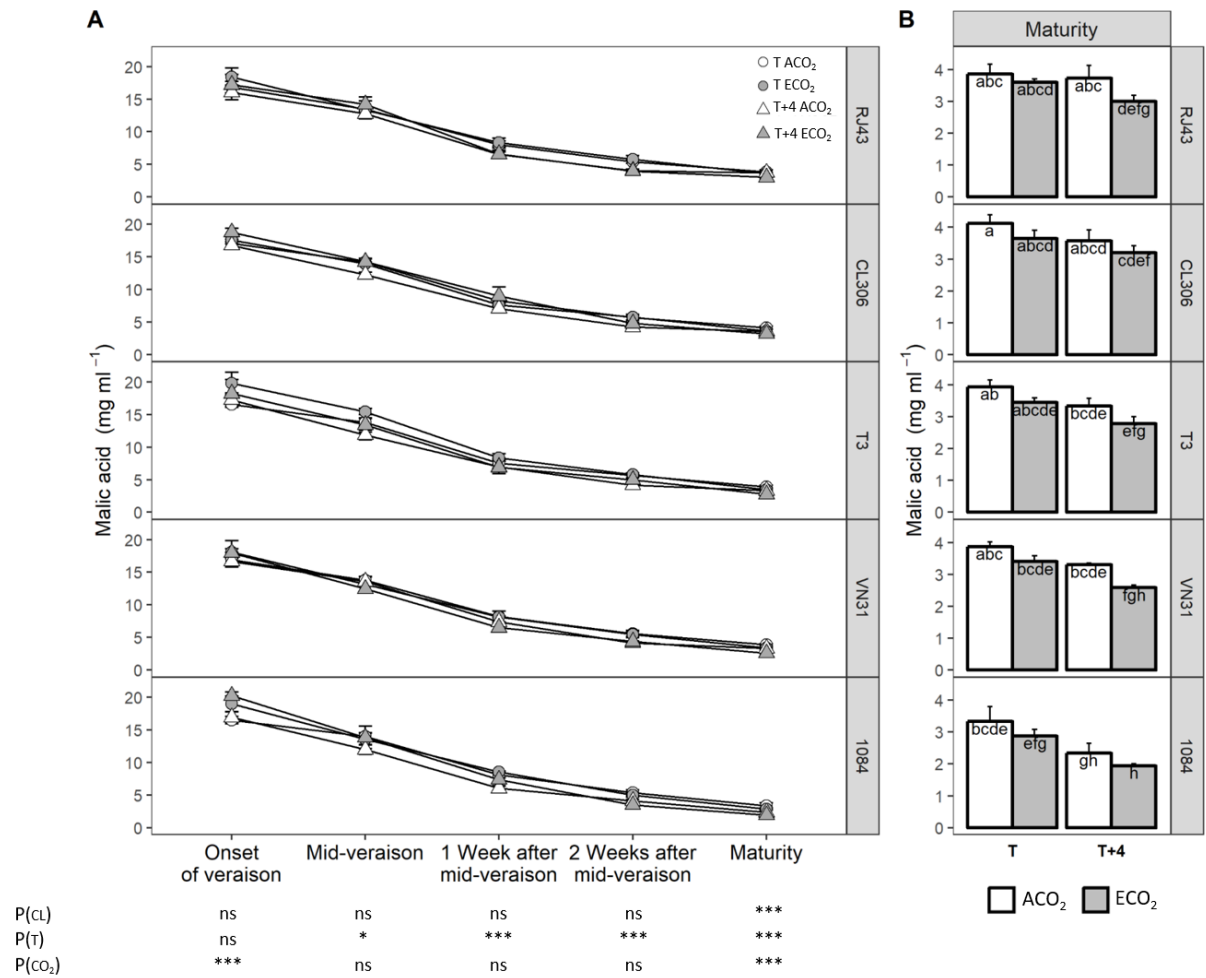


Figure 2. Malic acid concentration of the five Tempranillo clones grown under four temperature/CO<sub>2</sub> regimes: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). Data (values are means ± SE, n = 4) are presented according to the temperature (T or T+4) and CO<sub>2</sub> regime (ACO<sub>2</sub> or ECO<sub>2</sub>) (A) throughout ripening and (B) at maturity. Data are presented according to the temperature (T or T+4) and CO<sub>2</sub> regime (ACO<sub>2</sub> or ECO<sub>2</sub>) and considering each clone individually. Means with letters in common are not significantly different according to LSD test (P>0.05). Probability values (P) for the main effects of clone, P(CL); temperature, P(T); and CO<sub>2</sub>, P(CO<sub>2</sub>). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. All probability values for the interactions of factors (P(CL × T), P(CL × CO<sub>2</sub>), P(T × CO<sub>2</sub>) and P(CL × T × CO<sub>2</sub>)) were statistically not significant (P > 0.05).

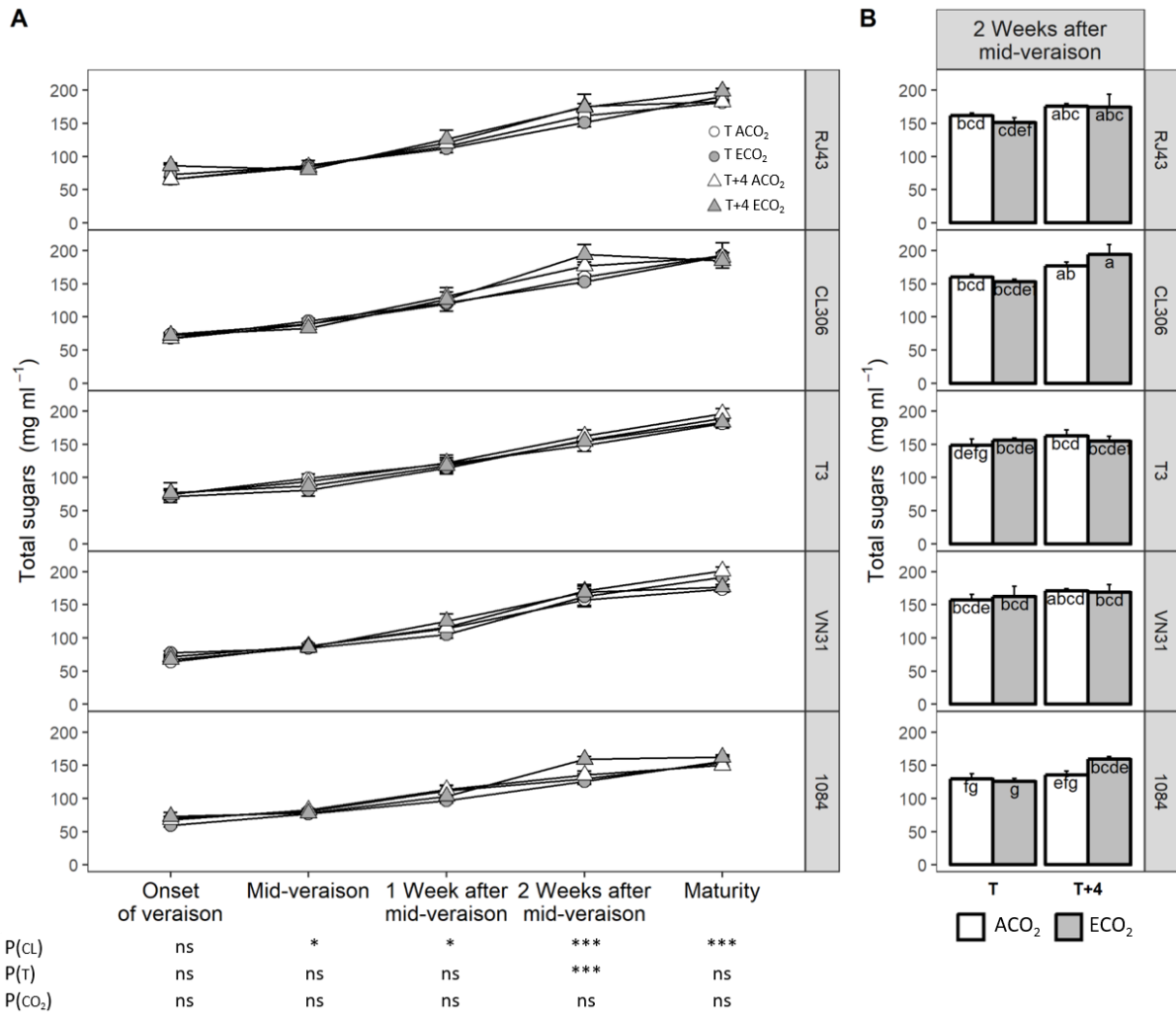


Figure 3. Sugar concentration in berries of the five Tempranillo clones grown under four temperature/CO<sub>2</sub> regimes: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). Data are presented according to the temperature (T or T+4) and CO<sub>2</sub> regime (ACO<sub>2</sub> or ECO<sub>2</sub>) and considering each clone individually (values are means ± SE, n = 4): (A) throughout ripening; and (B) 2 weeks after mid-veraison. Means with letters in common are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone, P(CL); temperature, P(T); and CO<sub>2</sub>, P(CO<sub>2</sub>). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. All probability values for the interactions of factors (P(CL × T), P(CL × CO<sub>2</sub>), P(T × CO<sub>2</sub>) and P(CL × T × CO<sub>2</sub>)) were statistically not significant (P > 0.05).

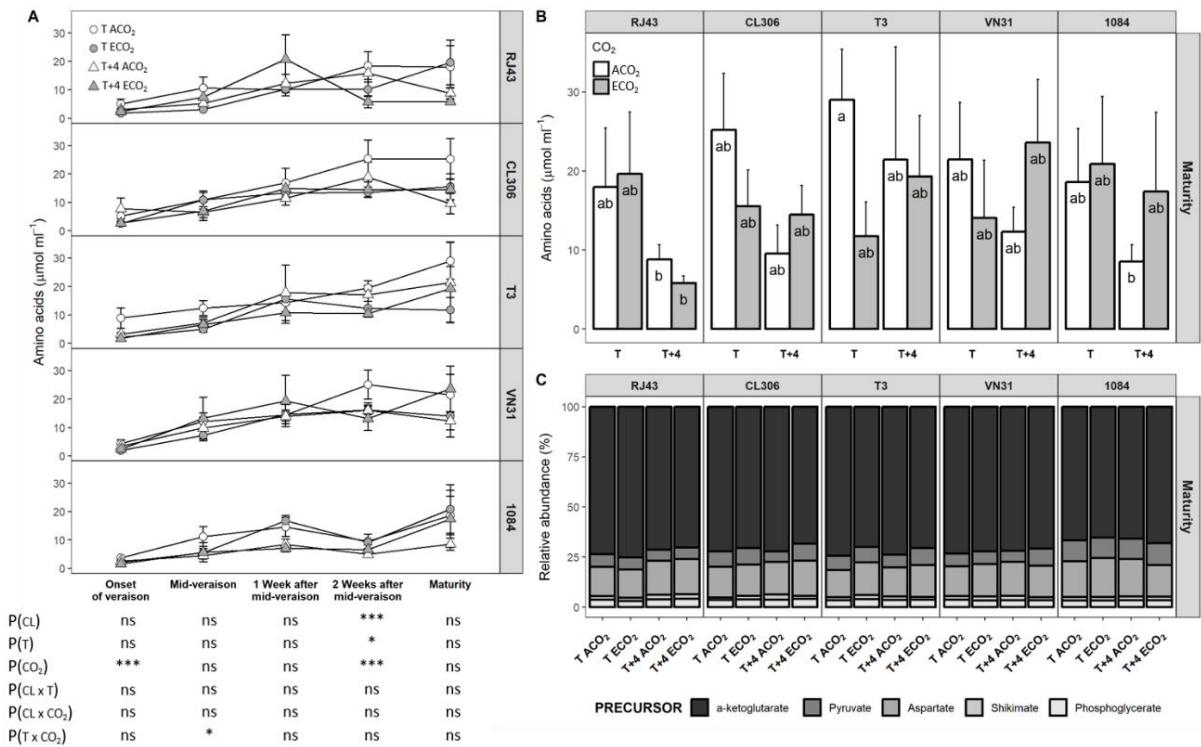


Figure 4. Amino acid concentration in berries of the five Tempranillo clones grown under four temperature/CO<sub>2</sub> regimes: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). Data are presented according to the temperature (T or T+4) and CO<sub>2</sub> regime (ACO<sub>2</sub> or ECO<sub>2</sub>) and considering each clone individually (values are means ± SE, n = 3-4). (A) throughout ripening, and (B) at maturity. Relative abundance of amino acids (C) grouped by their precursor (. Means with letters in common are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone P(CL); temperature, P(T); CO<sub>2</sub>, P(CO<sub>2</sub>); and their interactions, P(CL x T), P(CL x CO<sub>2</sub>) and P(T x CO<sub>2</sub>). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. All probability values for the interaction P(CL x T x CO<sub>2</sub>) were statistically not significant (P > 0.05).

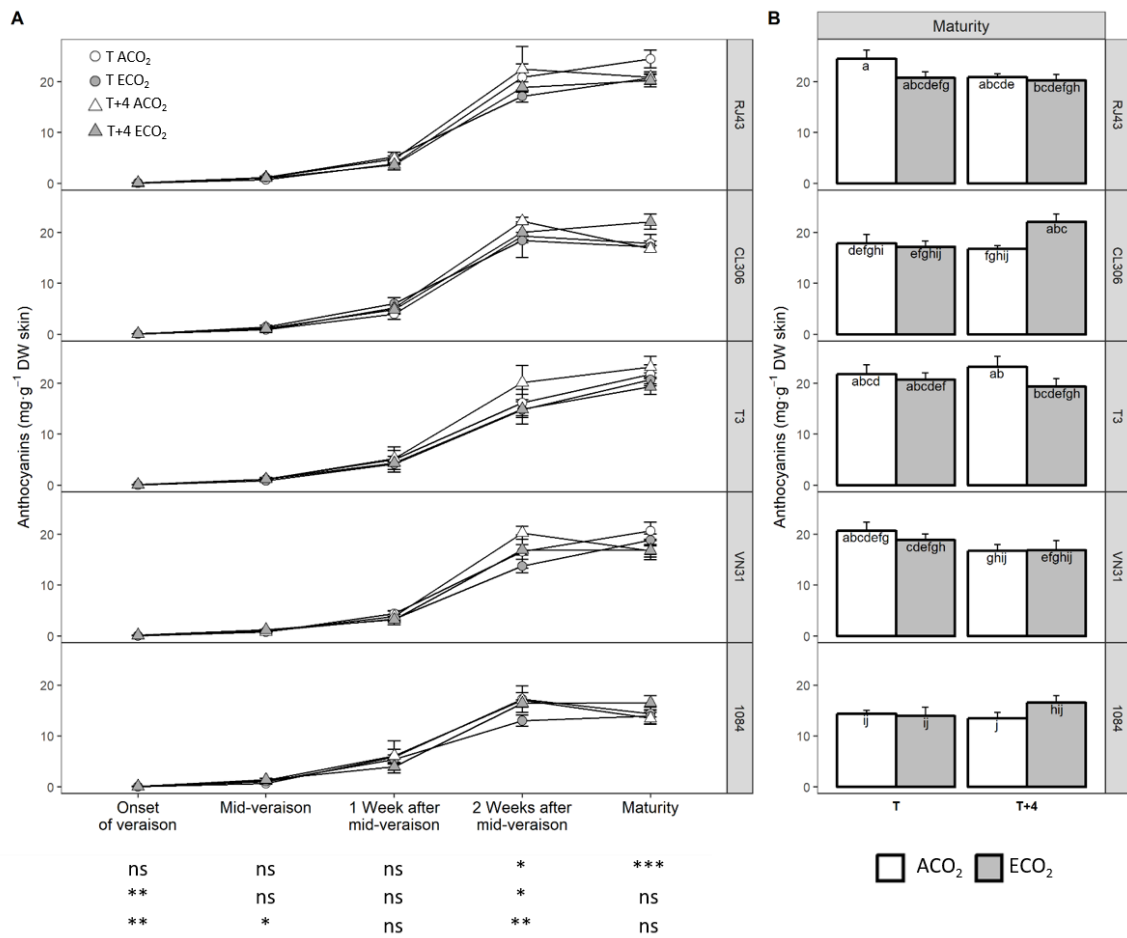


Figure 5. Total anthocyanin concentration in berries of the five Tempranillo clones grown under four temperature/CO<sub>2</sub> regimes: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). Data are presented according to the temperature (T or T+4) and CO<sub>2</sub> regime (ACO<sub>2</sub> or ECO<sub>2</sub>) and considering each clone individually (values are means ± SE, n = 4). (A) throughout ripening, and (B) at maturity. Means with letters in common are not significantly different (P > 0.05) according to LSD test. Probability values (P) for the main effects of clone, P(Cl); temperature, P(T); and CO<sub>2</sub>, P(CO<sub>2</sub>). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. All probability values for the interactions of factors (P(Cl × T), P(Cl × CO<sub>2</sub>), P(T × CO<sub>2</sub>) and P(Cl × T × CO<sub>2</sub>)) were statistically not significant (P > 0.05).

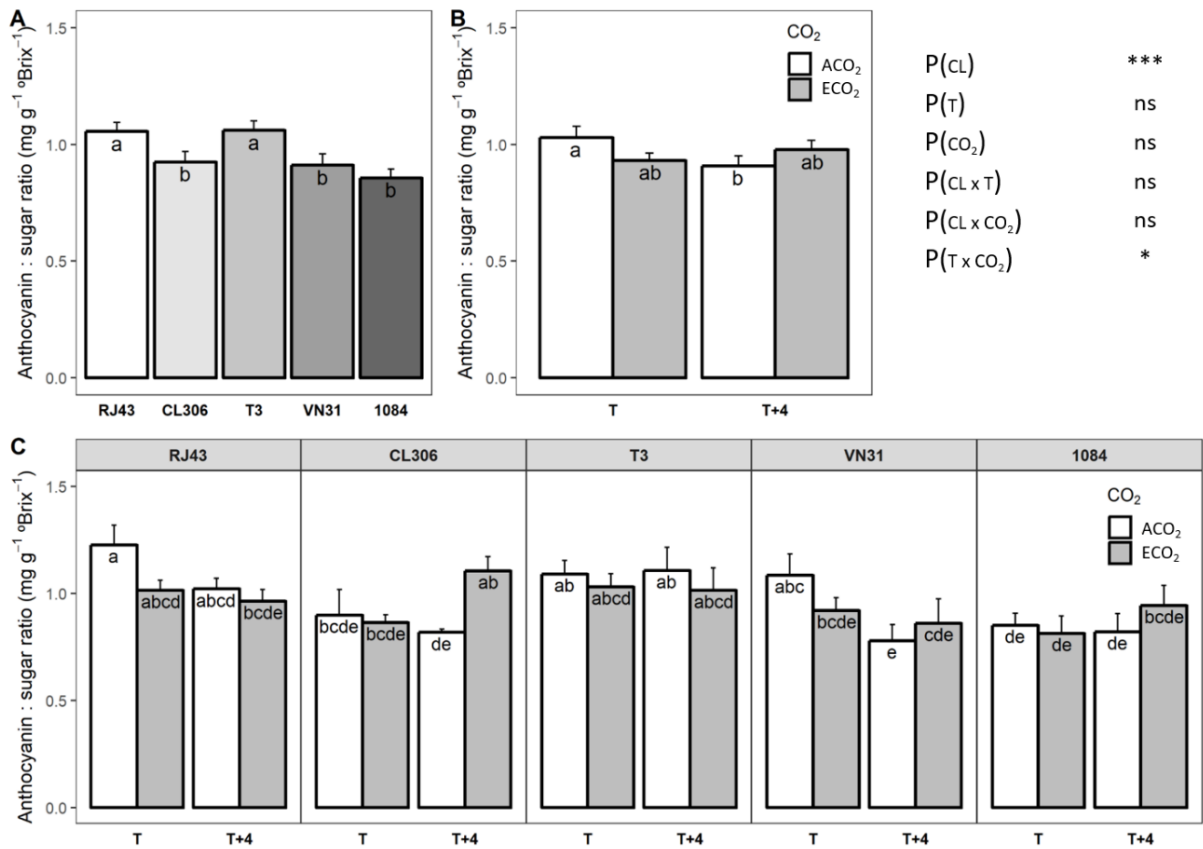


Figure 6. Anthocyanin to TSS ratio at maturity of the five Tempranillo clones grown under four temperature/CO<sub>2</sub> regimes: ambient temperature (T) or ambient temperature +4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). Results (values are means ± SE) are presented according to: (A) the clone identity (n = 15-16); (B) the temperature (T or T+4) and CO<sub>2</sub> regime (ACO<sub>2</sub> or ECO<sub>2</sub>) (n = 19-20); and (C) the clone identity, temperature and CO<sub>2</sub> regime (n = 3-4). Means with letters in common within each chart (A, B or C) are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone P(CL); temperature, P(T); CO<sub>2</sub>, P(CO<sub>2</sub>); and their interactions, P(CL x T), P(CL x CO<sub>2</sub>) and P(T x CO<sub>2</sub>). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. All probability values for the interaction P(CL x T x CO<sub>2</sub>) were statistically not significant (P > 0.05).

## SUPPLEMENTARY MATERIAL

Figure S1. Daily minimum and maximum temperatures recorded in the modules of ambient temperature (T) and ambient temperature +4 °C (T+4) of the TGGs during the experiment.

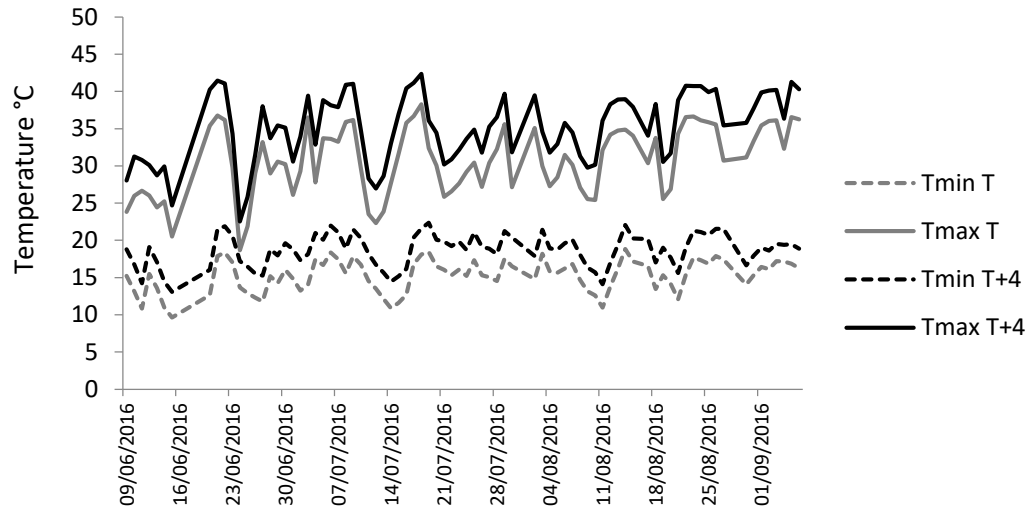


Table S1A. Total free amino acid content ( $\mu\text{mol}\cdot\text{mL}^{-1}$ ) and relative abundance of individual amino acids (%) at the onset of veraison in berries of the five Tempranillo clones grown under four temperature/ $\text{CO}_2$  regimes: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient  $\text{CO}_2$  (ca. 400 ppm,  $\text{ACO}_2$ ) or elevated  $\text{CO}_2$  (700 ppm,  $\text{ECO}_2$ ). Probability values (P) for the main effects of clone, P(Cl); temperature P(T); and  $\text{CO}_2$ , P( $\text{CO}_2$ ). \*\*,  $P < 0.001$ ; \*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant. All probability values for the interactions of factors (P(Cl x T), P(Cl x  $\text{CO}_2$ ), P(T x  $\text{CO}_2$ ) and P(Cl x T x  $\text{CO}_2$ ) were statistically not significant ( $P > 0.05$ ).

|                    |                | Relative abundance (%) |                         |         |          |               |           |      |           |          |         |        |         |           |           |               |            |            |            |        |           |          |               |                  |        |         |
|--------------------|----------------|------------------------|-------------------------|---------|----------|---------------|-----------|------|-----------|----------|---------|--------|---------|-----------|-----------|---------------|------------|------------|------------|--------|-----------|----------|---------------|------------------|--------|---------|
|                    |                | Total amino acid       | $\alpha$ -Ketoglutarate | Proline | Arginine | Glutamic acid | Glutamine | Gaba | Histidine | Pyruvate | Alanine | Valine | Leucine | Aspartate | Threonine | Aspartic acid | Asparagine | Isoleucine | Methionine | Lysine | Shikimate | Tyrosine | Phenylalanine | Phosphoglycerate | Serine | Glycine |
| R143               | $\text{ACO}_2$ | 3.0                    | 65.4                    | 3.37    | 25.92    | 2.32          | 28.13     | 2.74 | 2.93      | 6.48     | 4.83    | 0.93   | 0.73    | 14.0      | 10.91     | 0.87          | 1.15       | 0.63       | 0.41       | 0.07   | 3.69      | 1.05     | 2.64          | 10.37            | 9.61   | 0.77    |
|                    | $\text{ECO}_2$ | 4.5                    | 67.2                    | 3.74    | 27.71    | 2.58          | 28.55     | 2.50 | 2.07      | 6.18     | 4.52    | 0.96   | 0.71    | 13.5      | 10.19     | 1.10          | 1.17       | 0.59       | 0.37       | 0.11   | 3.39      | 0.92     | 2.48          | 9.73             | 9.00   | 0.73    |
| Cl306              | T              | 4.1                    | 67.3                    | 3.31    | 25.82    | 2.74          | 29.21     | 2.50 | 3.71      | 6.58     | 4.87    | 0.95   | 0.75    | 14.2      | 10.65     | 1.23          | 1.24       | 0.68       | 0.34       | 0.09   | 3.61      | 1.05     | 2.56          | 8.30             | 7.71   | 0.59    |
|                    | $\text{ACO}_2$ | 3.0                    | 66.6                    | 3.50    | 23.95    | 2.63          | 31.45     | 2.43 | 2.60      | 6.39     | 4.72    | 0.99   | 0.68    | 13.3      | 9.96      | 1.13          | 1.15       | 0.58       | 0.44       | 0.08   | 3.21      | 0.94     | 2.26          | 10.51            | 9.53   | 0.98    |
| VN31               | T              | 2.5                    | 65.4                    | 3.27    | 26.78    | 2.35          | 26.64     | 2.92 | 3.48      | 6.76     | 5.05    | 0.96   | 0.75    | 13.7      | 10.37     | 0.85          | 1.38       | 0.69       | 0.37       | 0.06   | 4.19      | 1.10     | 3.09          | 9.86             | 9.01   | 0.85    |
|                    | $\text{ECO}_2$ | 3.8                    | 66.3                    | 3.17    | 25.77    | 2.48          | 29.74     | 2.70 | 2.48      | 6.51     | 4.86    | 0.94   | 0.71    | 13.6      | 10.22     | 1.08          | 1.22       | 0.60       | 0.35       | 0.10   | 3.25      | 0.90     | 2.34          | 10.32            | 9.48   | 0.84    |
| T                  | T+4            | 3.1                    | 66.4                    | 3.72    | 26.31    | 2.56          | 27.82     | 2.53 | 3.43      | 6.44     | 4.73    | 0.98   | 0.73    | 14.0      | 10.60     | 0.99          | 1.22       | 0.67       | 0.42       | 0.06   | 4.00      | 1.12     | 2.88          | 9.21             | 8.48   | 0.73    |
|                    | $\text{ECO}_2$ | 4.7                    | 69.8                    | 3.93    | 25.66    | 2.67          | 32.69     | 2.91 | 1.92      | 6.22     | 4.72    | 0.86   | 0.65    | 12.6      | 9.43      | 1.21          | 0.99       | 0.52       | 0.29       | 0.13   | 2.50      | 0.76     | 1.74          | 8.93             | 8.18   | 0.75    |
| R143               | $\text{ECO}_2$ | 2.1                    | 62.9                    | 2.94    | 26.43    | 2.38          | 24.79     | 2.32 | 4.00      | 6.74     | 4.88    | 1.06   | 0.80    | 15.0      | 11.41     | 0.86          | 1.45       | 0.75       | 0.49       | 0.03   | 4.77      | 1.27     | 3.50          | 10.64            | 9.82   | 0.83    |
|                    | $\text{ACO}_2$ | 5.0                    | 71.4                    | 4.61    | 23.65    | 2.31          | 36.14     | 3.52 | 1.17      | 6.13     | 4.79    | 0.76   | 0.59    | 11.7      | 8.97      | 1.07          | 0.87       | 0.41       | 0.21       | 0.17   | 1.88      | 0.61     | 1.28          | 8.88             | 8.27   | 0.61    |
| T+4                | $\text{ACO}_2$ | 1.8                    | 60.4                    | 2.61    | 25.40    | 2.09          | 23.75     | 2.88 | 3.66      | 6.84     | 5.18    | 0.91   | 0.76    | 15.5      | 12.26     | 0.55          | 1.60       | 0.67       | 0.44       | 0.03   | 4.34      | 1.23     | 3.11          | 12.90            | 11.92  | 0.98    |
|                    | $\text{ECO}_2$ | 3.0                    | 68.6                    | 3.28    | 26.54    | 3.20          | 30.54     | 2.53 | 2.55      | 6.60     | 5.02    | 0.88   | 0.70    | 13.4      | 10.33     | 1.24          | 0.83       | 0.59       | 0.37       | 0.05   | 2.93      | 0.80     | 2.13          | 8.41             | 7.84   | 0.56    |
| Cl306              | T              | 2.4                    | 61.3                    | 2.99    | 28.11    | 1.67          | 22.11     | 2.04 | 4.36      | 6.35     | 4.32    | 1.17   | 0.86    | 15.5      | 12.08     | 0.61          | 1.29       | 0.86       | 0.61       | 0.03   | 5.59      | 1.55     | 4.05          | 11.31            | 10.39  | 0.91    |
|                    | $\text{ECO}_2$ | 5.1                    | 69.9                    | 3.48    | 27.43    | 2.79          | 32.61     | 2.29 | 1.35      | 6.28     | 4.83    | 0.85   | 0.60    | 12.7      | 9.17      | 1.25          | 1.33       | 0.54       | 0.24       | 0.14   | 2.33      | 0.67     | 1.66          | 8.76             | 8.06   | 0.70    |
| T3                 | T              | 2.6                    | 64.0                    | 2.56    | 28.97    | 2.68          | 24.90     | 2.33 | 2.51      | 6.98     | 5.03    | 1.11   | 0.84    | 14.3      | 10.95     | 0.72          | 1.38       | 0.72       | 0.51       | 0.04   | 4.00      | 1.06     | 2.94          | 10.74            | 9.96   | 0.78    |
|                    | $\text{ECO}_2$ | 7.8                    | 69.3                    | 5.60    | 25.14    | 3.00          | 30.21     | 4.15 | 1.23      | 6.50     | 4.98    | 0.91   | 0.61    | 12.9      | 9.58      | 1.62          | 0.77       | 0.44       | 0.23       | 0.24   | 1.98      | 0.66     | 1.32          | 9.32             | 8.63   | 0.69    |
| T+4                | $\text{ACO}_2$ | 2.7                    | 65.4                    | 3.32    | 29.32    | 1.87          | 26.50     | 1.23 | 3.17      | 4.98     | 3.23    | 0.98   | 0.77    | 14.3      | 11.05     | 0.81          | 1.21       | 0.67       | 0.50       | 0.02   | 5.27      | 1.27     | 4.00          | 10.07            | 9.33   | 0.74    |
|                    | $\text{ECO}_2$ | 8.9                    | 71.1                    | 4.12    | 23.37    | 2.42          | 36.84     | 3.04 | 1.31      | 5.70     | 4.14    | 0.87   | 0.70    | 12.7      | 9.48      | 1.36          | 0.81       | 0.54       | 0.29       | 0.23   | 2.01      | 0.65     | 1.37          | 8.49             | 8.05   | 0.44    |
| VN31               | T              | 2.1                    | 61.7                    | 2.68    | 26.05    | 3.03          | 22.89     | 2.42 | 4.59      | 7.87     | 5.79    | 1.19   | 0.90    | 15.9      | 11.64     | 1.51          | 1.36       | 0.86       | 0.45       | 0.05   | 4.93      | 1.20     | 3.73          | 9.67             | 8.99   | 0.68    |
|                    | $\text{ECO}_2$ | 3.2                    | 70.2                    | 3.70    | 27.19    | 2.54          | 31.16     | 2.55 | 3.10      | 5.90     | 4.51    | 0.76   | 0.62    | 13.2      | 10.19     | 1.02          | 1.16       | 0.55       | 0.23       | 0.05   | 2.95      | 1.00     | 1.95          | 7.72             | 6.96   | 0.76    |
| T+4                | $\text{ACO}_2$ | 1.7                    | 65.7                    | 2.56    | 26.94    | 3.04          | 24.86     | 1.80 | 6.54      | 6.92     | 5.11    | 1.00   | 0.80    | 15.5      | 11.49     | 0.98          | 1.77       | 0.79       | 0.42       | 0.02   | 4.87      | 1.45     | 3.43          | 7.00             | 6.57   | 0.43    |
|                    | $\text{ECO}_2$ | 4.4                    | 68.9                    | 3.59    | 23.92    | 2.59          | 34.30     | 2.80 | 1.70      | 6.47     | 4.46    | 1.01   | 0.69    | 12.3      | 8.57      | 1.72          | 0.93       | 0.51       | 0.32       | 0.22   | 2.31      | 0.70     | 1.61          | 10.37            | 9.03   | 1.34    |
| 1084               | T              | 2.0                    | 60.2                    | 2.67    | 23.15    | 2.45          | 27.23     | 2.64 | 2.10      | 7.01     | 5.29    | 1.03   | 0.68    | 14.8      | 11.65     | 0.77          | 1.31       | 0.62       | 0.46       | 0.02   | 3.70      | 1.06     | 2.64          | 14.22            | 12.93  | 1.29    |
|                    | $\text{ECO}_2$ | 3.4                    | 71.8                    | 3.65    | 23.32    | 2.50          | 38.42     | 1.97 | 1.98      | 5.41     | 4.04    | 0.80   | 0.57    | 11.9      | 8.95      | 0.98          | 1.10       | 0.44       | 0.03       | 0.03   | 2.42      | 0.78     | 1.64          | 8.38             | 7.80   | 0.58    |
| T+4                | $\text{ACO}_2$ | 2.3                    | 65.3                    | 4.11    | 25.41    | 2.97          | 25.84     | 2.32 | 4.62      | 6.96     | 5.08    | 1.11   | 0.78    | 14.3      | 10.66     | 1.07          | 1.25       | 0.74       | 0.53       | 0.06   | 4.40      | 1.23     | 3.17          | 9.07             | 8.36   | 0.71    |
|                    | $\text{ECO}_2$ | 3.7                    | 70.7                    | 2.75    | 30.90    | 2.08          | 29.81     | 2.62 | 2.55      | 5.98     | 4.55    | 0.78   | 0.65    | 11.6      | 8.97      | 0.94          | 0.80       | 0.52       | 0.23       | 0.10   | 2.92      | 0.81     | 2.11          | 8.83             | 7.94   | 0.89    |
| T+4                | $\text{ACO}_2$ | 2.6                    | 65.8                    | 4.52    | 25.16    | 3.21          | 26.91     | 3.68 | 2.28      | 7.54     | 5.87    | 0.95   | 0.72    | 13.4      | 10.13     | 0.88          | 1.33       | 0.64       | 0.30       | 0.09   | 3.23      | 0.94     | 2.30          | 10.10            | 9.21   | 0.89    |
|                    | $\text{ECO}_2$ | 1.6                    | 60.2                    | 3.20    | 26.17    | 1.76          | 20.89     | 2.89 | 5.29      | 7.35     | 5.23    | 1.20   | 0.92    | 15.7      | 11.81     | 0.69          | 1.60       | 0.96       | 0.56       | 0.03   | 6.58      | 1.61     | 4.97          | 10.20            | 9.25   | 0.95    |
| P(Cl)              |                | ns                     | ns                      | ns      | ns       | ns            | ns        | ns   | ns        | ns       | ns      | ns     | ns      | ns        | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns               | ns     | ns      |
| P(T)               |                | ns                     | ns                      | ns      | ns       | ns            | ns        | ns   | *         | ns       | ns      | ns     | ns      | ns        | ns        | ns            | ns         | ns         | ns         | *      | *         | *        | *             | ns               | ns     | ns      |
| P( $\text{CO}_2$ ) |                | ****                   | ****                    | ****    | ns       | ns            | ****      | *    | ****      | ns       | ns      | ****   | ****    | ****      | ****      | ****          | ****       | ****       | ****       | ****   | ****      | ****     | ****          | ****             | ns     | ns      |

Table S1B. Total free amino acid content ( $\mu\text{mol}\cdot\text{ml}^{-1}$ ) and relative abundance of individual amino acids (%) at mid-veraison in berries of the five Tempranillo clones grown under four temperature/ $\text{CO}_2$  regimes: ambient temperature (T) or ambient temperature +  $4^\circ\text{C}$  (T+4), combined with ambient  $\text{CO}_2$  (ca. 400 ppm,  $\text{ACO}_2$ ) or elevated  $\text{CO}_2$  (700 ppm,  $\text{ECO}_2$ ). Probability values (P) for the main effects of clone,  $P(\text{CL})$ ; temperature  $P(\text{T})$ ;  $\text{CO}_2$ ,  $P(\text{CO}_2)$ ; and their interactions,  $P(\text{CL}\times\text{T})$ ,  $P(\text{CL}\times\text{CO}_2)$ ,  $P(\text{T}\times\text{CO}_2)$  and  $P(\text{CL}\times\text{T}\times\text{CO}_2)$ . \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant.

|   |      | Relative abundance (%) |          |               |           |       |           |          |         |        |         |                  |           |               |            |            |            |        |           |          |               |        |         |      |
|---|------|------------------------|----------|---------------|-----------|-------|-----------|----------|---------|--------|---------|------------------|-----------|---------------|------------|------------|------------|--------|-----------|----------|---------------|--------|---------|------|
|   |      | Total amino acid       |          |               |           |       |           |          |         |        |         | Phosphoglycerate |           |               |            |            |            |        |           |          |               |        |         |      |
|   |      | Proline                | Arginine | Glutamic acid | Glutamine | Gaba  | Histidine | Pyruvate | Alanine | Valine | Leucine | Aspartate        | Threonine | Aspartic acid | Asparagine | Isoleucine | Methionine | Lysine | Shikimate | Tyrosine | Phenylalanine | Serine | Glycine |      |
| RI43  | 6.6  | 69.2                   | 5.57     | 24.20         | 2.84      | 31.69 | 3.31      | 1.55     | 6.14    | 1.12   | 0.79    | 13.6             | 10.13     | 1.39          | 0.86       | 0.76       | 0.42       | 0.08   | 2.60      | 0.80     | 1.80          | 6.55   | 6.06    | 0.50 |
| CL306   | 8.8  | 71.2                   | 5.86     | 27.05         | 3.30      | 30.88 | 3.04      | 1.06     | 8.00    | 6.22   | 1.04    | 12.9             | 9.11      | 1.71          | 0.89       | 0.58       | 0.44       | 0.16   | 2.11      | 0.71     | 1.40          | 5.82   | 5.35    | 0.47 |
| T3  | 7.8  | 69.1                   | 5.75     | 25.76         | 4.20      | 27.99 | 4.26      | 1.11     | 9.07    | 7.00   | 1.18    | 13.6             | 9.70      | 1.80          | 0.80       | 0.76       | 0.41       | 0.12   | 2.30      | 0.79     | 1.51          | 5.96   | 5.45    | 0.51 |
| VN31  | 10.6 | 71.2                   | 5.52     | 25.58         | 3.29      | 32.57 | 2.96      | 1.25     | 8.21    | 6.26   | 1.16    | 12.5             | 8.84      | 1.59          | 0.82       | 0.75       | 0.44       | 0.09   | 2.03      | 0.71     | 1.31          | 6.05   | 5.52    | 0.54 |
| 1084  | 6.7  | 66.4                   | 5.85     | 25.97         | 3.53      | 25.36 | 4.29      | 1.39     | 9.67    | 7.45   | 1.29    | 14.1             | 9.82      | 1.98          | 0.90       | 0.88       | 0.42       | 0.13   | 2.75      | 0.89     | 1.86          | 7.07   | 6.43    | 0.64 |
| T   | 8.9  | 69.7                   | 5.40     | 24.72         | 3.13      | 31.82 | 3.35      | 1.25     | 8.54    | 6.47   | 1.09    | 13.2             | 9.50      | 1.72          | 0.80       | 0.69       | 0.39       | 0.14   | 2.22      | 0.76     | 1.46          | 6.54   | 5.99    | 0.55 |
| T+4   | 7.3  | 69.1                   | 6.02     | 26.70         | 3.73      | 27.58 | 3.79      | 1.30     | 8.86    | 6.76   | 1.22    | 13.5             | 9.54      | 1.67          | 0.91       | 0.81       | 0.46       | 0.09   | 2.49      | 0.80     | 1.69          | 6.05   | 5.54    | 0.51 |
| $\text{ACO}_2$                                | 9.0  | 71.3                   | 6.10     | 24.10         | 3.55      | 32.96 | 3.90      | 0.73     | 8.59    | 6.62   | 1.04    | 12.4             | 8.68      | 1.98          | 0.67       | 0.61       | 0.39       | 0.09   | 1.89      | 0.66     | 1.23          | 5.98   | 5.50    | 0.48 |
| $\text{ECO}_2$                                | 7.2  | 67.5                   | 5.32     | 27.33         | 3.32      | 26.44 | 3.24      | 1.82     | 8.81    | 6.61   | 1.28    | 14.2             | 10.35     | 1.41          | 1.04       | 0.89       | 0.46       | 0.14   | 2.83      | 0.90     | 1.93          | 6.61   | 6.03    | 0.58 |
| RI43 T  | 10.6 | 74.9                   | 4.70     | 21.57         | 2.30      | 42.86 | 2.52      | 0.97     | 5.90    | 4.64   | 0.75    | 11.2             | 8.55      | 1.31          | 0.62       | 0.42       | 0.27       | 0.08   | 1.74      | 0.58     | 1.16          | 6.19   | 5.70    | 0.49 |
| $\text{ECO}_2$                                | 3.1  | 63.2                   | 6.02     | 21.75         | 2.66      | 26.18 | 4.44      | 2.09     | 10.29   | 8.06   | 1.33    | 15.9             | 11.74     | 1.88          | 0.79       | 0.93       | 0.47       | 0.07   | 2.93      | 0.91     | 2.02          | 7.75   | 7.21    | 0.54 |
| T+4   | 5.2  | 71.3                   | 6.71     | 22.91         | 3.24      | 33.52 | 3.81      | 1.10     | 7.62    | 5.93   | 0.99    | 12.5             | 9.35      | 1.49          | 0.58       | 0.60       | 0.45       | 0.07   | 2.27      | 0.72     | 1.55          | 6.30   | 5.79    | 0.51 |
| $\text{ECO}_2$                                | 7.4  | 67.3                   | 4.84     | 30.58         | 3.15      | 24.20 | 2.47      | 2.03     | 8.40    | 5.92   | 1.43    | 14.9             | 10.87     | 0.90          | 1.46       | 1.10       | 0.48       | 0.08   | 3.45      | 0.97     | 2.48          | 5.98   | 5.53    | 0.45 |
| CL306 T                                       | 10.9 | 74.3                   | 5.42     | 25.20         | 3.09      | 37.49 | 2.57      | 0.49     | 6.99    | 5.44   | 0.91    | 11.9             | 8.44      | 1.79          | 0.80       | 0.51       | 0.33       | 0.05   | 1.56      | 0.55     | 1.01          | 5.26   | 4.90    | 0.36 |
| $\text{ECO}_2$                                | 10.9 | 69.8                   | 6.17     | 25.18         | 3.33      | 29.62 | 3.71      | 1.76     | 9.28    | 7.29   | 1.15    | 13.0             | 8.77      | 1.89          | 0.90       | 0.56       | 0.45       | 0.46   | 2.02      | 0.76     | 1.26          | 5.89   | 5.36    | 0.53 |
| T+4   | 6.5  | 70.5                   | 7.03     | 23.97         | 3.90      | 30.93 | 4.22      | 0.50     | 8.78    | 6.82   | 1.16    | 12.9             | 8.76      | 2.12          | 0.80       | 0.67       | 0.49       | 0.05   | 1.85      | 0.67     | 1.18          | 5.95   | 5.50    | 0.45 |
| $\text{ECO}_2$                                | 6.9  | 70.2                   | 4.82     | 33.86         | 2.90      | 25.47 | 1.63      | 1.51     | 6.94    | 5.33   | 0.93    | 13.7             | 10.45     | 1.03          | 1.08       | 0.58       | 0.49       | 0.07   | 2.99      | 0.86     | 2.14          | 6.18   | 5.65    | 0.53 |
| T3  | 12.4 | 72.3                   | 6.28     | 21.99         | 3.78      | 36.07 | 3.84      | 0.39     | 8.74    | 7.09   | 0.97    | 11.7             | 8.21      | 2.00          | 0.59       | 0.56       | 0.28       | 0.10   | 1.77      | 0.61     | 1.16          | 5.42   | 4.90    | 0.52 |
| $\text{ECO}_2$                                | 5.0  | 67.2                   | 3.72     | 26.11         | 3.26      | 28.55 | 3.15      | 2.44     | 7.16    | 4.94   | 1.27    | 15.0             | 11.30     | 1.21          | 1.00       | 0.93       | 0.53       | 0.06   | 3.63      | 1.13     | 2.50          | 6.96   | 6.43    | 0.53 |
| T+4   | 7.1  | 67.4                   | 7.74     | 24.74         | 4.50      | 24.42 | 5.92      | 0.09     | 11.13   | 8.81   | 1.34    | 13.3             | 9.23      | 2.25          | 0.57       | 0.81       | 0.44       | 0.02   | 1.92      | 0.69     | 1.23          | 6.19   | 5.75    | 0.44 |
| $\text{ECO}_2$                                | 6.6  | 69.3                   | 5.28     | 30.20         | 5.24      | 22.91 | 4.13      | 1.51     | 9.23    | 7.16   | 1.15    | 14.3             | 10.06     | 1.76          | 1.06       | 0.75       | 0.41       | 0.31   | 1.90      | 0.74     | 1.16          | 5.26   | 4.72    | 0.54 |
| VN31 T  | 12.1 | 72.5                   | 6.02     | 24.03         | 3.71      | 35.25 | 3.09      | 0.43     | 7.80    | 6.12   | 0.99    | 12.0             | 8.18      | 2.19          | 0.62       | 0.54       | 0.36       | 0.06   | 1.57      | 0.58     | 1.00          | 6.13   | 5.66    | 0.47 |
| $\text{ECO}_2$                                | 7.3  | 67.3                   | 5.65     | 26.73         | 3.20      | 27.48 | 2.92      | 1.33     | 9.47    | 7.42   | 1.25    | 13.7             | 9.85      | 1.56          | 0.88       | 0.83       | 0.45       | 0.11   | 2.26      | 0.77     | 1.49          | 7.30   | 6.60    | 0.70 |
| T+4   | 9.8  | 72.9                   | 4.94     | 26.77         | 3.10      | 33.53 | 3.08      | 1.49     | 7.42    | 5.98   | 0.88    | 12.1             | 8.67      | 1.61          | 0.73       | 0.48       | 0.48       | 0.12   | 1.95      | 0.68     | 1.26          | 5.64   | 5.20    | 0.45 |
| $\text{ECO}_2$                                | 13.3 | 71.9                   | 5.49     | 24.79         | 3.13      | 34.02 | 2.74      | 1.77     | 8.17    | 5.53   | 1.52    | 12.4             | 8.68      | 0.99          | 1.05       | 1.16       | 0.48       | 0.07   | 2.33      | 0.82     | 1.51          | 5.14   | 4.61    | 0.53 |
| 1084 T  | 11.2 | 72.7                   | 5.07     | 28.00         | 3.03      | 31.70 | 3.96      | 0.91     | 8.14    | 6.39   | 0.91    | 11.9             | 8.63      | 1.48          | 0.68       | 0.56       | 0.28       | 0.28   | 1.70      | 0.67     | 1.03          | 5.59   | 5.11    | 0.48 |
| $\text{ECO}_2$                                | 5.5  | 62.5                   | 5.01     | 26.67         | 2.97      | 22.99 | 3.25      | 1.65     | 9.61    | 7.27   | 1.40    | 15.9             | 11.33     | 1.84          | 1.17       | 1.01       | 0.49       | 0.10   | 3.03      | 1.01     | 2.02          | 8.88   | 8.01    | 0.86 |
| T+4   | 4.5  | 64.5                   | 7.16     | 21.82         | 4.80      | 23.81 | 5.97      | 0.92     | 11.39   | 8.98   | 1.47    | 14.5             | 8.81      | 3.50          | 0.71       | 0.90       | 0.52       | 0.07   | 2.53      | 0.85     | 1.68          | 7.08   | 6.44    | 0.64 |
| $\text{ECO}_2$                                | 5.7  | 65.9                   | 6.18     | 27.39         | 3.31      | 22.93 | 3.97      | 2.08     | 9.52    | 7.16   | 1.36    | 14.1             | 10.51     | 1.08          | 1.05       | 1.06       | 0.38       | 0.08   | 3.74      | 1.04     | 2.70          | 6.73   | 6.15    | 0.58 |
| $P(\text{CL})$                                | ns   | ns                     | ns       | ns            | *         | ns    | *         | ns       | ns      | ns     | ns      | ns               | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns     | ns      | *    |
| $P(\text{T})$                                 | ns   | ns                     | ns       | *             | *         | ns    | ns        | ns       | ns      | *      | *       | ns               | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns     | ns      | ns   |
| $P(\text{CO}_2)$                              | ns   | **                     | ns       | **            | ns        | ***   | *         | ns       | ns      | ***    | ***     | ***              | ***       | **            | ***        | ***        | *          | ns     | ***       | ***      | ***           | ***    | ns      | **   |
| $P(\text{CL}\times\text{T})$                  | ns   | ns                     | ns       | ns            | ns        | ns    | ns        | ns       | ns      | ns     | ns      | ns               | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns     | ns      | ns   |
| $P(\text{CL}\times\text{CO}_2)$               | ns   | ns                     | ns       | ns            | ns        | ns    | ns        | ns       | *       | ns     | *       | ns               | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns     | ns      | ns   |
| $P(\text{T}\times\text{CO}_2)$                | *    | **                     | ns       | *             | ns        | **    | ns        | **       | *       | *      | *       | *                | *         | *             | ns         | ns         | ns         | ns     | ns        | ns       | ns            | *      | *       | ns   |
| $P(\text{CL}\times\text{T}\times\text{CO}_2)$ | ns   | ns                     | ns       | ns            | ns        | ns    | ns        | ns       | ns      | ns     | ns      | ns               | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns     | ns      | ns   |



Table S1C. Total free amino acid content ( $\mu\text{mol}\cdot\text{ml}^{-1}$ ) and relative abundance of individual amino acids (%) one week after mid-veraison in berries of the five Tempranillo clones grown under four temperature/ $\text{CO}_2$  regimes: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient  $\text{CO}_2$  (ca. 400 ppm,  $\text{ACO}_2$ ) or elevated  $\text{CO}_2$  (700 ppm,  $\text{ECO}_2$ ). Probability values (P) for the main effects of clone, P(Cl); temperature P(T);  $\text{CO}_2$ , P( $\text{CO}_2$ ); and their interactions, P(Cl x T), P(Cl x  $\text{CO}_2$ ) or P(T x  $\text{CO}_2$ ), \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. All probability values for the interaction P(Cl x T x  $\text{CO}_2$ ) were statistically not significant (P > 0.05).

| Clone                  | Temp           | $\text{CO}_2$  | Total amino acid | Relative abundance (%)  |         |          |               |           |      |           |          |         |        |         |           |           |               |            |            |            |        |           |          |               |                  |        |         |    |
|------------------------|----------------|----------------|------------------|-------------------------|---------|----------|---------------|-----------|------|-----------|----------|---------|--------|---------|-----------|-----------|---------------|------------|------------|------------|--------|-----------|----------|---------------|------------------|--------|---------|----|
|                        |                |                |                  | $\alpha$ -Ketoglutarate | Proline | Arginine | Glutamic acid | Glutamine | Gaba | Histidine | Pyruvate | Alanine | Valine | Leucine | Aspartate | Threonine | Aspartic acid | Asparagine | Isoleucine | Methionine | Lysine | Shikimate | Tyrosine | Phenylalanine | Phosphoglycerate | Serine | Glycine |    |
| R43                    | T              | $\text{ACO}_2$ | 13.3             | 76.2                    | 10.59   | 23.79    | 3.32          | 33.70     | 3.98 | 0.80      | 6.88     | 5.63    | 0.73   | 0.52    | 11.6      | 8.43      | 1.93          | 0.59       | 0.34       | 0.21       | 0.13   | 1.21      | 0.56     | 0.66          | 4.10             | 3.82   | 0.28    |    |
|                        |                | $\text{ECO}_2$ | 14.1             | 76.4                    | 10.66   | 22.58    | 3.84          | 35.18     | 3.73 | 0.43      | 8.06     | 6.69    | 0.77   | 0.61    | 10.8      | 7.42      | 2.10          | 0.64       | 0.34       | 0.24       | 0.10   | 0.99      | 0.47     | 0.52          | 3.70             | 3.44   | 0.27    |    |
|                        |                | T3             | 14.6             | 76.3                    | 10.93   | 22.33    | 3.47          | 35.03     | 3.74 | 0.76      | 7.48     | 6.14    | 0.77   | 0.57    | 11.6      | 8.36      | 2.03          | 0.47       | 0.38       | 0.19       | 0.12   | 1.11      | 0.52     | 0.59          | 3.60             | 3.32   | 0.28    |    |
| VN31                   | T              | $\text{ACO}_2$ | 15.5             | 77.2                    | 9.78    | 20.76    | 3.54          | 39.32     | 3.21 | 0.55      | 7.04     | 5.56    | 0.84   | 0.63    | 10.9      | 7.85      | 1.78          | 0.55       | 0.42       | 0.24       | 0.09   | 1.07      | 0.51     | 0.56          | 3.80             | 3.49   | 0.30    |    |
|                        |                | $\text{ECO}_2$ | 11.8             | 73.5                    | 9.92    | 25.02    | 3.58          | 30.18     | 4.07 | 0.78      | 8.18     | 6.82    | 0.78   | 0.58    | 12.9      | 9.16      | 2.26          | 0.72       | 0.41       | 0.22       | 0.13   | 1.24      | 0.59     | 0.66          | 4.13             | 3.78   | 0.36    |    |
|                        |                | T+4            | 14.1             | 76.4                    | 9.66    | 21.60    | 3.57          | 37.26     | 3.74 | 0.57      | 7.49     | 6.16    | 0.74   | 0.59    | 11.0      | 7.79      | 1.93          | 0.59       | 0.35       | 0.21       | 0.11   | 1.08      | 0.50     | 0.58          | 4.05             | 3.74   | 0.31    |    |
| $\text{ACO}_2$         | T              | $\text{ACO}_2$ | 13.4             | 76.1                    | 11.09   | 24.19    | 3.53          | 32.11     | 3.75 | 0.76      | 7.57     | 6.18    | 0.81   | 0.57    | 12.1      | 8.70      | 2.11          | 0.59       | 0.40       | 0.23       | 0.12   | 1.17      | 0.55     | 0.62          | 3.68             | 3.40   | 0.29    |    |
|                        |                | $\text{ECO}_2$ | 14.3             | 75.7                    | 9.82    | 23.38    | 3.49          | 34.22     | 4.04 | 0.74      | 7.44     | 6.00    | 0.82   | 0.62    | 11.0      | 8.63      | 1.91          | 0.65       | 0.43       | 0.21       | 0.13   | 1.18      | 0.56     | 0.62          | 3.73             | 3.41   | 0.32    |    |
|                        |                | T+4            | 14.3             | 75.9                    | 11.33   | 21.28    | 3.71          | 35.75     | 4.26 | 0.54      | 6.34     | 5.04    | 0.76   | 0.55    | 10.5      | 7.23      | 1.99          | 0.63       | 0.28       | 0.23       | 0.14   | 1.10      | 0.48     | 0.62          | 5.18             | 4.91   | 0.28    |    |
| R43                    | T              | $\text{ACO}_2$ | 10.2             | 74.5                    | 10.01   | 23.42    | 3.51          | 32.57     | 4.42 | 0.54      | 7.55     | 6.14    | 0.77   | 0.64    | 12.3      | 9.22      | 1.76          | 0.62       | 0.43       | 0.21       | 0.07   | 1.42      | 0.65     | 0.77          | 4.25             | 3.93   | 0.33    |    |
|                        |                | $\text{ECO}_2$ | 12.2             | 77.2                    | 9.62    | 23.98    | 3.14          | 35.81     | 3.75 | 0.93      | 7.08     | 6.02    | 0.66   | 0.40    | 11.1      | 7.73      | 2.36          | 0.39       | 0.26       | 0.20       | 0.18   | 1.00      | 0.45     | 0.56          | 3.57             | 3.29   | 0.28    |    |
|                        |                | T+4            | 20.8             | 76.2                    | 11.40   | 26.47    | 2.93          | 30.68     | 3.49 | 1.19      | 6.55     | 5.82    | 0.74   | 0.49    | 12.6      | 9.53      | 1.61          | 0.71       | 0.38       | 0.20       | 0.13   | 1.33      | 0.64     | 0.69          | 3.41             | 3.15   | 0.25    |    |
| CL306                  | T              | $\text{ACO}_2$ | 16.9             | 79.0                    | 9.44    | 22.46    | 4.01          | 39.34     | 3.47 | 0.24      | 7.33     | 6.68    | 0.69   | 0.56    | 8.4       | 4.96      | 2.26          | 0.63       | 0.29       | 0.23       | 0.03   | 0.91      | 0.41     | 0.51          | 3.80             | 3.57   | 0.22    |    |
|                        |                | $\text{ECO}_2$ | 13.3             | 75.5                    | 10.64   | 20.45    | 3.82          | 35.20     | 4.87 | 0.52      | 8.54     | 7.16    | 0.76   | 0.62    | 11.4      | 7.92      | 2.09          | 0.61       | 0.25       | 0.26       | 0.23   | 0.90      | 0.50     | 0.40          | 3.70             | 3.40   | 0.30    |    |
|                        |                | T+4            | 11.4             | 74.7                    | 12.32   | 22.28    | 3.90          | 33.58     | 2.57 | 0.06      | 8.08     | 6.72    | 0.77   | 0.59    | 11.9      | 8.48      | 2.06          | 0.72       | 0.33       | 0.31       | 0.01   | 1.14      | 0.52     | 0.62          | 4.17             | 3.89   | 0.28    |    |
| T3                     | T              | $\text{ACO}_2$ | 14.9             | 76.5                    | 10.25   | 25.12    | 3.64          | 32.61     | 4.00 | 0.89      | 7.70     | 6.19    | 0.86   | 0.65    | 11.6      | 8.31      | 1.99          | 0.59       | 0.49       | 0.16       | 0.11   | 1.01      | 0.44     | 0.56          | 3.14             | 2.88   | 0.26    |    |
|                        |                | $\text{ECO}_2$ | 14.3             | 77.5                    | 10.93   | 18.81    | 3.90          | 39.93     | 3.29 | 0.60      | 7.62     | 6.32    | 0.74   | 0.57    | 10.2      | 7.17      | 1.96          | 0.46       | 0.33       | 0.21       | 0.06   | 1.01      | 0.47     | 0.53          | 3.70             | 3.44   | 0.26    |    |
|                        |                | T+4            | 15.6             | 78.6                    | 7.84    | 20.99    | 3.14          | 42.49     | 3.55 | 0.59      | 6.42     | 5.38    | 0.57   | 0.47    | 10.8      | 8.27      | 1.56          | 0.46       | 0.29       | 0.14       | 0.12   | 1.02      | 0.46     | 0.56          | 3.12             | 2.87   | 0.25    |    |
| VN31                   | T              | $\text{ACO}_2$ | 10.7             | 76.3                    | 12.51   | 20.03    | 3.33          | 35.25     | 4.32 | 0.87      | 7.73     | 6.47    | 0.73   | 0.53    | 10.9      | 7.88      | 2.08          | 0.33       | 0.30       | 0.21       | 0.14   | 1.08      | 0.50     | 0.58          | 3.95             | 3.64   | 0.31    |    |
|                        |                | $\text{ECO}_2$ | 14.4             | 72.7                    | 12.45   | 29.51    | 3.50          | 22.44     | 3.79 | 0.99      | 8.14     | 6.39    | 1.04   | 0.70    | 14.2      | 10.12     | 2.54          | 0.62       | 0.61       | 0.21       | 0.15   | 1.32      | 0.63     | 0.69          | 3.62             | 3.33   | 0.29    |    |
|                        |                | T+4            | 14.4             | 74.8                    | 9.81    | 20.66    | 3.68          | 38.32     | 1.89 | 0.48      | 7.59     | 6.17    | 0.78   | 0.64    | 11.8      | 8.56      | 2.01          | 0.55       | 0.35       | 0.27       | 0.04   | 1.17      | 0.52     | 0.65          | 4.62             | 4.21   | 0.41    |    |
| 1084                   | T              | $\text{ACO}_2$ | 14.6             | 77.7                    | 9.08    | 21.45    | 3.32          | 39.12     | 4.15 | 0.58      | 6.96     | 5.62    | 0.73   | 0.61    | 10.4      | 7.60      | 1.49          | 0.66       | 0.37       | 0.22       | 0.08   | 1.01      | 0.51     | 0.50          | 3.90             | 3.60   | 0.30    |    |
|                        |                | $\text{ECO}_2$ | 13.9             | 78.4                    | 11.31   | 20.25    | 3.08          | 40.22     | 2.90 | 0.61      | 6.71     | 5.70    | 0.58   | 0.43    | 10.6      | 7.86      | 1.68          | 0.45       | 0.23       | 0.21       | 0.15   | 0.92      | 0.45     | 0.47          | 3.42             | 3.19   | 0.23    |    |
|                        |                | T+4            | 19.3             | 77.7                    | 8.91    | 20.69    | 4.09          | 39.61     | 3.90 | 0.53      | 6.89     | 4.76    | 1.29   | 0.84    | 11.0      | 7.38      | 1.92          | 0.56       | 0.72       | 0.29       | 0.09   | 1.17      | 0.56     | 0.62          | 3.24             | 2.97   | 0.28    |    |
| T+4                    | $\text{ACO}_2$ | $\text{ACO}_2$ | 14.6             | 72.6                    | 11.08   | 26.19    | 3.75          | 27.04     | 3.83 | 0.70      | 9.25     | 7.48    | 1.05   | 0.72    | 13.0      | 8.75      | 2.60          | 0.74       | 0.58       | 0.21       | 0.14   | 1.13      | 0.51     | 0.63          | 4.00             | 3.74   | 0.26    |    |
|                        |                | $\text{ECO}_2$ | 16.8             | 76.9                    | 6.45    | 20.32    | 2.82          | 42.83     | 3.62 | 0.88      | 6.68     | 5.59    | 0.59   | 0.50    | 11.0      | 8.20      | 1.55          | 0.57       | 0.34       | 0.14       | 0.20   | 1.13      | 0.52     | 0.61          | 4.26             | 3.75   | 0.31    |    |
|                        |                | T+4            | 8.5              | 73.9                    | 10.95   | 28.15    | 3.63          | 26.18     | 4.20 | 0.80      | 7.87     | 6.72    | 0.66   | 0.48    | 13.3      | 9.91      | 2.30          | 0.50       | 0.28       | 0.24       | 0.13   | 1.24      | 0.67     | 0.57          | 3.64             | 3.37   | 0.27    |    |
| P(Cl)                  | ns             | $\text{ECO}_2$ | 7.1              | 70.7                    | 11.19   | 25.42    | 4.11          | 26.67     | 4.61 | 0.74      | 8.94     | 7.50    | 0.80   | 0.63    | 14.2      | 9.77      | 2.59          | 1.06       | 0.44       | 0.28       | 0.07   | 1.48      | 0.65     | 0.83          | 4.64             | 4.24   | 0.40    |    |
|                        |                |                | ns               | *                       | ns      | *        | ns            | *         | ns   | *         | ns       | *       | ns     | *       | ns        | *         | ns            | *          | ns         | *          | ns     | *         | ns       | *             | ns               | *      | ns      | *  |
| P(T)                   | ns             | $\text{ECO}_2$ | ns               | ns                      | *       | ns       | *             | ns        | *    | ns        | *        | ns      | *      | ns      | *         | ns        | *             | ns         | *          | ns         | *      | ns        | *        | ns            | *                | ns     | *       | ns |
|                        |                |                | ns               | ns                      | *       | ns       | *             | ns        | *    | ns        | *        | ns      | *      | ns      | *         | ns        | *             | ns         | *          | ns         | *      | ns        | *        | ns            | *                | ns     | *       | ns |
| P( $\text{CO}_2$ )     | ns             | $\text{ECO}_2$ | ns               | ns                      | *       | ns       | *             | ns        | *    | ns        | *        | ns      | *      | ns      | *         | ns        | *             | ns         | *          | ns         | *      | ns        | *        | ns            | *                | ns     | *       | ns |
|                        |                |                | ns               | ns                      | *       | ns       | *             | ns        | *    | ns        | *        | ns      | *      | ns      | *         | ns        | *             | ns         | *          | ns         | *      | ns        | *        | ns            | *                | ns     | *       | ns |
| P(Cl x T)              | ns             | $\text{ECO}_2$ | ns               | ns                      | *       | ns       | *             | ns        | *    | ns        | *        | ns      | *      | ns      | *         | ns        | *             | ns         | *          | ns         | *      | ns        | *        | ns            | *                | ns     | *       | ns |
|                        |                |                | ns               | ns                      | *       | ns       | *             | ns        | *    | ns        | *        | ns      | *      | ns      | *         | ns        | *             | ns         | *          | ns         | *      | ns        | *        | ns            | *                | ns     | *       | ns |
| P(Cl x $\text{CO}_2$ ) | ns             | $\text{ECO}_2$ | ns               | ns                      | *       | ns       | *             | ns        | *    | ns        | *        | ns      | *      | ns      | *         | ns        | *             | ns         | *          | ns         | *      | ns        | *        | ns            | *                | ns     | *       | ns |
|                        |                |                | ns               | ns                      | *       | ns       | *             | ns        | *    | ns        | *        | ns      | *      | ns      | *         | ns        | *             | ns         | *          | ns         | *      | ns        | *        | ns            | *                | ns     | *       | ns |
| P(T x $\text{CO}_2$ )  | ns             | $\text{ECO}_2$ | ns               | ns                      | *       | ns       | *             | ns        | *    | ns        | *        | ns      | *      | ns      | *         | ns        | *             | ns         | *          | ns         | *      | ns        | *        | ns            | *                | ns     | *       | ns |
|                        |                |                | ns               | ns                      | *       | ns       | *             | ns        | *    | ns        | *        | ns      | *      | ns      | *         | ns        | *             | ns         | *          | ns         | *      | ns        | *        | ns            | *                | ns     | *       | ns |

Table S1D. Concentration of total free amino acid ( $\mu\text{mol ml}^{-1}$ ) and relative abundance of individual amino acids (%) two weeks after mid-veraison of the five Tempranillo clones grown under four temperature/ $\text{CO}_2$  regimes: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient  $\text{CO}_2$  (ca. 400 ppm,  $\text{ACO}_2$ ) or elevated  $\text{CO}_2$  (700 ppm,  $\text{ECO}_2$ ). Probability values (P) for the main effects of clone,  $P(\text{Cl})$ ;  $\text{CO}_2$ ,  $P(\text{CO}_2)$ ; temperature (T);  $\text{CO}_2$ ,  $P(\text{CO}_2)$ ; and their interactions,  $P(\text{Cl} \times \text{T})$ ,  $P(\text{Cl} \times \text{CO}_2)$  and  $P(\text{T} \times \text{CO}_2)$ . \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns, not significant. All probability values for the interaction  $P(\text{Cl} \times \text{T} \times \text{CO}_2)$  were statistically not significant ( $P > 0.05$ ).

|                                   | Relative abundance (%) |                         |         |          |               |           |      |           |          |         |        |         |           |           |               |            |            |            |        |           |          |               |                  |        |         |
|-----------------------------------|------------------------|-------------------------|---------|----------|---------------|-----------|------|-----------|----------|---------|--------|---------|-----------|-----------|---------------|------------|------------|------------|--------|-----------|----------|---------------|------------------|--------|---------|
|                                   | Total amino acid       | $\alpha$ -ketoglutarate | Proline | Arginine | Glutamic acid | Glutamine | Gaba | Histidine | Pyruvate | Alanine | Valine | Leucine | Aspartate | Threonine | Aspartic acid | Asparagine | Isoleucine | Methionine | Lysine | Shikimate | Tyrosine | Phenylalanine | Phosphoglycerate | Serine | Glycine |
| R143                              | 12.5                   | 74.2                    | 18.52   | 22.16    | 4.22          | 24.29     | 3.75 | 1.30      | 8.39     | 7.11    | 0.82   | 0.46    | 12.8      | 8.93      | 2.57          | 0.48       | 0.26       | 0.31       | 0.31   | 1.08      | 0.72     | 0.36          | 3.44             | 3.19   | 0.25    |
| CL306                             | 18.0                   | 74.7                    | 16.86   | 19.51    | 4.52          | 29.98     | 2.92 | 0.92      | 8.59     | 7.27    | 0.84   | 0.49    | 12.7      | 8.71      | 2.68          | 0.48       | 0.28       | 0.28       | 0.26   | 0.90      | 0.58     | 0.33          | 3.11             | 2.91   | 0.20    |
| T3                                | 14.8                   | 72.9                    | 17.46   | 20.17    | 4.49          | 26.09     | 3.72 | 1.00      | 9.41     | 7.93    | 0.96   | 0.52    | 13.4      | 9.15      | 2.82          | 0.42       | 0.28       | 0.37       | 0.34   | 0.94      | 0.61     | 0.32          | 3.34             | 3.11   | 0.23    |
| VN31                              | 17.6                   | 75.5                    | 15.64   | 19.56    | 4.12          | 31.71     | 3.22 | 1.20      | 7.98     | 6.64    | 0.86   | 0.48    | 12.3      | 8.34      | 2.46          | 0.52       | 0.32       | 0.34       | 0.30   | 0.99      | 0.65     | 0.34          | 3.30             | 3.04   | 0.26    |
| 1084                              | 7.6                    | 70.4                    | 14.03   | 31.80    | 4.99          | 14.89     | 3.32 | 1.32      | 8.43     | 6.90    | 0.90   | 0.64    | 16.3      | 12.55     | 2.26          | 0.67       | 0.40       | 0.33       | 0.12   | 1.68      | 1.07     | 0.60          | 3.21             | 2.94   | 0.27    |
| T                                 | 15.9                   | 73.6                    | 15.24   | 21.03    | 4.19          | 28.95     | 3.26 | 0.94      | 8.60     | 7.20    | 0.86   | 0.54    | 13.4      | 9.42      | 2.59          | 0.52       | 0.30       | 0.32       | 0.25   | 1.07      | 0.66     | 0.41          | 3.32             | 3.09   | 0.23    |
| T+4                               | 12.3                   | 73.5                    | 17.77   | 24.25    | 4.75          | 21.83     | 3.51 | 1.36      | 8.52     | 7.13    | 0.89   | 0.50    | 13.6      | 9.65      | 2.53          | 0.50       | 0.32       | 0.33       | 0.28   | 1.16      | 0.79     | 0.37          | 3.24             | 2.98   | 0.26    |
| $\text{ACO}_2$                    | 17.1                   | 74.3                    | 16.79   | 21.21    | 4.26          | 27.98     | 3.11 | 0.97      | 8.51     | 7.13    | 0.88   | 0.51    | 12.7      | 8.60      | 2.73          | 0.49       | 0.28       | 0.33       | 0.29   | 1.02      | 0.69     | 0.34          | 3.41             | 3.17   | 0.24    |
| $\text{ECO}_2$                    | 11.2                   | 72.7                    | 16.21   | 24.07    | 4.67          | 22.80     | 3.66 | 1.33      | 7.85     | 6.70    | 0.87   | 0.53    | 14.3      | 10.47     | 2.39          | 0.54       | 0.33       | 0.32       | 0.24   | 1.21      | 0.77     | 0.44          | 3.15             | 2.91   | 0.24    |
| R143 T                            | 18.4                   | 76.7                    | 15.25   | 17.33    | 4.21          | 36.00     | 3.17 | 0.78      | 8.61     | 7.21    | 0.71   | 0.44    | 11.1      | 7.70      | 2.33          | 0.40       | 0.27       | 0.27       | 0.19   | 0.92      | 0.49     | 0.43          | 3.36             | 3.13   | 0.23    |
| $\text{ECO}_2$                    | 10.1                   | 73.3                    | 20.80   | 22.75    | 4.50          | 20.09     | 4.43 | 0.75      | 8.57     | 7.28    | 0.78   | 0.50    | 13.6      | 9.73      | 2.60          | 0.39       | 0.24       | 0.26       | 0.33   | 1.07      | 0.74     | 0.33          | 3.50             | 3.25   | 0.25    |
| T+4 $\text{ACO}_2$                | 15.8                   | 74.4                    | 21.96   | 19.53    | 4.93          | 22.95     | 3.89 | 1.15      | 9.01     | 7.75    | 0.82   | 0.43    | 12.4      | 7.61      | 3.55          | 0.38       | 0.22       | 0.22       | 0.43   | 0.70      | 0.60     | 0.09          | 3.47             | 3.22   | 0.25    |
| $\text{ECO}_2$                    | 5.7                    | 72.5                    | 16.06   | 29.04    | 3.24          | 18.13     | 3.51 | 2.53      | 8.13     | 6.70    | 0.97   | 0.46    | 14.3      | 10.67     | 1.79          | 0.75       | 0.32       | 0.47       | 0.29   | 1.64      | 1.05     | 0.58          | 3.43             | 3.17   | 0.26    |
| CL306 T                           | 25.3                   | 74.5                    | 16.97   | 17.96    | 4.82          | 32.11     | 2.11 | 0.47      | 8.76     | 7.29    | 0.91   | 0.55    | 12.5      | 7.90      | 2.96          | 0.70       | 0.31       | 0.32       | 0.32   | 0.80      | 0.56     | 0.24          | 3.47             | 3.29   | 0.18    |
| $\text{ECO}_2$                    | 13.5                   | 73.9                    | 15.13   | 20.07    | 4.04          | 30.82     | 3.10 | 0.75      | 8.89     | 7.61    | 0.76   | 0.52    | 12.9      | 9.41      | 2.45          | 0.48       | 0.27       | 0.25       | 0.09   | 0.98      | 0.55     | 0.43          | 3.28             | 3.06   | 0.22    |
| T+4 $\text{ACO}_2$                | 18.8                   | 75.8                    | 17.96   | 18.90    | 4.64          | 30.21     | 3.08 | 0.98      | 8.07     | 6.88    | 0.77   | 0.42    | 12.3      | 8.21      | 2.92          | 0.33       | 0.23       | 0.30       | 0.29   | 0.94      | 0.62     | 0.33          | 2.93             | 2.74   | 0.19    |
| $\text{ECO}_2$                    | 14.5                   | 74.7                    | 17.39   | 21.12    | 4.58          | 26.77     | 3.37 | 1.49      | 8.66     | 7.28    | 0.90   | 0.47    | 13.0      | 9.33      | 2.38          | 0.40       | 0.31       | 0.24       | 0.33   | 0.88      | 0.58     | 0.30          | 2.76             | 2.54   | 0.22    |
| T3 $\text{ACO}_2$                 | 19.4                   | 74.5                    | 14.82   | 14.41    | 4.52          | 37.05     | 3.07 | 0.62      | 9.60     | 7.69    | 1.33   | 0.58    | 11.7      | 6.83      | 2.84          | 0.48       | 0.21       | 0.62       | 0.71   | 0.73      | 0.52     | 0.22          | 3.47             | 3.31   | 0.16    |
| $\text{ECO}_2$                    | 12.3                   | 71.2                    | 17.64   | 20.72    | 4.78          | 22.99     | 4.19 | 0.91      | 9.75     | 8.41    | 0.82   | 0.51    | 14.7      | 10.23     | 3.28          | 0.48       | 0.25       | 0.27       | 0.22   | 1.05      | 0.65     | 0.40          | 3.26             | 3.04   | 0.22    |
| T+4 $\text{ACO}_2$                | 17.1                   | 75.0                    | 20.44   | 20.28    | 4.15          | 25.63     | 3.22 | 1.27      | 8.94     | 7.65    | 0.80   | 0.49    | 11.6      | 8.04      | 2.48          | 0.35       | 0.26       | 0.30       | 0.15   | 0.96      | 0.58     | 0.37          | 3.55             | 3.24   | 0.31    |
| $\text{ECO}_2$                    | 10.5                   | 71.0                    | 16.96   | 25.26    | 4.51          | 18.68     | 4.38 | 1.21      | 9.33     | 7.95    | 0.87   | 0.51    | 15.6      | 11.50     | 2.70          | 0.35       | 0.42       | 0.30       | 0.30   | 1.00      | 0.70     | 0.31          | 3.09             | 2.85   | 0.25    |
| VN31 T                            | 25.1                   | 75.6                    | 14.42   | 17.26    | 4.27          | 35.95     | 2.92 | 0.82      | 8.01     | 6.87    | 0.72   | 0.42    | 11.7      | 7.82      | 2.63          | 0.45       | 0.24       | 0.33       | 0.20   | 0.86      | 0.52     | 0.34          | 3.82             | 3.50   | 0.32    |
| $\text{ECO}_2$                    | 16.2                   | 77.2                    | 12.92   | 19.70    | 3.77          | 37.06     | 2.80 | 0.95      | 7.19     | 6.01    | 0.70   | 0.47    | 11.8      | 8.47      | 2.04          | 0.56       | 0.30       | 0.27       | 0.14   | 0.97      | 0.56     | 0.41          | 2.87             | 2.65   | 0.22    |
| T+4 $\text{ACO}_2$                | 16.0                   | 74.4                    | 20.36   | 18.94    | 4.91          | 25.21     | 3.85 | 1.14      | 8.93     | 7.59    | 0.91   | 0.44    | 12.4      | 7.63      | 3.37          | 0.42       | 0.24       | 0.29       | 0.49   | 0.77      | 0.68     | 0.09          | 3.47             | 3.18   | 0.29    |
| $\text{ECO}_2$                    | 13.1                   | 74.6                    | 14.87   | 22.33    | 3.54          | 28.63     | 3.30 | 1.89      | 7.80     | 6.08    | 1.11   | 0.60    | 13.2      | 9.42      | 1.80          | 0.66       | 0.49       | 0.48       | 0.37   | 1.36      | 0.85     | 0.50          | 3.06             | 2.83   | 0.23    |
| 1084 T                            | 9.5                    | 71.0                    | 10.82   | 29.51    | 3.23          | 23.06     | 2.76 | 1.64      | 8.33     | 6.67    | 0.98   | 0.68    | 15.8      | 12.06     | 2.05          | 0.74       | 0.52       | 0.30       | 0.12   | 1.76      | 1.13     | 0.64          | 3.11             | 2.84   | 0.26    |
| $\text{ECO}_2$                    | 9.1                    | 68.1                    | 13.60   | 30.62    | 3.72          | 14.39     | 4.06 | 1.73      | 9.06     | 7.51    | 0.88   | 0.68    | 18.2      | 14.02     | 2.70          | 0.56       | 0.39       | 0.28       | 0.22   | 1.55      | 0.93     | 0.62          | 3.11             | 2.85   | 0.26    |
| T+4 $\text{ACO}_2$                | 5.0                    | 71.3                    | 14.93   | 37.98    | 2.94          | 11.63     | 3.00 | 0.86      | 7.65     | 6.19    | 0.84   | 0.63    | 15.7      | 12.18     | 2.15          | 0.64       | 0.33       | 0.40       | 0.04   | 1.79      | 1.16     | 0.63          | 3.50             | 3.24   | 0.25    |
| $\text{ECO}_2$                    | 6.6                    | 70.9                    | 16.79   | 29.09    | 10.06         | 10.48     | 3.47 | 1.04      | 8.70     | 7.24    | 0.90   | 0.56    | 15.6      | 11.93     | 2.13          | 0.76       | 0.36       | 0.35       | 0.09   | 1.60      | 1.08     | 0.52          | 3.13             | 2.82   | 0.31    |
| $P(\text{Cl})$                    | ***                    | ***                     | *       | ***      | ns            | ***       | ns   | ns        | ns       | ns      | ns     | *       | ***       | ***       | ns            | ns         | ns         | ns         | ns     | ***       | ***      | *             | ns               | ns     | ns      |
| $P(\text{T})$                     | *                      | ns                      | **      | *        | ns            | ***       | ns   | **        | ns       | ns      | ns     | ns      | ns        | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | *             | ns               | ns     | ns      |
| $P(\text{CO}_2)$                  | ***                    | *                       | ns      | *        | ns            | **        | *    | ***       | ns       | ns      | ns     | ns      | ***       | ***       | *             | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns               | *      | ns      |
| $P(\text{Cl} \times \text{T})$    | ns                     | ns                      | ns      | ns       | ns            | ns        | ns   | ***       | ns       | ns      | ns     | ns      | ns        | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns               | ns     | ns      |
| $P(\text{Cl} \times \text{CO}_2)$ | ns                     | ns                      | ns      | ns       | ns            | ns        | ns   | ns        | ns       | ns      | ns     | ns      | ns        | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns               | ns     | ns      |
| $P(\text{T} \times \text{CO}_2)$  | ns                     | ns                      | *       | ns       | ns            | ns        | ns   | ns        | ns       | ns      | ns     | ns      | ns        | ns        | ***           | ***        | ns         | ns         | ns     | ns        | ns       | ns            | ns               | ns     | ns      |

Table S1E. Total free amino acid content ( $\mu\text{mol}\cdot\text{ml}^{-1}$ ) and relative abundance of individual amino acids (%) at maturity in berries of the five Tempranillo clones grown under four temperature/ $\text{CO}_2$  regimes: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient  $\text{CO}_2$  (ca. 400 ppm,  $\text{ACO}_2$ ) or elevated  $\text{CO}_2$  (700 ppm,  $\text{ECO}_2$ ). Probability values (P) for the main effects of clone, P(Cl); temperature P(T);  $\text{CO}_2$ , P( $\text{CO}_2$ ); and their interactions, P(Cl x T), P(Cl x  $\text{CO}_2$ ) and P(T x  $\text{CO}_2$ ). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. All probability values for the interaction P(Cl x T x  $\text{CO}_2$ ) were statistically not significant (P > 0.05).

| Clone                 | Temp           | $\text{CO}_2$  | Total amino acid | Relative abundance (%)  |         |          |               |           |      |           |          |         |        |         |           |           |               |            |            |            |        |           |          |               |                  |        |         |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|-----------------------|----------------|----------------|------------------|-------------------------|---------|----------|---------------|-----------|------|-----------|----------|---------|--------|---------|-----------|-----------|---------------|------------|------------|------------|--------|-----------|----------|---------------|------------------|--------|---------|-----------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
|                       |                |                |                  | $\alpha$ -Ketoglutarate | Proline | Arginine | Glutamic acid | Glutamine | Gaba | Histidine | Pyruvate | Alanine | Valine | Leucine | Aspartate | Threonine | Aspartic acid | Asparagine | Isoleucine | Methionine | Lysine | Shikimate | Tyrosine | Phenylalanine | Phosphoglycerate | Serine | Glycine |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| R43                   | T              | $\text{ACO}_2$ | 13.5             | 72.7                    | 24.60   | 25.74    | 2.78          | 15.00     | 1.44 | 3.12      | 6.00     | 4.37    | 0.95   | 0.68    | 15.6      | 12.50     | 1.69          | 0.56       | 0.36       | 0.41       | 0.09   | 2.02      | 1.11     | 0.90          | 3.70             | 3.46   | 0.24    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | $\text{ECO}_2$ | 16.2             | 70.8                    | 24.23   | 24.87    | 3.06          | 14.19     | 1.78 | 2.70      | 7.37     | 5.40    | 1.20   | 0.76    | 16.2      | 11.99     | 2.10          | 0.92       | 0.41       | 0.42       | 0.33   | 1.79      | 1.05     | 0.74          | 3.83             | 3.59   | 0.24    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | T              | 21.0             | 72.3                    | 26.88   | 21.98    | 3.25          | 15.65     | 2.00 | 2.53      | 7.34     | 5.29    | 1.38   | 0.67    | 15.0      | 11.23     | 1.85          | 0.53       | 0.35       | 0.62       | 0.46   | 1.66      | 1.03     | 0.63          | 3.68             | 3.46   | 0.22    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| VN31                  | T              | $\text{ACO}_2$ | 17.9             | 72.0                    | 23.90   | 24.11    | 2.90          | 16.56     | 1.68 | 2.87      | 6.72     | 5.00    | 1.10   | 0.62    | 15.8      | 12.22     | 1.81          | 0.64       | 0.32       | 0.58       | 0.27   | 1.91      | 1.13     | 0.78          | 3.50             | 3.35   | 0.15    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | $\text{ECO}_2$ | 16.4             | 66.4                    | 22.10   | 30.15    | 2.69          | 6.59      | 2.90 | 1.98      | 10.45    | 6.91    | 2.08   | 1.46    | 17.9      | 13.71     | 2.19          | 0.63       | 0.95       | 0.29       | 0.16   | 1.76      | 1.04     | 0.73          | 3.43             | 3.20   | 0.23    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | T              | 19.6             | 71.3                    | 24.73   | 23.84    | 3.16          | 15.15     | 1.95 | 2.49      | 7.68     | 5.60    | 1.27   | 0.81    | 15.7      | 11.82     | 2.07          | 0.72       | 0.46       | 0.41       | 0.25   | 1.74      | 1.04     | 0.70          | 3.54             | 3.34   | 0.20    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| T+4                   | $\text{ACO}_2$ | $\text{ACO}_2$ | 17.3             | 70.3                    | 23.89   | 26.99    | 2.70          | 11.96     | 1.98 | 2.79      | 7.52     | 5.22    | 1.42   | 0.88    | 16.5      | 12.86     | 1.79          | 0.60       | 0.50       | 0.51       | 0.27   | 1.91      | 1.10     | 0.81          | 3.72             | 3.48   | 0.24    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | $\text{ECO}_2$ | 14.3             | 71.5                    | 23.50   | 24.24    | 2.69          | 16.61     | 1.62 | 2.83      | 7.05     | 4.97    | 1.30   | 0.78    | 15.9      | 12.17     | 1.83          | 0.74       | 0.47       | 0.51       | 0.23   | 1.90      | 1.13     | 0.77          | 3.60             | 3.42   | 0.19    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | T              | 16.6             | 70.1                    | 25.16   | 26.64    | 3.19          | 10.33     | 2.33 | 2.44      | 8.17     | 5.87    | 1.39   | 0.91    | 16.3      | 12.52     | 2.04          | 0.57       | 0.49       | 0.41       | 0.30   | 1.75      | 1.01     | 0.74          | 3.65             | 3.40   | 0.25    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| R43                   | T              | $\text{ACO}_2$ | 18.0             | 73.4                    | 25.53   | 20.39    | 3.20          | 20.10     | 1.33 | 2.85      | 6.54     | 4.75    | 1.11   | 0.68    | 14.5      | 11.25     | 1.55          | 0.56       | 0.44       | 0.46       | 0.20   | 1.82      | 1.07     | 0.74          | 3.78             | 3.50   | 0.20    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | $\text{ECO}_2$ | 19.6             | 75.1                    | 26.97   | 26.70    | 3.59          | 13.10     | 2.32 | 2.37      | 6.12     | 4.63    | 0.84   | 0.64    | 14.1      | 10.65     | 2.31          | 0.45       | 0.31       | 0.28       | 0.08   | 1.64      | 0.96     | 0.68          | 3.08             | 2.88   | 0.20    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | T              | 8.8              | 71.5                    | 22.86   | 24.89    | 2.24          | 16.94     | 1.04 | 3.49      | 5.49     | 4.14    | 0.84   | 0.51    | 16.8      | 14.02     | 1.57          | 0.55       | 0.25       | 0.42       | 0.04   | 2.34      | 1.29     | 1.05          | 3.88             | 3.70   | 0.18    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| CL306                 | T              | $\text{ACO}_2$ | 5.8              | 70.2                    | 22.54   | 32.75    | 1.85          | 8.15      | 0.94 | 3.97      | 5.78     | 3.81    | 1.02   | 0.95    | 17.5      | 14.63     | 1.21          | 0.71       | 0.45       | 0.48       | 0.00   | 2.35      | 1.13     | 1.22          | 4.19             | 3.73   | 0.45    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | $\text{ECO}_2$ | 25.2             | 72.1                    | 25.13   | 20.42    | 3.72          | 18.41     | 1.85 | 2.55      | 7.66     | 5.51    | 1.35   | 0.80    | 15.4      | 9.95      | 2.66          | 1.41       | 0.56       | 0.42       | 0.38   | 1.31      | 0.85     | 0.46          | 3.55             | 3.35   | 0.20    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | T              | 15.6             | 70.6                    | 25.96   | 22.44    | 2.88          | 14.68     | 2.28 | 2.40      | 8.24     | 6.41    | 1.21   | 0.62    | 15.5      | 11.36     | 2.25          | 0.72       | 0.33       | 0.38       | 0.47   | 1.74      | 0.96     | 0.78          | 3.87             | 3.66   | 0.21    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| T+4                   | $\text{ACO}_2$ | $\text{ACO}_2$ | 9.5              | 72.3                    | 19.55   | 29.01    | 1.84          | 17.36     | 0.89 | 3.63      | 5.17     | 3.83    | 0.81   | 0.53    | 16.2      | 13.37     | 1.13          | 0.97       | 0.26       | 0.47       | 0.02   | 2.53      | 1.49     | 1.04          | 3.79             | 3.63   | 0.17    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | $\text{ECO}_2$ | 14.5             | 68.3                    | 26.30   | 27.62    | 3.80          | 6.30      | 2.10 | 2.21      | 8.41     | 5.87    | 1.44   | 1.10    | 17.6      | 13.30     | 2.35          | 0.57       | 0.49       | 0.42       | 0.46   | 1.56      | 0.90     | 0.66          | 4.12             | 3.73   | 0.39    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | T              | 29.0             | 74.3                    | 25.84   | 18.39    | 3.44          | 22.79     | 1.67 | 2.15      | 7.08     | 5.32    | 1.08   | 0.67    | 13.6      | 10.20     | 1.98          | 0.55       | 0.42       | 0.39       | 0.10   | 1.55      | 0.98     | 0.57          | 3.44             | 3.26   | 0.18    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| VN31                  | T              | $\text{ACO}_2$ | 21.4             | 73.9                    | 28.90   | 20.75    | 2.05          | 18.28     | 1.19 | 2.70      | 6.25     | 3.65    | 1.95   | 0.66    | 14.5      | 9.99      | 1.41          | 0.59       | 0.27       | 1.26       | 1.00   | 1.71      | 1.02     | 0.69          | 3.64             | 3.45   | 0.19    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | $\text{ECO}_2$ | 19.3             | 70.5                    | 24.65   | 26.71    | 4.09          | 9.58      | 3.08 | 2.37      | 8.42     | 6.42    | 1.26   | 0.73    | 15.9      | 12.12     | 2.18          | 0.52       | 0.38       | 0.37       | 0.35   | 1.45      | 0.89     | 0.56          | 3.72             | 3.44   | 0.28    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | T              | 21.5             | 73.3                    | 24.12   | 22.05    | 2.95          | 20.12     | 1.44 | 2.61      | 6.40     | 4.77    | 1.02   | 0.61    | 14.8      | 11.33     | 1.91          | 0.61       | 0.30       | 0.44       | 0.24   | 1.75      | 1.08     | 0.67          | 3.73             | 3.57   | 0.16    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| T+4                   | $\text{ACO}_2$ | $\text{ACO}_2$ | 14.0             | 72.2                    | 21.29   | 26.66    | 2.67          | 17.03     | 1.53 | 3.04      | 6.31     | 4.50    | 1.10   | 0.70    | 16.0      | 12.35     | 1.53          | 0.81       | 0.25       | 0.69       | 0.41   | 2.12      | 1.22     | 0.90          | 3.42             | 3.28   | 0.13    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | $\text{ECO}_2$ | 12.3             | 71.8                    | 23.22   | 25.12    | 2.19          | 16.55     | 1.24 | 3.51      | 5.49     | 4.02    | 0.94   | 0.53    | 17.0      | 13.73     | 1.47          | 0.76       | 0.36       | 0.59       | 0.05   | 2.31      | 1.31     | 1.00          | 3.42             | 3.28   | 0.13    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | T              | 23.6             | 70.7                    | 26.98   | 22.64    | 3.77          | 12.56     | 2.49 | 2.32      | 8.69     | 6.72    | 1.33   | 0.64    | 15.6      | 11.46     | 2.35          | 0.38       | 0.38       | 0.59       | 0.41   | 1.46      | 0.92     | 0.54          | 3.54             | 3.36   | 0.18    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| 1084                  | T              | $\text{ACO}_2$ | 18.6             | 66.7                    | 22.06   | 29.12    | 3.00          | 7.67      | 2.53 | 2.31      | 10.32    | 7.36    | 1.72   | 1.24    | 17.9      | 13.43     | 2.48          | 0.84       | 0.69       | 0.33       | 0.15   | 1.77      | 1.07     | 0.71          | 3.32             | 3.09   | 0.23    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | $\text{ECO}_2$ | 20.9             | 65.2                    | 22.80   | 29.64    | 2.79          | 5.77      | 2.52 | 1.68      | 10.41    | 6.88    | 2.07   | 1.44    | 19.3      | 14.96     | 2.14          | 0.75       | 0.91       | 0.32       | 0.21   | 1.72      | 1.01     | 0.71          | 3.48             | 3.16   | 0.23    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | T              | 8.6              | 65.8                    | 17.84   | 32.31    | 2.28          | 7.92      | 3.00 | 2.44      | 10.13    | 6.38    | 2.22   | 1.53    | 18.7      | 14.46     | 2.10          | 0.58       | 1.19       | 0.32       | 0.08   | 1.88      | 1.09     | 0.79          | 3.40             | 3.24   | 0.24    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| T+4                   | $\text{ACO}_2$ | $\text{ACO}_2$ | 17.4             | 68.1                    | 25.73   | 29.56    | 2.71          | 5.02      | 3.55 | 1.51      | 10.93    | 6.99    | 2.32   | 1.53    | 15.8      | 12.01     | 2.03          | 0.34       | 0.99       | 0.21       | 0.22   | 1.67      | 0.98     | 0.69          | 3.53             | 3.30   | 0.22    |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | $\text{ECO}_2$ | ns               | ***                     | ns      | *        | ns            | ***       | ns   | ***       | ns       | ***     | ns     | ***     | ns        | **        | ns            | ns         | ***        | ns         | ns     | ns        | ns       | ns            | ns               | ns     | ns      | ns        |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                | P(Cl)          | ns               | ns                      | ns      | *        | ns            | ns        | ns   | ns        | ns       | ns      | ns     | ns      | ns        | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns               | ns     | ns      | ns        |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| P( $\text{CO}_2$ )    | ns             | ns             | ns               | *                       | ns      | ns       | ns            | ***       | ns   | ns        | ns       | ns      | ns     | ns      | ns        | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns               | ns     | ns      |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|                       |                |                |                  |                         |         |          |               |           |      |           |          |         |        |         |           |           |               |            |            |            |        |           |          |               |                  |        |         | P(Cl x T) | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns | ns |
|                       |                |                |                  |                         |         |          |               |           |      |           |          |         |        |         |           |           |               |            |            |            |        |           |          |               |                  |        |         |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| P(T x $\text{CO}_2$ ) | ns             | ns             | ns               | ns                      | ns      | ns       | ns            | ns        | ns   | ns        | ns       | ns      | ns     | ns      | ns        | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            |                  |        |         |           |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

## **CHAPTER 5**

Impact of environmental conditions projected for 2100 on grape primary and secondary metabolites of different Tempranillo clones



# Impact of environmental conditions projected for 2100 on grape primary and secondary metabolites of different Tempranillo clones

## ABSTRACT

The potential impact of climate change on grape berry composition is a great concern among grape growers and oenologists. The exploration of the intra-varietal diversity of grapevine (*Vitis vinifera* L.) can be an interesting approach for the adaptation of varieties in their traditional growing regions. The response of four Tempranillo clones to simulated year-2100-expected air temperature, CO<sub>2</sub> concentration and relative humidity (RH): climate change (CC; 28 °C/18 °C, 700 ppm CO<sub>2</sub>, and 35 %/53 % RH) vs. current situation conditions (CS; 24 °C/14°C, 400 ppm CO<sub>2</sub> and 45 %/63 %); under two irrigation regimes (“well-watered” vs. “water-deficit”) was evaluated, focusing on the evolution of grape quality components (organic acids, sugars, amino acids and anthocyanins), over the ripening period. The treatments were applied to fruit-bearing cuttings from fruit set to maturity in growth chamber greenhouses. CC increased sugar accumulation and hastened grape phenology, but such effect was mitigated by water deficit. Both CC and water deficit modified amino acid concentration and profile with different intensity depending on the clone. When CC and water deficit were applied simultaneously, decreased anthocyanin concentration and the anthocyanin to TSS ratio. The results suggest differences in the response of the clones studied to the environmental conditions projected for 2100, although the responses not always depended on the differences observed in the ripening period.

**Keywords:** Tempranillo; Climate change; Grapevine; Water deficit; Sugars; Organic acids; Amino acids; Anthocyanins

## 1. INTRODUCTION

Nowadays, one of the most worrying environmental issues is the modification of air composition and the consequences that this swift is expected to provoke in the near future. The levels of greenhouse gases (GHG) have increased especially since the industrial revolution and between 2000 and 2010 the GHG emission rates reached the highest values of the last decades [1]. High levels of GHG contribute to the so-called “greenhouse effect” and to global warming. The Intergovernmental Panel on Climate Change (IPCC) considers different scenarios for the coming years. In the worst cases (scenarios RCP 6.0 and RCP 8.5) the IPCC estimates a rise of the global mean temperature between  $2.2 \pm 0.5$  °C and  $3.7 \pm 0.7$  °C and an atmospheric CO<sub>2</sub> concentration between 669.7 and 935.9 ppm for 2100 [2]. Besides, a swift in the precipitation regime around the globe is expected. In some areas, such as the Mediterranean region, medium or long drought periods are forecast to be 3-8 times more frequent than nowadays [3]. In addition, near-surface land relative humidity (RH) is expected to be reduced in coming years as a result of climate change [4]. These environmental changes are expected to severely affect in a severe manner crop performance, due to its great dependency on abiotic factors.

Fruit crops are affected by different environmental factors including air temperature, atmospheric CO<sub>2</sub> and water availability. In the case of grapevine, the research done so far points towards an earlier harvest, mainly associated with the increase in air temperature [5–9]. In addition, changes in grape composition have been reported in response to warm temperatures, with reductions in total must acidity and malic acid concentration [10–12], concomitant with higher sugar accumulation rates [13]. High temperatures are also known to reduce anthocyanin accumulation [10,14,15], as well as to produce an imbalance between anthocyanins and sugars [16]. However, the impact of elevated CO<sub>2</sub> on grape composition has been less studied. Although some authors report a decrease in must acidity and an increase in total soluble solids (TSS) [12], other studies conclude that the expected rise in CO<sub>2</sub> may not cause negative repercussions on the quality of grapes [17,18].

Research on water scarcity effects has determined that drought might provoke a reduction of the final fruit production [19], berry size [20] and organic acid concentration [21], increase in sugars in Cabernet Sauvignon [22], linked to the decline in berry size [23], and the promotion of the anthocyanin biosynthetic route [24]. Furthermore, Deluc et al. reported an impact of water stress on the amino acid content of a red cultivar (Cabernet Sauvignon) but not on a white one (Chardonnay) [25].

Although the literature about the combined effects of these environmental factors is not so extensive, studies suggest that CO<sub>2</sub> does not seem to modify the impact of elevated temperature on malic acid, sugars and anthocyanins [8,9]. Additionally, elevated temperature high CO<sub>2</sub> and drought applied simultaneously reduced malic acid and total polyphenol index, increasing colour density in grapes of Tempranillo [26]. In the case of amino acids, some authors have reported that both drought [19,27] and its combination with high temperature [13] increased amino acid concentration. However, studies in recent literature are scarce to draw a clear picture of the interaction effects of higher temperature, elevated CO<sub>2</sub> and water deficit on the accumulation of grape nutritional components.

Different strategies have been suggested to mitigate the potential negative impact of the projected future environmental conditions on grape composition. Among them, plant material has been proposed as one of the most powerful tools to face with climate change [28]. Clonal diversity within grapevine cultivars has been studied for a broad range of characteristics, including phenological development, probable alcohol and titratable acidity, colour for the red varieties or aroma for the white ones, as well as disease resistance [29]. For example, the use of clones with long ripening periods, thus compensating the shortening of the ripening period produced by warmer conditions [30–32], has been proposed as an adaptive approach to be explored.

The present work is focused on Tempranillo, a variety well-settled in Spain [33], more specifically in La Rioja, País Vasco and Navarra [34]. Despite this cultivar has a large number of clones characterised and commercialised [29], few studies have explored their performance under the projected environmental conditions, including changes in the most important climate change-related factors such as temperature, air CO<sub>2</sub> or water availability. The objective of this research was to evaluate the response of four Tempranillo clones with different reproductive cycle lengths to a combination of high temperature, elevated air CO<sub>2</sub> concentration and low relative humidity, under different water availabilities, focusing on plant phenology and the evolution over the ripening period of must characteristics (organic acids, sugars and amino acids) and skin anthocyanin levels.



## 2. MATERIAL AND METHODS

### 2.1. *Plant material: origin and development*

Four clones of grapevine (*V. vinifera*) cv. Tempranillo were used in the experiment: RJ43, CL306, VN31 and 1084. The plant material was obtained from the germplasm bank of Estación de Viticultura y Enología de Navarra (EVENA, Navarra, Spain), RJ43 and CL306 (the most widely used Tempranillo clones in Spain); Vitis Navarra (Navarra, Spain), VN31; and the Institute of Sciences of Vine and Wine (La Rioja, Spain), 1084 (non-commercialised). These clones were selected on the basis of differences in their reproductive cycle length, previously characterised by the provider organisms as intermediate-reproductive cycle (RJ43) [35], short-reproductive cycle (CL306) [36,37]; and long-reproductive cycle (both VN31 and 1084) [38,39].

The selection, activation and growth of 400-500 mm-long dormant cuttings were carried out according to an adapted protocol from Mullins and Rajasekaran [40] described in detail in Arrizabalaga et al. and Morales et al. [38,41]. Only a single inflorescence per plant was allowed to grow and the irrigation throughout all the experiment was done with the nutritive solution described in Ollat et al. [42].

### 2.2. *Experimental design*

At fruit set, plants of the four clones with similar phenological stage and bunch size characteristics were divided homogeneously and placed in growth chamber-greenhouses (GCGs) [41] set at different temperature, CO<sub>2</sub> concentration and relative humidity (T/CO<sub>2</sub>/RH) regimes: 24 °C/14 °C (day/night), 400 ppm of CO<sub>2</sub> and RH of 45 %/63 % (day/night) (Current Situation conditions, CS) vs. 28 °C/18 °C (day/night), 700 ppm of CO<sub>2</sub> and RH of 35 %/53 % (day/night) (Climate Change conditions, CC). Within each greenhouse, plants were subjected to two irrigation regimes: well-watered (WW) *versus* water deficit (WD, receiving 60% the water applied to the WW plants). IPCC predictions were considered for setting the temperature and CO<sub>2</sub> conditions of the CC treatment [2] while the RH conditions were set according to ENSEMBLES models developed based on the MPI-ECHAM5 Max Planck Institute model and IPCC data. According to those models, in 2100 the RH during summer for the area of Navarra and La Rioja will be 12 % lower [43]. The WD treatment was determined according to the expected conditions predicted by the model of the Max Planck Institute for the North of Spain at the end of the present century, referring a summer precipitation 40 % lower [43]. Soil water content was monitored with soil moisture sensors (EC-5 Soil Moisture Sensors, Decagon Devices Inc., Pullman, WA, USA). Plants under WD treatment were watered when the correspondent sensor marked a soil humidity lower than 10 % (m<sup>3</sup> H<sub>2</sub>O x 100 m<sup>-3</sup> substrate) [26]. Then, they received 60 % of the equivalent volume of solution used for watering WW plants during the corresponding drought period. In the case of WW

plants, moisture levels were kept at ca. 80-90 % of the substrate field capacity (sensor value between 30-40 %,  $\text{m}^3 \text{H}_2\text{O} \times 100 \text{m}^{-3}$  substrate). Pre-dawn leaf water potential was measured at mid-veraison and 2 weeks after mid-veraison using a pressure chamber SKYE SKPM 1400, Skye Instruments Ltd, Llandrindod, Wales, UK) and according to the methodology described by Scholander et al. [44]. Average values are included in Table S1. Plants of all the treatments received the same amount of nutrients watering them by combining nutrient solution and plain water. Each clone was represented with among 7 and 8 plants per treatment.

### *2.3. Phenological development*

The dates of fruit set, mid-veraison (half of the berries in the bunch had started to change colour) and maturity (total soluble solid content, TSS, of ca. 22 °Brix) were annotated for each plant individually, making it possible to calculate the elapsed time between fruit set and mid-veraison, and between mid-veraison and maturity. The determination of both fruit set and mid-veraison was accomplished visually. Maturity was determined by measuring periodically the levels of TSS of two berries per bunch during the last weeks of development (every 2 or 3 days) until they reached a TSS level of ca. 22 °Brix.

### *2.4. Sample collection*

Berries were harvested at mid-veraison (defined in section 2.3), 1 week after mid-veraison, 2 weeks after mid-veraison and maturity (defined in section 2.3), frozen and stored at -80 °C. The number of berries per bunch sampled was either 3 or 4, except at maturity, when 10 berries per bunch were taken. Berry volume was estimated by measuring the diameters of 3 berries per bunch (10 berries at maturity) and applying the formula of the volume of a spheroid ( $Volume = \frac{4}{3} \pi \times r_1^2 \times r_2$ ; being  $r_1$  the equatorial radio and  $r_2$  the polar radio).

For carrying out the analysis, pools of berries taken from two or three different plants (3-4 berries per plant) were prepared and berries were handled according to an adapted protocol from Bobeica et al. [45] and Torres et al. [13]. Berries were weighed and the skin, pulp and seeds were separated. The skin and seeds were weighed and the relative skin mass determined using the quotient between skin fresh weight (FW) and berry FW, expressed as a percentage. Frozen pulp was ground using an MM200 ball grinder (Retsch, Haan, Germany). The skin was ground with a MM200 ball grinder (Retsch, Haan, Germany) after freeze drying (Alph1-4, CHRIST, Osterode, Germany) to carry out the anthocyanin analysis.

## 2.5. *Sugars, organic acids and amino acids profiles*

Primary metabolites in berries were extracted according to an adapted protocol from Torres et al. [13]. 250 mg of frozen powdered pulp were extracted with decreasing concentrations of ethanol (80 %, 50 % and 0 % ethanol (v/v)), dried using a Speed-Vac [SAVANT SC 110A, Thermo Fisher Scientific, Waltham, MA USA] and resuspended in ultrapure water. The obtained extracts were used for the analysis of sugars, organic acids and amino acids.

Sugar analysis was carried out according to the manufacturer, by measuring enzymatically both the glucose and fructose concentration with an automated absorbance microplate reader (Elx800UV, Biotek Instruments Inc., Winooski, VT, USA) using the Glucose/Fructose kit from BioSenTec (Toulouse, France). The results were presented as the sum of glucose and fructose and referred to as total sugars.

Malic and tartaric acid were analysed with automated colorimetric methods using a Bran and Luebbe TRAACS 800 autoanalyser (Bran & Luebbe, Plaisir, France) as previously described by Pereira et al. [46]. Malic acid determination was based on the detection of NADH at 340 nm, formed by the reduction of NAD<sup>+</sup> during the enzymatic conversion of L-malate to oxaloacetate by L-malate dehydrogenase (L-MDH). Tartaric acid determination was based on a colorimetric method with ammonium vanadate reactions. The results were presented as the concentration of malic acid and the sum of malic and tartaric acid (referred to as total acidity according to Iland et al. [47]).

For the amino acid determination at maturity, samples were derivatized with 6-aminoquinolyl-N-hydroxy-succinimidyl-carbamate (AccQ-Tag derivatization reagent, Waters, Milford, MA, USA) according to Hilbert et al. [48] with minor modifications, and analysed with an UltiMate 3000 UHPLC system (Thermo Electron SAS, Waltham, MA USA) equipped with an FLD-3000 Fluorescence Detector (Thermo Electron SAS, Waltham, MA USA). As described by Cohen and Michaud [49] and with modifications of Habran et al. [50], free amino acid separation was achieved by using a AccQ•Tag Ultra column, 2.1 × 100 mm, 1.7 μm (Waters, Milford, MA, USA) at 37 °C with elution at 0.5 ml min<sup>-1</sup> (eluent A, sodium acetate buffer, 140 mM at pH 5.7; eluent B, acetonitrile; eluent C, water). Chromatographic analyses were carried out using an excitation wavelength of 250 nm and an emission wavelength of 395 nm. The identification and quantification of nineteen amino acids (excluding tryptophan) was done as described by Pereira et al. [46]. In order to keep a stable baseline and a consistent retention time over the analysis, a control analysis was carried out as described in Torres et al. [13].

## 2.6. Total anthocyanins

Anthocyanin concentration in berry skins was determined according to Torres et al. and using the analysis described by Acevedo De la Cruz et al. and Hilbert et al. [13,51,52]. Briefly, ground dried skins were extracted with methanol containing 0.1 % HCl (v/v) and the obtained solution was filtered using a polypropylene syringe filter of 0.45 µm (Pall Gelman Corp., Ann Arbor, USA). The separation of the different compounds was carried out with a Synchronis C18, 2.1 × 100 mm, 1.7 µm Column (Thermo Fisher Scientific, Waltham, MA USA) meanwhile they were analysed with an UltiMate 3000 UHPLC system (Thermo Electron SAS, Waltham, MA USA) equipped with DAD-3000 diode array detector (Thermo Electron SAS, Waltham, MA USA). The chromatographic analysis was done with a detection wavelength of 520 nm and malvidin-3-O-glucoside as the standard sample (Extrasynthese, Genay, France). The obtained chromatograms were analysed with the Chromeleon software (version 7.1) (Thermo Electron SAS, Waltham, MA USA) in order to calculate the peak area of each of them. The concentration of total anthocyanins was calculated as the sum of the concentration of the individual anthocyanins determined.

## 2.7. Statistical analysis

The statistical analysis carried out for each parameter consisted in a three-way ANOVA (clone, T/CO<sub>2</sub>/RH regime and irrigation regime) and a Fisher's least significant difference (LSD) as a post-hoc test when statistically significant differences were found (P < 0.05). The analyses were done using the software R (3.5.3).

# 3. RESULTS

## 3.1. Phenological development

The length of the period between fruit set and mid-veraison and, especially, between mid-veraison and maturity was significantly different among clones, the 1084 accession having the longest ripening period (mid-veraison to maturity) (Figure 1A). Considering the clones altogether, CC significantly reduced the number of days to reach mid-veraison compared with CS plants, but it did not affect the length between mid-veraison and maturity (Figure 1B). Also, WD slowed down, in general, the phenological development. However, a significant interaction between clone and irrigation regime was observed for the elapsed time between mid-veraison and maturity. In this way, the 1084 accession was much less affected by drought than the other clones (Figure 1A).

### 3.2. *Berry volume and relative skin mass*

The volume of the grapes differed among clones from mid-veraison onwards, the 1084 and RJ43 accessions showing the biggest berries and VN31 the smallest at maturity (Table 1). CC significantly reduced berry volume at mid-veraison, but this effect disappeared during the rest of the ripening period. Drought reduced significantly berry volume at every developmental stage considered. A significant interaction among clone, T/CO<sub>2</sub>/RH regime and irrigation was observed at maturity for berry volume. Notably, the RJ43 accession was very affected by drought, especially under CC conditions, while the impact of WD on CL306 and VN31 was similar under CC and CS conditions. Also, berry volume of 1084 was strongly affected by drought under CS but not under CC conditions (Table 1).

In general terms, WD significantly increased the grape relative skin mass mid-veraison, 1 week after mid-veraison and at maturity, and CC conditions had a similar effect 2 weeks after mid-veraison and at maturity (Table 2). However, at maturity, a significant interaction between clone and T/CO<sub>2</sub>/RH regime, as well as between clone and irrigation regime was observed, which was reflected in the significant reduction in the relative skin mass of the 1084 grapes when CC and WD were combined, contrary to what happened in the rest of the clones. At maturity, the 1084 accession showed the lowest relative skin mass values, meanwhile RJ43 and CL306 presented the highest, this effect being more evident under CC and WD conditions

### 3.3. *Malic acid and total acidity*

Malic acid concentration and total acidity (sum of malic and tartaric acid) in the must decreased throughout the ripening process and differed among clones (Figures 2A and 2B). The 1084 accession had significantly lower malic acid levels compared with the rest of the clones at mid-veraison and maturity, regardless of the T/CO<sub>2</sub>/RH and irrigation regimes (Figure 2A), whereas VN31 had lower total acidity than the rest of the clones at mid-veraison, 2 weeks after mid-veraison and at maturity (Figure 2B). When analysed the clones altogether, grapes grown under CC conditions showed a higher malic acid concentration and total acidity at mid-veraison, compared with CS. However, in later stages these levels dropped faster in the CC treatment, reaching lower values than in CS plants both 2 weeks after mid-veraison and at maturity (Figure 2A and 2B). WD did not affect significantly the concentration of malic acid in grapes. However, total acidity at maturity was, in general terms, lower in WD plants compared with WW plants (Figure 2B and 2C), due to a significant reduction in tartaric acid from  $4.42 \pm 0.14 \text{ mg g}^{-1}$  pulp FW to  $3.97 \pm 0.13 \text{ mg g}^{-1}$  pulp FW ( $P < 0.001$ , data not shown). At maturity, total acidity of grapes ripened under CC/WD was lower than in grapes ripened at CS/WW (Figure 2C).

### 3.4. Sugars

The concentration of total sugars (sum of glucose and fructose) was lower in the berries of the 1084 accession, from 1 week after mid-veraison onwards, compared with the other clones (Figure 3A). Considering the clones altogether, in relation to CS, CC significantly increased the sugar concentration 1 and 2 weeks after mid-veraison (from  $72.73 \pm 2.96$  mg g<sup>-1</sup> pulp FW to  $89.03 \pm 3.70$  and from  $142.20 \pm 4.28$  to  $162.89 \pm 4.22$  mg g<sup>-1</sup> pulp FW, respectively) (Figure 3A). WD reduced sugar levels during all the ripening process compared with WW plants. One week after mid-veraison there was a significant interaction between the T/CO<sub>2</sub>/RH and irrigation regimes, the plants grown under combined CC and WW conditions showing the highest sugar values at this stage (Figure 3A). Total sugar concentration 2 weeks after mid-veraison was similar in plants grown at CS/WW and CC/WD (Figure 3B).

### 3.5. Amino Acids

The concentration of total amino acids at maturity was significantly different among clones, the 1084 having the lowest values (Figure 4A). As the significant interaction among factors indicates, the effect of CC depended on the water availability in a different manner depending on the clone, while WD raised amino acid concentration differently among clones. Thus, CC tended to reduce the amino acid levels relative to CS conditions in the RJ43, VN31 and 1084 accessions regardless of water availability, and especially in VN31 plants grown at WD conditions. Conversely, in the case of CL306, amino acid concentration in grape significantly increased in plants grown under CC/WD conditions in comparison to CS/WW (Figure 4A). Finally, the 1084 accession was the one least affected by the T/CO<sub>2</sub>/RH regime and water availability.

The amino acid profile at maturity also varied among clones and treatments (Figure 4B, Table S2). Amino acids originated from  $\alpha$ -ketoglutarate were the most abundant in every case, but its proportion in 1084 was significantly lower than in the other clones, mainly because of the relatively low glutamine content. Aspartate derivatives were the second most abundant group in all the clones, wherein threonine and asparagine had the highest relative abundance. The 1084 accession showed a significantly higher relative abundance of aspartate derivatives compared with other accessions. Pyruvate, phosphoglycerate and shikimate derivatives were the least abundant groups and their proportions were similar among the clones studied. The relative abundance of aspartate derivatives significantly increased with WD, especially in the 1084 accession (Figure 4B, Table S2). In contrast, WD reduced the relative abundance of shikimate and phosphoglycerate derivatives (except for 1084 accession under CC conditions), this effect being more evident in the CL306 accession. A significant interaction between clone and irrigation regime was observed for the relative abundance of  $\alpha$ -

ketoglutarate derivatives, as it was reduced by WD in 1084, in opposition to VN31, where WD tended to increase it (Figure 4B, Table S2). CC conditions increased the proportion of valine, leucine, isoleucine, tyrosine and serine, and reduced the relative abundance of asparagine and histidine, the latter under WW conditions in RJ43, CL306 and under both WW and WD in VN31 and 1084 (Table S2).

### *3.6. Total Anthocyanins and anthocyanin to TSS ratio*

Clones showed differences in their anthocyanin levels only at maturity, the 1084 accession having the lowest concentration (Figure 5A and B). CC conditions increased the levels of anthocyanins at mid-veraison and 1 week after mid-veraison. Grapes ripened under drought conditions had lower levels of anthocyanins 2 weeks after mid-veraison (Figure 5A). At maturity, there was an interaction between the CO<sub>2</sub>/T/RH and irrigation regime as, considering the clones altogether, CC significantly reduced the anthocyanin levels when combined with WD conditions but not with WW (Figure 5C).

Regarding the relationship between anthocyanins and TSS at maturity, RJ43 and VN31 had the highest ratios and 1084 the lowest ones (Figure 6A). Considering the clones altogether, the CC/WD treatment significantly reduced the anthocyanin to TSS ratio with respect to the CS/WW treatment (Figure 6B). When studied independently for each clone, this reduction was more evident in RJ43 and less marked in other clones such as VN31 or 1084 (Figure 6C).

## **4. DISCUSSION**

In the future, changes in grape composition are expected in the Mediterranean area, as a result of one or more stress factors related to climate change. In the present study, the response of four Tempranillo clones to the foreseen T/CO<sub>2</sub>/RH conditions by the end of the present century, combined or not with water deficit, was studied, focusing on the evolution of grape components throughout the ripening period.

Tartaric and malic acid are the principal organic acids of grape berry and represent the most significant influences on the acidity and pH of the juice [47]. Organic acids (especially malic acid) are degraded along the ripening period, thus decreasing their concentration up to maturity [53,54]. In the present study, the degradation of malic acid was enhanced by CC conditions; consequently, the levels of malic acid and total acidity were lower in CC compared to CS at maturity. The results agree with previous studies in red Tempranillo that report a lower concentration of malic acid in berries ripened under combined high temperature and elevated CO<sub>2</sub> [6,8,26]. A higher malate export rate from the vacuole to the cytoplasm and altered expression and activity of enzymes involved in malate catabolism have been described as responsible for the enhancement of malate degradation under high temperature

[11,55]. Among the clones studied, 1084 was the accession that showed the lowest levels of malic acid at maturity. Rather than a higher sensitivity of this clone to the projected environmental conditions assayed, this result might be a consequence of the longer ripening period of this accession, since phenology is the first source of genetic variation of grape acidity at harvest [56].

Decrease in malic acid in response to a limitation in water supply has been described on various grapevine cultivars [57]. Some authors justify this decrease by respiration due to an increase of cluster temperature, caused by a reduced vegetative growth under drought conditions [58]. In the present study, however, we did not observe a significant effect of drought on malic acid concentration, as was also reported by Berdeja et al. [59]. The controlled environmental conditions within the greenhouse, which minimised differences in cluster temperature between WW and WD plants grown under the same T/CO<sub>2</sub>/RH regime, may explain the lack of differences in malic acid between irrigation regimes. In contrast, total acidity measured at maturity was lower in the plants subjected to WD, due to a significant reduction in tartaric acid. Although tartaric acid is less sensitive to climatic conditions during ripening [56], some authors have reported a reduction in tartaric acid levels in grapes of cv. Tempranillo ripened under water deficit conditions and ambient temperature [26].

The phenology of grape development has been described as a process highly dependent on environmental factors, especially on temperature [6,60]. In the present study, the results suggest a higher impact of the CC treatment on the period before veraison, shortening the elapsed time between fruit set and mid-veraison. In contrast, WD had a stronger effect after mid-veraison, slowing down ripening. Therefore, the results indicate that WD compensated the impact of CC on grape sugar accumulation and phenological development. Although mild water deficit has proven to enhance ripening through several processes, such as altering plant abscisic acid signalling, reduction in berry size or concentrating berry sugar content [25,60–62], severe water deficit can induce stomatal closure, thus limiting carbon fixation and extending berry ripening [6,63]. In our case, the lower sugar concentration observed from 1 week after mid-veraison onwards in the grapes subjected to WD suggests a lower sugar accumulation rate, probably associated with a limited photosynthetic activity under these conditions. Clones showed differences in their phenological development; however, differences between mid-veraison and maturity were greater than between fruit set and mid-veraison, in a similar way that seen in previous experiments considering several Tempranillo clones [39].

The effect of CC/WD treatment on berry volume varied among clones, being 1084 and VN31 the least affected when comparing the volume of plants grown at CS/WW with the volume of plants grown at CC/WD. However, big berries are not desirable at viticulture, being the small size an interesting trait



[64–66]. CC modified the relative skin mass, increasing it. This means that berry skins were thicker in the CC than in CS treatment, even though 1084 relative skin mass was reduced by CC.

In grapes, nitrogen is present in inorganic (ammonium and ammonium salts) and organic (proteins and amino acids) forms. Together with ammonia, free amino acids (except proline and hydroxyproline) are components of the yeast assimilable nitrogen (YAN), thus having important implication for must fermentation [67]. In this sense, the lower levels of total amino acids in the 1084 accession may involve a lower N availability during the fermentation process. Regarding the impact of environmental factors, the concentration of total free amino acids decreased under CC, considering the clones altogether. This result agrees with Martínez-Lüscher et al., who reported a reduction in  $\alpha$ -amino nitrogen in grapes of Tempranillo developed under high temperature combined with elevated CO<sub>2</sub> [9]. Decreases in amino acid concentration have also been reported in wheat plant exposed to elevated CO<sub>2</sub> [68], while it increased in basil and peppermint plants under elevated CO<sub>2</sub> [69]. Studies of Torres et al. and Sweetman et al. on the effect of high temperature applied as a single factor show an increase in the amino acid content in grapes [11,13]. However, Torres et al. performed the anthocyanins analysis on the skin, and, in the study of Sweetman et al., the temperatures assayed were much more extreme than in the present work (35 °C/28 °C day/night as high temperature treatment compared with 28 °C/18 °C of the present study). These differences in the methodology may explain the contradictory results.

It should be noted that the response of total amino acids to CC conditions depended on the clone studied as well as on the irrigation regime, as indicates the significant interaction among the three factors. In particular, the combination of CC and WD conditions increased the amino acid concentration in RJ43 and CL306, two of the most widely distributed Tempranillo clones, but did not modified de levels in VN31 and 1084. Disparity of responses to drought have already seen among different grapevine cultivars [25,70], but the present results also suggest variability in the response of amino acids to changes in the T/CO<sub>2</sub>/RH regime and water availability among clones within the same cultivar.

Qualitative differences in the grape amino acid profile were noticeable at maturity among clones, with the 1084 accession having a higher relative abundance of aspartate derivatives at the expense of  $\alpha$ -ketoglutarate derivatives. Even though the relative abundance of some individual amino acids was affected by CC conditions, the proportions of amino acid families according to their precursor were not modified. These results may indicate that the T/CO<sub>2</sub>/RH regime did not impact the relative accumulation of precursors but it affected later stages of the biosynthetic route. WD had a more marked impact on amino acid profile than CC conditions. Despite relative abundance of proline was

reduced under WD conditions, its concentration increased, matching its osmoprotector role [71] and other authors' observations [25,72]. Moreover, WD effect was in some cases dependent on the clone. The reduction in the relative abundance of shikimate and phosphoglycerate derivatives, for example, was more evident in CL306 and less marked in 1084. This amplitude of the response of Tempranillo genotypes to the projected environmental conditions may have implications in the organoleptic properties of the wine produced from these grapes, since the shikimate route is responsible for the biosynthesis of aromatic amino acids (tyrosine and phenylalanine) [73] and the precursor of the phenylpropanoid pathway (phenylalanine) [74].

Together with sugars and acids, phenolic compounds (anthocyanins among them) are the most abundant constituents present in grapes. In red varieties, anthocyanins play an essential role in the grape and wine colour [60]. The lower values in anthocyanin concentration, as well as in the anthocyanin to TSS ratio in the berries of 1084, reveal the existence of intra-varietal diversity among Tempranillo clones for this trait and agree with previous studies [39]. The decrease in the accumulation of anthocyanins in grapes due to high temperature has been widely described and it has been associated to a reduction in anthocyanin biosynthesis, through the inhibition of mRNA transcription of the biosynthetic genes, as well as to an enhancement of anthocyanin degradation [75]. Also, elevated temperature has been shown to decouple berry sensory traits such as the accumulation of anthocyanins and sugars, thus decreasing the anthocyanins to sugars ratio [16]. Conversely, high CO<sub>2</sub> applied as a single environmental factor did not affect total anthocyanins in cv. Touriga Franca [17]. In our study, the combination of elevated temperature and elevated CO<sub>2</sub> did not have a great impact either on anthocyanin concentration or on the anthocyanins to TSS ratio when applied under WW conditions. Decreases occurred when the CO<sub>2</sub>/T/RH conditions foreseen for 2100 were combined with WD. The results suggest that the effect of CC conditions on anthocyanin levels as well as on the anthocyanins to TSS ratio may be more intense in a future with low water availability. Similarly, Zarrouk et al., in one of the two years of a field experiment, observed no differences in the anthocyanin content of east- and west-exposed berries (temperature 5 °C - 9 °C higher in the west-exposed berries) in the treatment with low water deficit [76]. However, under most severe water stress, anthocyanin levels decreased in the west-exposed berries. In the same line, Kizildeniz et al. reported no consistent decreases in anthocyanins and anthocyanins to sugars ratio in response to elevated temperature, but decreases in both parameters in response to water stress [12]. In general, a moderate water stress is reported to increase anthocyanins, through the up regulation of genes involved in their biosynthetic pathway [25,62]. However, when a certain threshold of water stress is surpassed, up to a threshold, anthocyanin concentration can be negatively affected [12,26,76–78]. Such negative impact of water stress on anthocyanins can result from the repression of biosynthesis at the onset of ripening and from

degradation at later stages [76]. Regarding the performance of the clones, the RJ43 and CL306 clones studied, come of the most widely cultivated Tempranillo accessions, were the most affected by CC/WD conditions. In contrast, the VN31 clone sustained relatively high anthocyanin concentration with low effects in anthocyanin to TSS ratio.

## **5. CONCLUSION**

Considering the environmental conditions projected for 2100, the results point towards a swift in grape berry composition that might affect wine quality. Climate change conditions (elevated temperature, elevated CO<sub>2</sub> and reduce RH) hastened grape sugar accumulation and consequently advanced maturity, whereas water deficit compensated such effects slowing-down the ripening period and sugar accumulation. The impact of climate change on grape amino acids depended on the irrigation level and on the clone, the 1084 accession being less affected. Water deficit was the factor that the most impacted amino acid profile, decreasing shikimate and phosphoglycerate derivatives at the expense of aspartate derivatives, with different intensity depending on the clone studied. The combination of climate change and water deficit reduced the anthocyanin concentration as well as the anthocyanins to TSS ratio. The results suggest differences in the response of the clones studied to the future expected environmental conditions projected for 2100, not always being necessarily associated with differences in the length of their reproductive period. this study also adds information that can be useful in order to design adaptive strategies in the vineyard.

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## TABLES

Table 1. Grape berry volume of the *Vitis vinifera* cv. Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation (“CS”; 24 °C/14 °C, 400 ppm and 45 %/65 % RH) and climate change (“CC”; 28 °C/18 °C, 700 ppm and 33 %/53 % RH), combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Results (values are means ± SE) are shown according to the clone identity (n = 12-16), T/CO<sub>2</sub>/RH regime (n = 29-30), irrigation regime (n = 29-30) and the three factors together (n = 3-4). Means with letters in common within the same stage and factor (clone, T/CO<sub>2</sub>/RH, irrigation regime, or their interaction) are not significantly different (P > 0.05) according to LSD test. Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I), P(T/CO<sub>2</sub>/RH x I) and P(CL x T/CO<sub>2</sub>/RH x I).\*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant.

|                                   |    |         | Berry volume (ml) |                           |                            |                  |
|-----------------------------------|----|---------|-------------------|---------------------------|----------------------------|------------------|
|                                   |    |         | Mid-veraison      | 1 week after mid-veraison | 2 weeks after mid-veraison | Maturity         |
| RJ43                              |    |         | 0.68 ± 0.03 b     | 0.80 ± 0.04 b             | 0.87 ± 0.03 a              | 0.97 ± 0.04 ab   |
| CL306                             |    |         | 0.60 ± 0.05 c     | 0.76 ± 0.04 bc            | 0.85 ± 0.04 ab             | 0.93 ± 0.04 b    |
| VN31                              |    |         | 0.62 ± 0.03 c     | 0.71 ± 0.03 c             | 0.78 ± 0.03 b              | 0.86 ± 0.03 c    |
| 1084                              |    |         | 0.83 ± 0.04 a     | 0.87 ± 0.04 a             | 0.92 ± 0.05 a              | 1.00 ± 0.04 a    |
| CS                                |    |         | 0.75 ± 0.03 a     | 0.80 ± 0.03 a             | 0.86 ± 0.02 a              | 0.93 ± 0.03 a    |
| CC                                |    |         | 0.62 ± 0.03 b     | 0.77 ± 0.03 a             | 0.84 ± 0.03 a              | 0.95 ± 0.03 a    |
| Water                             |    |         | 0.78 ± 0.02 a     | 0.90 ± 0.02 a             | 0.95 ± 0.02 a              | 1.02 ± 0.02 a    |
| Drought                           |    |         | 0.59 ± 0.02 b     | 0.67 ± 0.02 b             | 0.75 ± 0.02 b              | 0.86 ± 0.02 b    |
| RJ43                              | CS | Water   | 0.81 ± 0.04 bc    | 0.96 ± 0.06 a             | 0.92 ± 0.03 abcd           | 1.06 ± 0.05 ab   |
|                                   |    | Drought | 0.71 ± 0.03 cd    | 0.73 ± 0.04 def           | 0.84 ± 0.04 cdef           | 0.93 ± 0.03 cdef |
|                                   | CC | Water   | 0.72 ± 0.01 cd    | 0.92 ± 0.03 ab            | 1.01 ± 0.03 a              | 1.12 ± 0.04 a    |
|                                   |    | Drought | 0.49 ± 0.02 gh    | 0.59 ± 0.02 g             | 0.71 ± 0.04 efg            | 0.79 ± 0.02 gh   |
| CL306                             | CS | Water   | 0.81 ± 0.04 bc    | 0.88 ± 0.04 abc           | 0.99 ± 0.05 abc            | 1.11 ± 0.09 a    |
|                                   |    | Drought | 0.57 ± 0.06 efg   | 0.68 ± 0.03 efg           | 0.80 ± 0.02 defg           | 0.89 ± 0.03 defg |
|                                   | CC | Water   | 0.62 ± 0.01 def   | 0.86 ± 0.01 abcd          | 0.92 ± 0.06 abcd           | 0.95 ± 0.01 bcde |
|                                   |    | Drought | 0.41 ± 0.03 h     | 0.61 ± 0.03 fg            | 0.68 ± 0.01 fg             | 0.76 ± 0.05 h    |
| VN31                              | CS | Water   | 0.75 ± 0.05 bc    | 0.82 ± 0.04 bcd           | 0.87 ± 0.02 abcd           | 0.90 ± 0.02 defg |
|                                   |    | Drought | 0.59 ± 0.02 efg   | 0.61 ± 0.03 fg            | 0.70 ± 0.02 efg            | 0.75 ± 0.02 h    |
|                                   | CC | Water   | 0.64 ± 0.06 de    | 0.80 ± 0.05 bcde          | 0.89 ± 0.05 abcd           | 0.97 ± 0.05 bcd  |
|                                   |    | Drought | 0.51 ± 0.03 fgh   | 0.62 ± 0.06 fg            | 0.66 ± 0.07 g              | 0.83 ± 0.03 efg  |
| 1084                              | CS | Water   | 1.00 ± 0.02 a     | 0.99 ± 0.04 a             | 1.00 ± 0.02 ab             | 1.03 ± 0.06 abc  |
|                                   |    | Drought | 0.73 ± 0.10 bcd   | 0.73 ± 0.09 defg          | 0.77 ± 0.10 defg           | 0.81 ± 0.05 fgh  |
|                                   | CC | Water   | 0.85 ± 0.05 b     | 0.95 ± 0.03 a             | 1.01 ± 0.07 ab             | 1.07 ± 0.03 ab   |
|                                   |    | Drought | 0.71 ± 0.03 cd    | 0.77 ± 0.07 cde           | 0.85 ± 0.13 bcde           | 1.06 ± 0.06 ab   |
| P(CL)                             |    |         | ***               | **                        | *                          | ***              |
| P(T/CO <sub>2</sub> /RH)          |    |         | ***               | ns                        | ns                         | ns               |
| P(I)                              |    |         | ***               | ***                       | ***                        | ***              |
| P(CL x T/CO <sub>2</sub> /RH)     |    |         | ns                | ns                        | ns                         | ***              |
| P(CL x I)                         |    |         | ns                | ns                        | ns                         | ns               |
| P(T/CO <sub>2</sub> /RH x I)      |    |         | ns                | ns                        | ns                         | ns               |
| P(CL x T/CO <sub>2</sub> /RH x I) |    |         | ns                | ns                        | ns                         | *                |

Table 2. Relative skin mass (%) of the *Vitis vinifera* cv. Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation (“CS”; 24 °C/14 °C, 400 ppm and 45 %/65 % RH) and climate change (“CC”; 28 °C/18 °C, 700 ppm and 33 %/53 % RH), combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Results (values are means ± SE) are shown according to the clone identity (n = 12-16), T/CO<sub>2</sub>/RH regime (n = 29-30), irrigation regime (n = 29-30) and the three factors together (n = 3-4). Means with letters in common within the same stage and factor (clone, T/CO<sub>2</sub>/RH, irrigation regime, or their interaction) are not significantly different (P > 0.05) according to LSD test. Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I) and P(T/CO<sub>2</sub>/RH x I). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. Interaction of all factors P(CL x T/CO<sub>2</sub>/RH x I) was statistically not significant (P > 0.05).

|                               |    |         |                   | Relative skin mass (%) |                           |                            |                 |
|-------------------------------|----|---------|-------------------|------------------------|---------------------------|----------------------------|-----------------|
|                               |    |         |                   | Mid-veraison           | 1 week after mid-veraison | 2 weeks after mid-veraison | Maturity        |
| RJ43                          |    |         |                   | 11.16 ± 0.23 a         | 10.14 ± 0.24 a            | 9.22 ± 0.26 a              | 11.86 ± 0.45 a  |
| CL306                         |    |         |                   | 11.05 ± 0.29 a         | 9.72 ± 0.23 a             | 8.74 ± 0.18 ab             | 11.61 ± 0.37 ab |
| VN31                          |    |         |                   | 11.21 ± 0.23 a         | 10.01 ± 0.14 a            | 8.71 ± 0.27 ab             | 11.05 ± 0.21 bc |
| 1084                          |    |         |                   | 10.49 ± 0.41 a         | 9.90 ± 0.41 a             | 8.35 ± 0.24 b              | 10.36 ± 0.33 c  |
| CS                            |    |         |                   | 11.00 ± 0.21 a         | 9.85 ± 0.24 a             | 8.43 ± 0.17 b              | 10.86 ± 0.17 b  |
| CC                            |    |         |                   | 10.96 ± 0.22 a         | 10.06 ± 0.13 a            | 9.09 ± 0.18 a              | 11.55 ± 0.32 a  |
| Water                         |    |         |                   | 10.42 ± 0.13 b         | 9.64 ± 0.14 b             | 8.89 ± 0.18 a              | 10.88 ± 0.15 b  |
| Drought                       |    |         |                   | 11.55 ± 0.23 a         | 10.28 ± 0.22 a            | 8.63 ± 0.19 a              | 11.55 ± 0.34 a  |
| RJ43                          | CS | Water   | 10.89 ± 0.31 d    | 9.77 ± 0.39 b          | 8.61 ± 0.54 cd            | 10.65 ± 0.26 cd            |                 |
|                               |    | Drought | 11.37 ± 0.56 abc  | 10.55 ± 0.63 ab        | 8.28 ± 0.20 d             | 11.69 ± 0.80 bcd           |                 |
|                               | CC | Water   | 10.50 ± 0.17 d    | 9.58 ± 0.23 ab         | 9.77 ± 0.39 abcd          | 11.04 ± 0.40 cd            |                 |
|                               |    | Drought | 11.86 ± 0.55 abcd | 10.67 ± 0.49 a         | 10.21 ± 0.28 cd           | 14.07 ± 0.89 a             |                 |
| CL306                         | CS | Water   | 10.08 ± 0.33 cd   | 9.33 ± 0.25 ab         | 9.07 ± 0.29 abcd          | 10.57 ± 0.29 cd            |                 |
|                               |    | Drought | 11.25 ± 0.65 abcd | 9.59 ± 0.62 ab         | 8.63 ± 0.53 bcd           | 10.76 ± 0.30 cd            |                 |
|                               | CC | Water   | 10.73 ± 0.14 abcd | 9.29 ± 0.30 ab         | 8.40 ± 0.26 bcd           | 11.91 ± 0.72 bcd           |                 |
|                               |    | Drought | 12.16 ± 0.39 a    | 10.66 ± 0.12 a         | 8.85 ± 0.41 abcd          | 13.19 ± 0.10 ab            |                 |
| VN31                          | CS | Water   | 10.93 ± 0.18 abcd | 10.20 ± 0.43 ab        | 8.74 ± 0.78 bcd           | 10.39 ± 0.33 d             |                 |
|                               |    | Drought | 11.84 ± 0.53 abcd | 10.26 ± 0.22 a         | 8.17 ± 0.39 cd            | 11.10 ± 0.21 cd            |                 |
|                               | CC | Water   | 10.36 ± 0.33 bcd  | 9.84 ± 0.25 ab         | 9.20 ± 0.31 ab            | 10.76 ± 0.48 cd            |                 |
|                               |    | Drought | 11.69 ± 0.37 ab   | 9.74 ± 0.20 a          | 8.73 ± 0.69 a             | 11.97 ± 0.20 bc            |                 |
| 1084                          | CS | Water   | 9.90 ± 0.19 abcd  | 8.75 ± 0.42 ab         | 8.28 ± 0.47 bcd           | 10.91 ± 0.44 cd            |                 |
|                               |    | Drought | 11.75 ± 1.37 ab   | 10.27 ± 1.93 ab        | 7.64 ± 0.21 cd            | 10.65 ± 0.81 cd            |                 |
|                               | CC | Water   | 9.99 ± 0.67 bcd   | 10.22 ± 0.34 ab        | 8.94 ± 0.62 abc           | 10.95 ± 0.33 cd            |                 |
|                               |    | Drought | 10.64 ± 0.94 abc  | 10.45 ± 0.39 ab        | 8.36 ± 0.34 bcd           | 8.98 ± 0.63 e              |                 |
| P(CL)                         |    |         |                   | ns                     | ns                        | ns                         | ***             |
| P(T/CO <sub>2</sub> /RH)      |    |         |                   | ns                     | ns                        | *                          | **              |
| P(I)                          |    |         |                   | ***                    | *                         | ns                         | *               |
| P(CL x T/CO <sub>2</sub> /RH) |    |         |                   | ns                     | ns                        | ns                         | **              |
| P(CL x I)                     |    |         |                   | ns                     | ns                        | ns                         | ***             |
| P(T/CO <sub>2</sub> /RH x I)  |    |         |                   | ns                     | ns                        | ns                         | ns              |

**FIGURES**

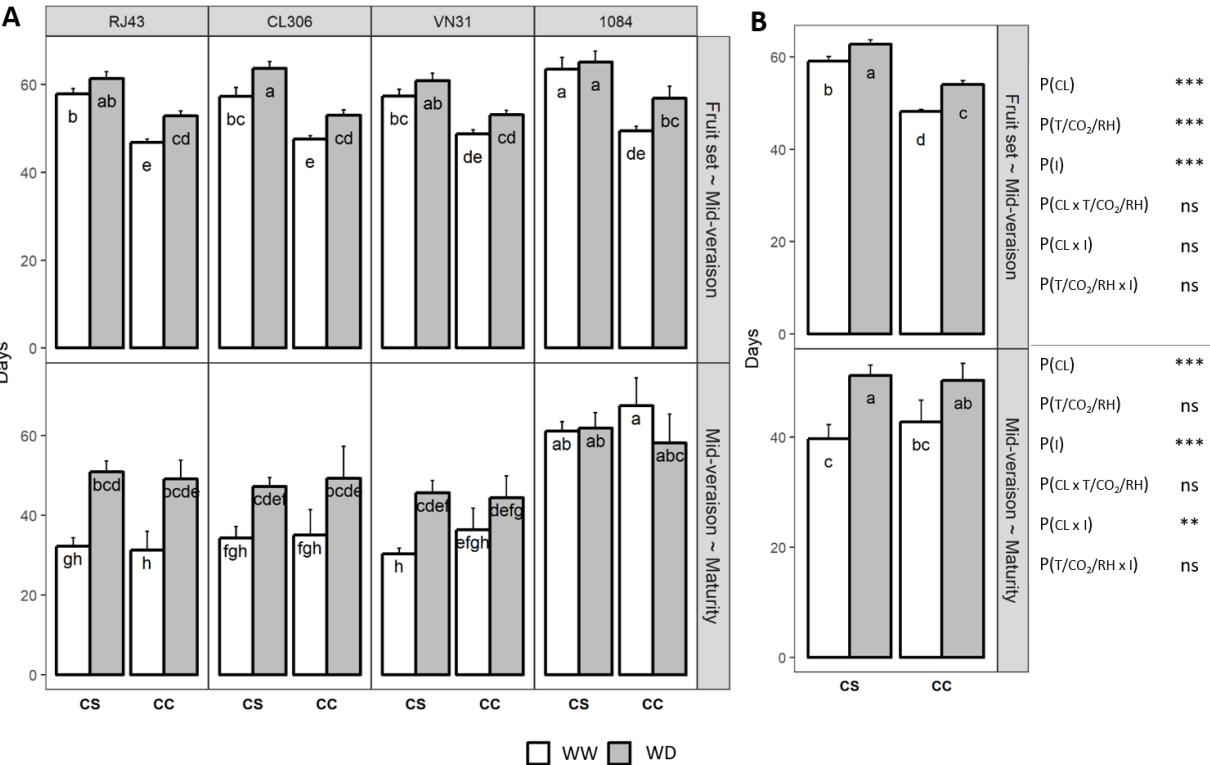


Figure 1. Elapsed time between fruitset and mid-veraison and between mid-veraison and maturity of Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation (“CS”; 24 °C/14 °C, 400 ppm and 45 %/65 % RH) and climate change (“CC”; 28 °C/18 °C, 700 ppm and 33 %/53 % RH) combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Data (values are means ± SE) are represented according to the T/CO<sub>2</sub>/RH and irrigation regimes (A) considering each clone individually (n = 6-8) and (B) considering all the clones as a whole (n = 28-31). Means with letters in common within the same chart (A or B) and parameter are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I) and P(T/CO<sub>2</sub>/RH x I). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. Interaction of all factors P(CL x T/CO<sub>2</sub>/RH x I) was statistically not significant (P > 0.05).

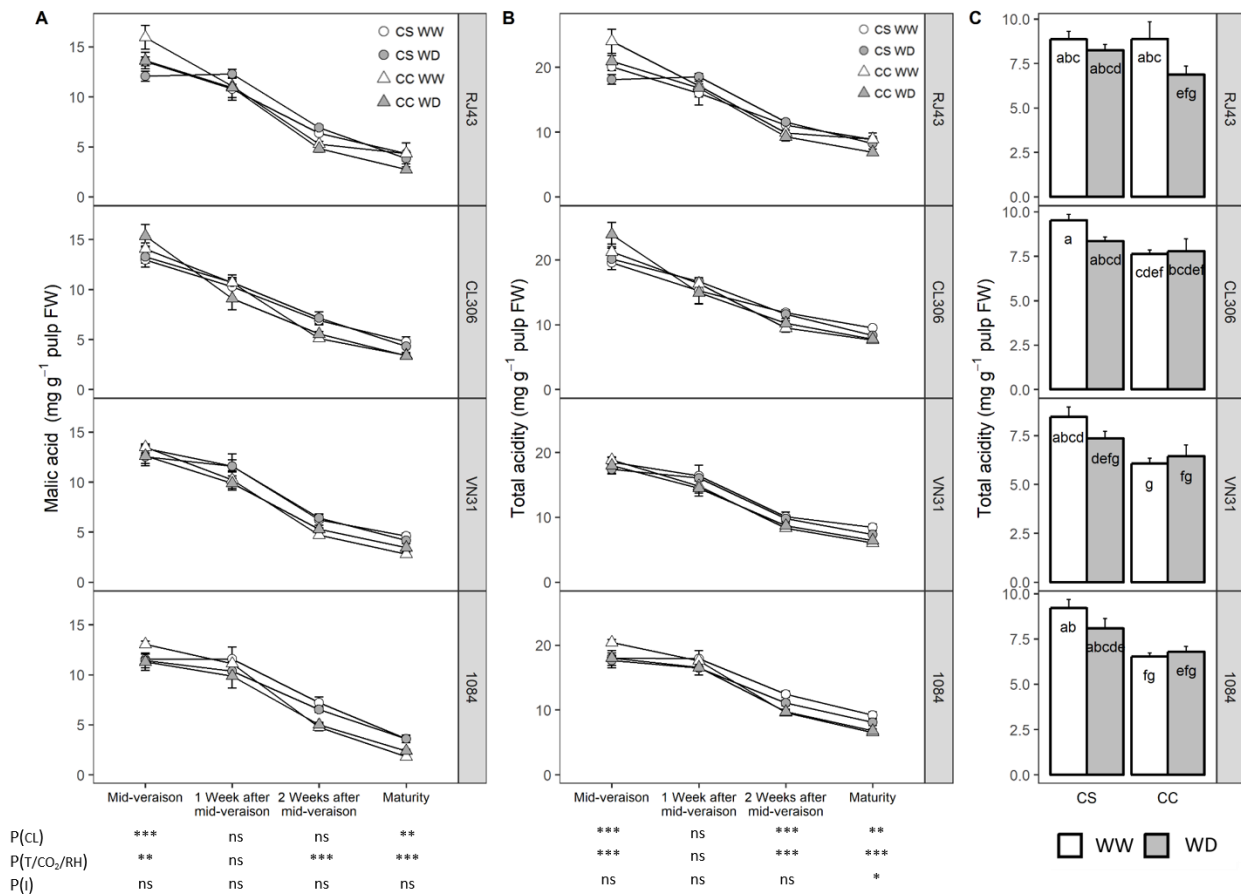


Figure 2. Evolution of the concentration of malic acid (A) and total acidity (B), and detail of total acidity at maturity (C) of Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation ("CS"; 24 °C/14 °C, 400 ppm and 45 %/65 % RH) and climate change ("CC"; 28 °C/18 °C, 700 ppm and 33 %/53 % RH) combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Results (values are means ± SE) are represented according to the T/CO<sub>2</sub>/RH and irrigation regimes and considering clones independently (n = 3-4). Means with letters in common are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); and irrigation regime, P(I); and their interactions. \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. All probability values for the interactions of factors (P(CL x T), P(CL x CO<sub>2</sub>), P(T x CO<sub>2</sub>) and P(CL x T x CO<sub>2</sub>)) were statistically not significant (P > 0.05).

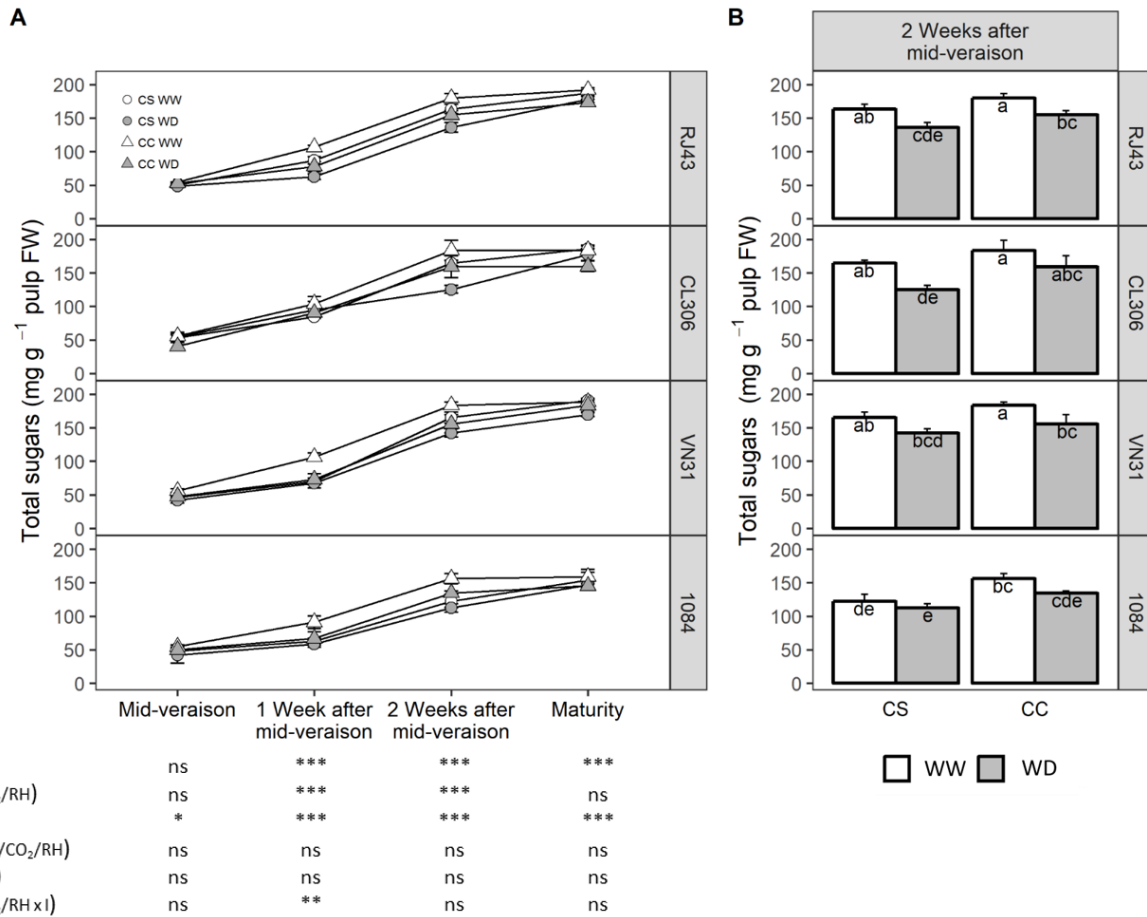


Figure 3. Evolution of the concentration of total sugars (sum of glucose and fructose) in berries (A) and detail of sugar concentration 2 weeks after mid-veraison (B) of Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation (“CS”; 24 °C/14 °C, 400 ppm and 45 %/65 % RH) and climate change (“CC”; 28 °C/18 °C, 700 ppm and 33 %/53 % RH) combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Results (values are means ± SE) are represented according to the T/CO<sub>2</sub>/RH and irrigation regimes, considering each clone individually (n = 3-4). Means with letters in common are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I) and P(T/CO<sub>2</sub>/RH x I).\*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. Interaction of all factors P(CL x T/CO<sub>2</sub>/RH x I) was statistically not significant (P > 0.05).

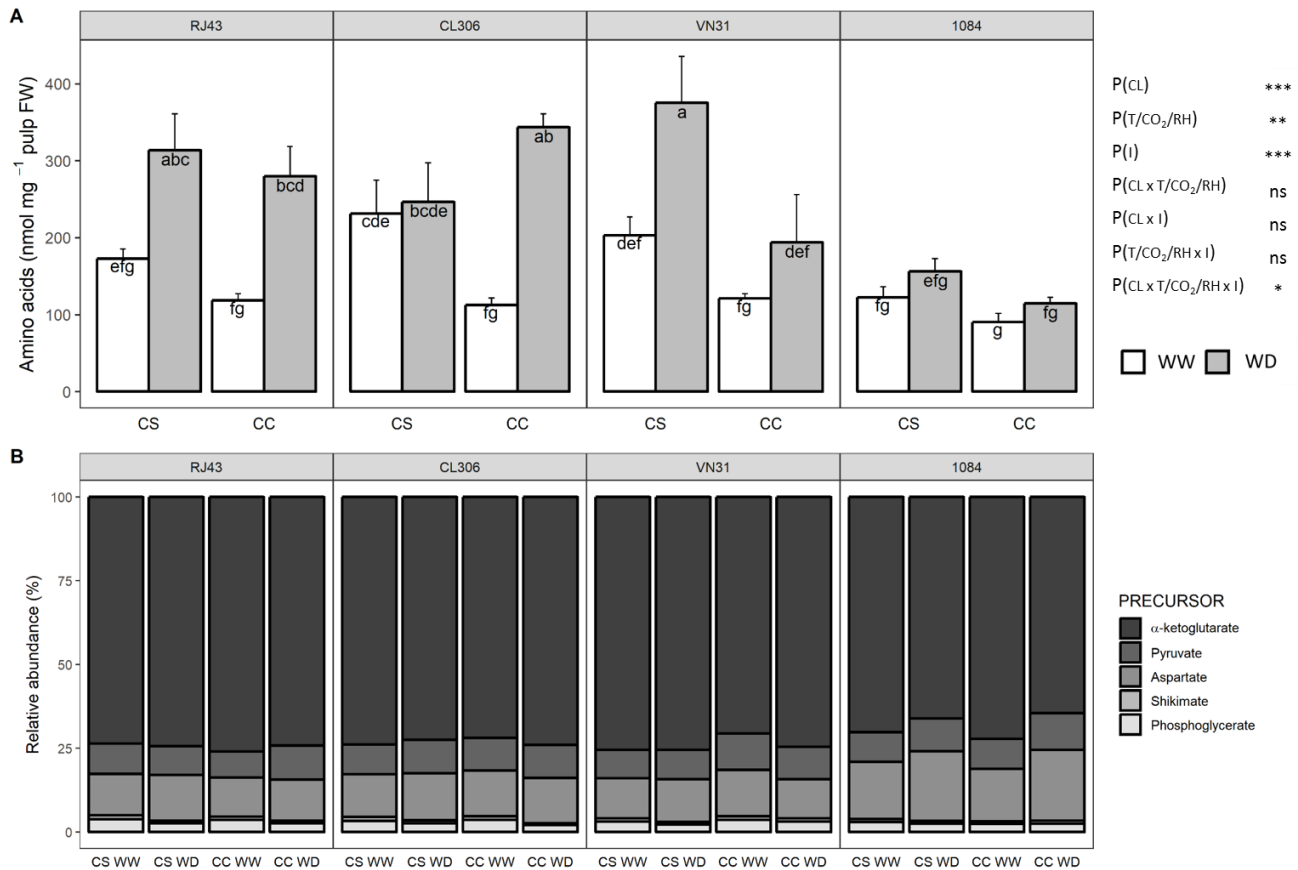


Figure 4. Total concentration of grape amino acids at maturity (A) and relative abundance of amino acids grouped according to their precursor (B) in Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation (“CS”; 24 °C/14 °C, 400 ppm, and 45 %/65 % RH) and climate change (“CC”; 28 °C/18 °C, 700 ppm, and 33 %/53 % RH) combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Results (values are means ± SE) are represented according to the T/CO<sub>2</sub>/RH and irrigation regimes, considering each clone individually (n = 3-4). In chart A, means with letters in common are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I), P(T/CO<sub>2</sub>/RH x I) and P(CL x T/CO<sub>2</sub>/RH x I).\*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant.

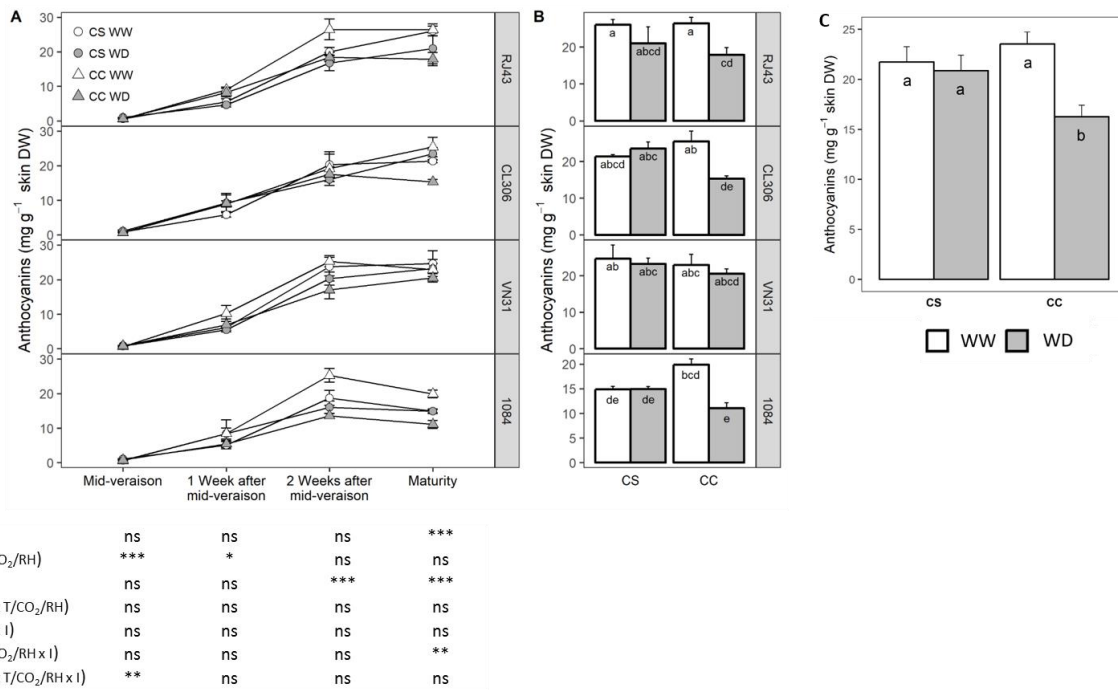


Figure 5. Total skin anthocyanins of *Vitis vinifera* cv. Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation (“CS”; 24 °C/14 °C, 400 ppm and 45 %/65 % RH) and climate change (“CC”; 28 °C/18 °C, 700 ppm and 33 %/53 % RH) combined with two irrigation regimes: well-watered (WW) and water deficit (WD) Results (values are means ± SE) are represented according to the T/CO<sub>2</sub>/RH and irrigation regimes, considering each clone individually (n = 3-4), (A) during ripening evolution and (B) at maturity. Figure C shows total skin anthocyanins at maturity considering clones altogether (n = 14-15). Means with letters in common within the same chart (B or C) are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I), P(T/CO<sub>2</sub>/RH x I) and P(CL x T/CO<sub>2</sub>/RH x I). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant.



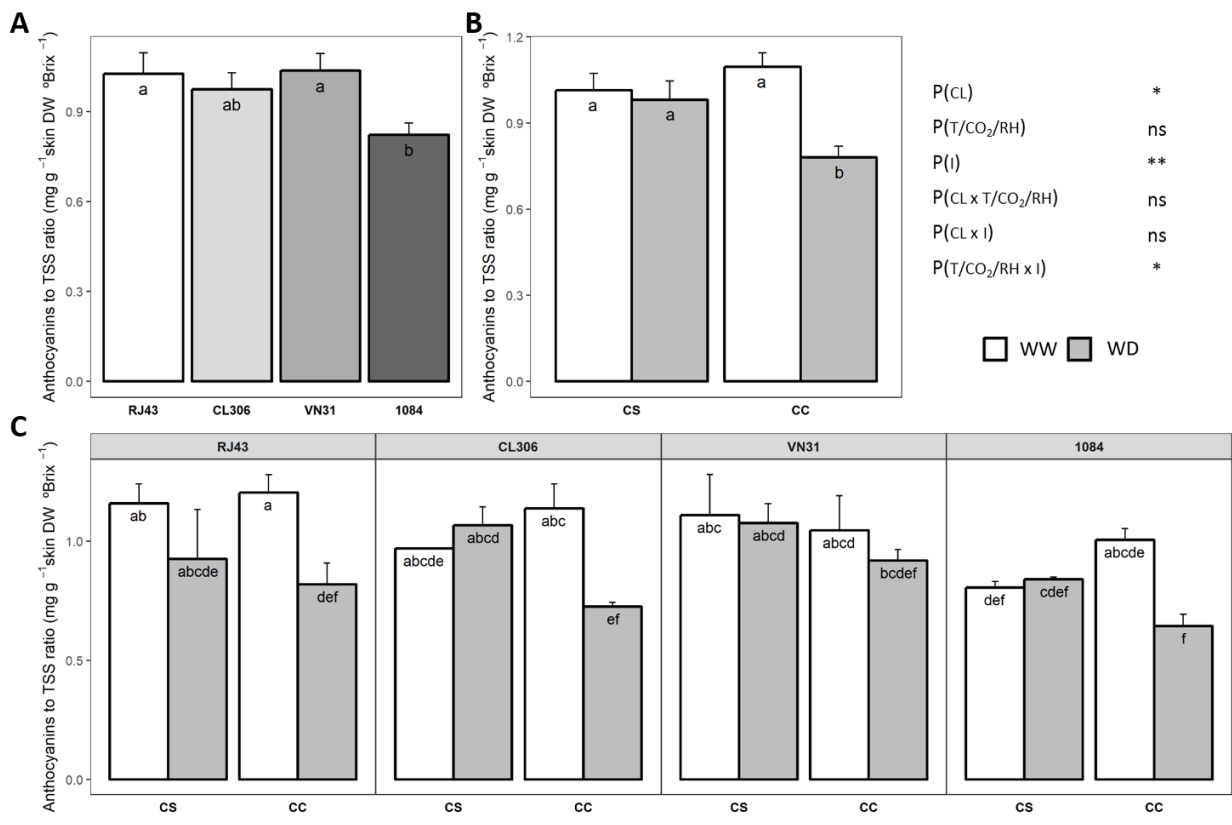


Figure 6. Anthocyanin to TSS ratio at maturity of Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation (“CS”; 24 °C/14 °C, 400 ppm, and 45 %/65 % RH) and climate change (“CC”; 28 °C/18 °C, 700 ppm, and 33 %/53 % RH) combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Results (values are means ± SE) are represented according to: (A) the clone identity (n = 12-16), (B) the T/CO<sub>2</sub>/RH and irrigation regimes (n = 14-15) and (C) the three factors together (n = 3-4). Means with letters in common within the same chart (A, B or C) are not significantly different according to LSD test (P > 0.05). Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I) and P(T/CO<sub>2</sub>/RH x I).\*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. Interaction of all factors P(CL x T/CO<sub>2</sub>/RH x I) was statistically not significant (P > 0.05).

## SUPPLEMENTARY MATERIAL

Table S1. Pre-dawn leaf water potential at mid-veraison and 2 weeks after mid-veraison of the *Vitis vinifera* L. cv. Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation (“CS”; 24 °C/14 °C, 400 ppm, and 45 %/65% RH) and climate change (“CC”; 28 °C/18 °C, 700 ppm, and 33 %/53% RH), combined with two irrigation regimes: well-watered (WW) and water deficit (WD). Results (values are means ± SE) are represented according to the T/CO<sub>2</sub>/RH and irrigation regimes (n = 14-19). Means with letters in common within the same stage are not significantly different (P > 0.05) according to LSD test. Probability values (P) for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I) and P(T/CO<sub>2</sub>/RH x I).\*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant. Interaction of all factors P(CL x T/CO<sub>2</sub>/RH x I) was statistically not significant (P > 0.05).

|                               | Pre-dawn water potential (MPa) |   |      |                            |       |   |      |   |
|-------------------------------|--------------------------------|---|------|----------------------------|-------|---|------|---|
|                               | Mid-veraison                   |   |      | 2 weeks after mid-veraison |       |   |      |   |
| CS-Water                      | -0.63                          | ± | 0.03 | a                          | -0.59 | ± | 0.03 | a |
| CS-Drought                    | -1.48                          | ± | 0.08 | c                          | -1.48 | ± | 0.09 | b |
| CC-Water                      | -0.77                          | ± | 0.06 | a                          | -0.71 | ± | 0.04 | a |
| CC-Drought                    | -1.28                          | ± | 0.07 | b                          | -1.37 | ± | 0.07 | b |
| P(CL)                         | ns                             |   |      | ns                         |       |   |      |   |
| P(T/CO <sub>2</sub> /RH)      | ns                             |   |      | ns                         |       |   |      |   |
| P(I)                          | ***                            |   |      | ***                        |       |   |      |   |
| P(CL x T/CO <sub>2</sub> /RH) | ns                             |   |      | ns                         |       |   |      |   |
| P(CL x I)                     | ns                             |   |      | ns                         |       |   |      |   |
| P(T/CO <sub>2</sub> /RH x I)  | **                             |   |      | ns                         |       |   |      |   |

Table S2. Total free amino acid content (nmol·mg<sup>-1</sup> pulp FW) and relative abundance of individual amino acids (%) at maturity in berries of the *Vitis vinifera* cv. Tempranillo clones grown under two T/CO<sub>2</sub>/RH conditions: current situation ("CS"; 24 °C/14 °C, 400 ppm and 45%/65% RH) and water deficit (WD). Probability values (P) for the main effects of clone, P(Cl); T/CO<sub>2</sub>/RH regime, P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(Cl x T/CO<sub>2</sub>/RH), P(Cl x I), P(T/CO<sub>2</sub>/RH x I) and P(Cl x T/CO<sub>2</sub>/RH x I). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant.

|                                   | Total amino acid | Relative abundance (%) |         |          |               |           |      |           |          |         |        |         |           |           |               |            |            |            |        |           |          |               |                  |        |         |
|-----------------------------------|------------------|------------------------|---------|----------|---------------|-----------|------|-----------|----------|---------|--------|---------|-----------|-----------|---------------|------------|------------|------------|--------|-----------|----------|---------------|------------------|--------|---------|
|                                   |                  | Ketoglutarate          | Proline | Arginine | Glutamic acid | Glutamine | Gaba | Histidine | Pyruvate | Alanine | Valine | Leucine | Aspartate | Threonine | Aspartic acid | Asparagine | Isoleucine | Methionine | Lysine | Shikimate | Tyrosine | Phenylalanine | Phosphoglycerate | Serine | Glycine |
| R43                               | 221.1            | 74.5                   | 30.61   | 21.63    | 2.93          | 15.13     | 3.65 | 0.57      | 8.90     | 7.17    | 1.00   | 0.72    | 12.5      | 7.53      | 2.19          | 2.07       | 0.43       | 0.17       | 0.10   | 0.93      | 0.47     | 0.46          | 3.16             | 2.66   | 0.50    |
| CL306                             | 233.4            | 73.1                   | 30.26   | 16.85    | 3.30          | 18.96     | 2.97 | 0.71      | 9.63     | 7.22    | 1.31   | 1.11    | 13.5      | 7.18      | 2.73          | 2.50       | 0.75       | 0.25       | 0.06   | 0.92      | 0.45     | 0.46          | 2.92             | 2.61   | 0.31    |
| VN31                              | 223.1            | 74.0                   | 34.48   | 13.92    | 3.29          | 18.98     | 2.89 | 0.47      | 9.42     | 7.50    | 1.10   | 0.82    | 12.6      | 7.21      | 2.51          | 2.03       | 0.51       | 0.20       | 0.10   | 0.96      | 0.50     | 0.47          | 3.02             | 2.74   | 0.28    |
| 1084                              | 118.5            | 68.4                   | 35.42   | 21.87    | 2.10          | 5.01      | 3.40 | 0.61      | 9.60     | 7.17    | 1.21   | 1.22    | 18.5      | 10.09     | 2.31          | 5.13       | 0.65       | 0.19       | 0.15   | 0.82      | 0.50     | 0.32          | 2.64             | 2.17   | 0.47    |
| CS                                | 229.2            | 72.9                   | 31.12   | 18.07    | 2.94          | 16.65     | 3.35 | 0.77      | 8.98     | 7.16    | 0.99   | 0.83    | 14.3      | 7.75      | 2.44          | 3.32       | 0.49       | 0.19       | 0.10   | 0.93      | 0.45     | 0.49          | 2.91             | 2.32   | 0.59    |
| CC                                | 167.9            | 72.2                   | 34.45   | 19.17    | 2.84          | 12.19     | 3.14 | 0.41      | 9.74     | 7.38    | 1.29   | 1.07    | 14.2      | 8.29      | 2.39          | 2.54       | 0.66       | 0.21       | 0.11   | 0.88      | 0.51     | 0.37          | 2.97             | 2.77   | 0.21    |
| Water                             | 144.7            | 73.0                   | 35.85   | 20.27    | 2.99          | 9.79      | 3.73 | 0.37      | 9.05     | 7.40    | 0.89   | 0.76    | 13.9      | 8.44      | 2.20          | 2.47       | 0.33       | 0.11       | 0.10   | 1.02      | 0.55     | 0.47          | 3.30             | 2.85   | 0.45    |
| Drought                           | 253.3            | 72.1                   | 29.67   | 16.94    | 2.79          | 19.13     | 2.74 | 0.80      | 9.70     | 7.12    | 1.41   | 1.16    | 14.9      | 7.60      | 2.64          | 3.39       | 0.83       | 0.29       | 0.11   | 0.79      | 0.41     | 0.38          | 2.57             | 2.28   | 0.34    |
| R43                               | 172.8            | 73.6                   | 36.00   | 19.02    | 2.87          | 10.75     | 4.50 | 0.43      | 9.06     | 7.72    | 0.76   | 0.58    | 12.4      | 8.01      | 1.60          | 2.35       | 0.22       | 0.12       | 0.09   | 1.18      | 0.59     | 0.59          | 3.80             | 2.85   | 0.96    |
| Drought                           | 313.7            | 74.4                   | 29.01   | 16.06    | 3.48          | 21.72     | 3.45 | 0.69      | 8.57     | 6.97    | 0.94   | 0.66    | 13.6      | 7.08      | 2.78          | 3.09       | 0.43       | 0.19       | 0.06   | 0.81      | 0.36     | 0.44          | 2.59             | 2.05   | 0.54    |
| CC                                | 118.3            | 76.0                   | 27.45   | 34.93    | 2.63          | 7.45      | 3.47 | 0.05      | 7.76     | 6.37    | 0.79   | 0.60    | 11.7      | 8.35      | 1.70          | 1.20       | 0.27       | 0.08       | 0.11   | 0.97      | 0.55     | 0.42          | 3.59             | 3.34   | 0.25    |
| Drought                           | 279.6            | 74.2                   | 29.99   | 16.51    | 2.76          | 20.61     | 3.18 | 1.13      | 10.21    | 7.63    | 1.53   | 1.05    | 12.2      | 6.68      | 2.69          | 1.62       | 0.82       | 0.28       | 0.13   | 0.75      | 0.37     | 0.38          | 2.65             | 2.42   | 0.23    |
| CL306                             | 231.1            | 73.9                   | 32.81   | 17.70    | 3.11          | 16.29     | 3.40 | 0.55      | 8.85     | 7.48    | 0.76   | 0.62    | 12.7      | 7.33      | 2.50          | 2.49       | 0.23       | 0.10       | 0.08   | 1.22      | 0.55     | 0.66          | 3.33             | 2.81   | 0.51    |
| Drought                           | 246.5            | 72.5                   | 27.12   | 14.17    | 3.22          | 25.08     | 2.35 | 0.52      | 9.99     | 7.05    | 1.55   | 1.39    | 14.1      | 6.31      | 2.50          | 3.77       | 1.06       | 0.39       | 0.04   | 0.86      | 0.35     | 0.51          | 2.63             | 2.21   | 0.41    |
| CC                                | 112.4            | 71.9                   | 36.50   | 17.80    | 4.36          | 9.37      | 3.64 | 0.28      | 9.76     | 8.14    | 0.93   | 0.69    | 13.6      | 9.16      | 2.52          | 1.46       | 0.31       | 0.10       | 0.07   | 1.09      | 0.63     | 0.46          | 3.60             | 3.49   | 0.11    |
| Drought                           | 343.4            | 74.0                   | 24.61   | 17.75    | 2.50          | 25.09     | 2.50 | 1.50      | 9.93     | 6.21    | 1.99   | 1.74    | 13.5      | 5.91      | 3.41          | 2.28       | 1.41       | 0.40       | 0.06   | 0.50      | 0.27     | 0.23          | 2.15             | 1.93   | 0.21    |
| VN31                              | 202.8            | 75.5                   | 33.77   | 16.40    | 3.44          | 17.97     | 3.34 | 0.56      | 8.46     | 7.23    | 0.71   | 0.52    | 11.9      | 7.42      | 2.37          | 1.76       | 0.20       | 0.11       | 0.07   | 1.07      | 0.48     | 0.59          | 3.06             | 2.64   | 0.42    |
| Drought                           | 375.0            | 75.5                   | 27.56   | 13.45    | 2.98          | 28.12     | 2.26 | 1.09      | 8.74     | 6.52    | 1.27   | 0.94    | 12.8      | 5.75      | 2.90          | 2.97       | 0.76       | 0.26       | 0.16   | 0.74      | 0.36     | 0.38          | 2.25             | 2.00   | 0.25    |
| CC                                | 120.9            | 70.6                   | 40.10   | 14.50    | 3.65          | 8.49      | 3.82 | 0.06      | 10.84    | 8.98    | 1.03   | 0.84    | 13.8      | 9.13      | 2.62          | 1.48       | 0.38       | 0.12       | 0.10   | 1.06      | 0.64     | 0.42          | 3.64             | 3.48   | 0.17    |
| Drought                           | 193.8            | 74.5                   | 36.49   | 11.32    | 3.08          | 21.34     | 2.14 | 0.17      | 9.66     | 7.29    | 1.39   | 0.98    | 11.7      | 6.54      | 2.15          | 1.91       | 0.70       | 0.32       | 0.05   | 0.98      | 0.51     | 0.48          | 3.14             | 2.84   | 0.30    |
| 1084                              | 156.3            | 66.1                   | 29.62   | 23.50    | 2.45          | 6.35      | 2.96 | 1.24      | 9.76     | 7.24    | 1.22   | 1.30    | 20.9      | 10.32     | 2.68          | 6.64       | 0.82       | 0.28       | 0.12   | 0.72      | 0.41     | 0.31          | 2.54             | 1.97   | 0.57    |
| Drought                           | 90.4             | 72.2                   | 47.45   | 15.94    | 2.26          | 3.15      | 3.41 | 0.00      | 8.95     | 6.42    | 1.24   | 1.30    | 15.6      | 8.04      | 2.16          | 4.50       | 0.63       | 0.14       | 0.11   | 0.79      | 0.53     | 0.27          | 2.45             | 2.30   | 0.15    |
| CC                                | 114.7            | 64.6                   | 31.08   | 23.88    | 1.81          | 4.54      | 2.95 | 0.29      | 10.89    | 7.94    | 1.51   | 1.44    | 21.2      | 12.12     | 2.20          | 5.50       | 0.84       | 0.27       | 0.23   | 0.87      | 0.60     | 0.28          | 2.52             | 2.30   | 0.21    |
| P(Cl)                             | ***              | ***                    | ns      | ns       | *             | ***       | *    | ns        | ns       | ns      | ns     | ***     | ***       | ***       | ns            | ***        | *          | ns         | *      | ns        | ns       | ***           | ns               | **     | ***     |
| P(T/CO <sub>2</sub> /RH)          | ***              | ns                     | ns      | ns       | ns            | ns        | ns   | **        | ns       | ns      | ***    | *       | ns        | ns        | ns            | ***        | *          | ns         | ns     | ns        | ***      | ***           | ns               | **     | ***     |
| P(I)                              | ***              | ns                     | ***     | ns       | ns            | ns        | ns   | ***       | ns       | ns      | ***    | ***     | ***       | ***       | *             | ***        | ***        | ***        | ***    | ***       | ***      | ***           | ***              | ***    | ***     |
| P(Cl x T/CO <sub>2</sub> /RH)     | ns               | ns                     | ns      | ns       | ns            | ns        | ns   | ***       | ns       | ns      | ns     | ns      | ns        | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns               | ns     | ns      |
| P(Cl x I)                         | ns               | ***                    | ns      | ns       | *             | ns        | ns   | ns        | ns       | ns      | ns     | *       | ***       | ***       | ns            | ns         | *          | ns         | ns     | *         | *        | *             | *                | *      | *       |
| P(T/CO <sub>2</sub> /RH x I)      | ns               | ns                     | ns      | ns       | **            | ns        | ns   | *         | ns       | ns      | ns     | ns      | ns        | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns               | ns     | ns      |
| P(Cl x T/CO <sub>2</sub> /RH x I) | *                | ns                     | ns      | ns       | ns            | ns        | ns   | *         | ns       | ns      | ns     | ns      | ns        | ns        | ns            | ns         | ns         | ns         | ns     | ns        | ns       | ns            | ns               | ns     | ns      |

## **CHAPTER 6**

Anthocyanin profile is affected by climate change related environmental conditions (elevated temperature, CO<sub>2</sub> and water scarcity) differentially in grapevine Tempranillo clones



# **Anthocyanin profile is affected by climate change related environmental conditions (elevated temperature, CO<sub>2</sub> and water scarcity) differentially in grapevine Tempranillo clones**

## **ABSTRACT**

Anthocyanin potential of grape berries is an important quality factor in wine production because of both their role determining wine organoleptic characteristics and their health promoting effects. Anthocyanin concentration and profile differ among varieties and it also depends on the environmental conditions. Growing conditions of Tempranillo, one of the most important red grape (*Vitis vinifera* L.) cultivars in Spain, are expected to be greatly modified by climate change. The aim of this paper was to determine the effects of individual and combined factors associated to climate change (increase of temperature, rise of CO<sub>2</sub> concentration and water deficit) on the anthocyanin profile of different clones of Tempranillo that differ in the length of their reproductive cycle. The study tries to highlight those clones more adapted for future climatic conditions and to maintain specific Tempranillo typicity in the future. With this purpose, fruit-bearing cuttings were grown under different scenarios of climate change in temperature gradient greenhouses (TGGs) and growth chamber greenhouses (GCGs). Elevated temperature increased anthocyanin acylation, whereas elevated CO<sub>2</sub> and water deficit favoured the accumulation of malvidin derivatives, as well as the acylation and tri-hydroxylation level of anthocyanins. Climate change conditions (understood as elevated temperature and high CO<sub>2</sub>) and water scarcity showed additive effects on the grape anthocyanin profile. The results obtained point towards an anthocyanin regulation mechanism induced by the expected environmental conditions, which may favour the enrichment of the berry in more stable anthocyanin forms. Such impact of environmental conditions was especially noticeable in one of the most widely distributed Tempranillo clones, the accession RJ43.

**Keywords:** Climate change; Tempranillo; Temperature; CO<sub>2</sub>; Water deficit; Anthocyanin profile

## 1. INTRODUCTION

Anthocyanins, together with tannins, are an important class of phenolic compounds [1] and, quantitatively, they are the most represented flavonoid sub-families in grapes. The colour of the red grapes is due to the accumulation of anthocyanins [2,3], which are generally located in the berry skin and more precisely in the vacuoles of the cells [4–6], although they have also been detected in the epidermal cells [7]. Beyond colour, anthocyanins are important in wine production as they contribute to certain flavours. Besides, they participate in colloidal stability due to their interactions with other constituents of wine (phenolic compounds, proteins and polysaccharides) or to their self-condensation [8,9]. Anthocyanins have been also related to wine health benefits as they reduce the risk of cardiovascular diseases [10] and have a high antioxidant activity [11].

Anthocyanins are products of the secondary metabolism, synthesized through the phenylpropanoid biosynthetic pathway from mid-veraison onwards in the case of grape berries [12,13]. Five anthocyanin families are present in berries of *V. vinifera*: cyanidin, delphinidin, malvidin, peonidin and petunidin. The differences between individual anthocyanins are related to the number of hydroxyl and methyl groups, to the nature and number of sugars, to the position of sugar attachment and to the nature and number of acids [13–15]. Therefore, anthocyanins can be grouped according to the level of hydroxylation. The activity of the flavonoid 3'-hydroxylase (F3'H) originates the precursors of the di-hydroxylated forms (cyanidins and peonidins), meanwhile the activity of the flavonoid 3'5'-hydroxylase (F3'5'H) originates the precursors of the tri-hydroxylated forms (delphinidins, petunidins and malvidins). In addition, they can also be grouped according to their methylation level (carried out by a methyltransferase, MT), in non-methylated (cyanidin and delphinidin), mono-methylated (peonidin and petunidin) and di-methylated anthocyanins (malvidin). Moreover, anthocyanins can be modified by processes of acylation of their glucosyl group, generating a higher level of diversity within each family: non-acylated (3-monoglucosided), acetyl and coumaroyl derivatives and, in some cases, caffeoyl derivatives. The nature of the glycosyl units or of the acyl groups, as well as the site of their bonding have a significant effect on the stability and reactivity of anthocyanin molecules, which can greatly affect wine quality in terms of colour or storage. Thereby, the increased hydroxylation of the aglycone stabilizes anthocyanins [16], just as methylation enhances the stability by decreasing the chemical reactivity of hydroxyl clusters and the acylation decreases the chemical reactivity of glucoside anthocyanins [17–19]. Moreover, acylated anthocyanins are deemed to contribute to colour stability of red wines during storage [20]. Differences in colour and stability among anthocyanin forms have been studied by different authors [21–25]. The colour of anthocyanins depends on the number and structure of benzene ring substituents, the acylation state, and the environmental conditions: pH,

sulphur dioxide (SO<sub>2</sub>), temperature and light. Therefore, the profile of anthocyanins in the berries used for vinification is a significant factor for the quality of the wine produced.

The accumulation of anthocyanins and their profile depend on the cultivar [2,26,27], the clone [28], as well as on the environmental conditions. Light exposure can modify the anthocyanin profile [29] and N supply can change both the concentration of anthocyanins and their profile [27]. Elevated temperature has been reported to decrease total anthocyanins [2,14,30,31], while the increase in air CO<sub>2</sub> concentration increased anthocyanins in some cases [24] and had no effect in others [25]. Studies on the combined effect of high temperature and elevated CO<sub>2</sub> concentration reveal a decrease in anthocyanin concentration [32–34]. Besides, water scarcity had a rising effect on anthocyanin concentration in Syrah [35] and Tempranillo cultivars [32], although lower anthocyanin levels have also been reported under drought conditions in Tempranillo [36] and under drought combined with high temperature and elevated CO<sub>2</sub> [32]. Changes in total anthocyanin concentrations are often concomitant with changes in anthocyanin profile. Malvidin derivatives, highly methylated anthocyanin forms, as well as anthocyanins with high acylation level, seem to be the least affected by high temperatures [31]. Water deficit has been shown to enhance *p*-coumaroylated derivatives in Shiraz [37] and the expression of the genes *F3'5'H* and *O-methyltransferase (OMT)*, thus increasing the proportion of tri-hydroxylated and methylated anthocyanins in Cabernet Sauvignon and Tempranillo respectively [38,39].

Some of the environmental factors that can affect anthocyanin concentration and profile are expected to change in a near future due to the on-going climate change. According to the RCP6.0 and RCP8.5 scenarios proposed by the Intergovernmental Panel on Climate Change (IPCC) a rise in global mean temperature between  $2.2 \pm 0.5$  °C and  $3.7 \pm 0.7$  °C and an increase in the atmospheric CO<sub>2</sub> concentration up to levels between 669.7 and 935.9 ppm are expected [40]. Increase in the frequency and intensity of drought events in some regions, such as the Mediterranean area, are also predicted as a consequence of changes in the rainfall distribution and periodicity [40], as well as to a higher evapotranspiration associated with warm temperatures [41]. In this sense, the impact of climate-change related factors on anthocyanin profile has been assessed individually. However, whereas in the future all these factors will act in combination and their effect on anthocyanins profile cannot be extrapolated from the sum of their individual effects, few studies have addressed this issue. In this line, Zarrouk et al. concluded that anthocyanin methoxylation might represent a strategy to cope with the combined effects of water shortage and heat stress [42].



Attending to these predictions, knowing in advance the impact of climate change on grapevine production is of special interest to determine possible strategies to mitigate its potential negative effects on grape composition. The approaches proposed to limit the impact of elevated temperature include, among others, the exploration of intra-varietal diversity, looking for clones more adapted to the projected environmental conditions [43,44]. In particular, the use of late-ripening clones within the same variety, may allow to delay the ripening period to the ideal ripening window in terms of temperature, without changing wine typicity [42,45]. Tempranillo, a grapevine cultivar largely cultivated in Spain [46], is especially suitable for clonal selection as there is a great number of clones already characterised [47] presenting a diversity in terms of phenological development. However, there are few studies assessing the response of different clones to climate change-related factors considering their anthocyanin profiles.

The aim of this paper was to determine the effects of individual and combined factors associated to climate change (increase of temperature, rise of CO<sub>2</sub> concentration, reduction of RH and water availability decline) on the anthocyanin profile of different clones of grapevine cv. Tempranillo, which differ in the length of their reproductive cycle.

## **2. MATERIAL AND METHODS**

### *2.1. Experimental design and plant material*

Two experiments were conducted using fruit-bearing cuttings of *Vitis vinifera* (cv. Tempranillo). One experiment was carried out in temperature gradient greenhouses (TGGs), in which temperature and CO<sub>2</sub> concentration were studied as single environmental factors or in combination (TGG experiment). TGGs maintain natural fluctuations in temperature, RH and radiation, therefore the impact of elevated temperature and elevated CO<sub>2</sub> can be assessed under more realistic conditions. Another experiment was performed in growth chamber greenhouses (GCGs), with a higher control of environmental factors, in which the combined effect of elevated CO<sub>2</sub>, elevated temperature and low relative humidity was assessed under different water availabilities (GCG experiment).

Five clones of grapevine (*V. vinifera* L.) cv. Tempranillo were used in the TGG experiment (RJ43, CL306, T3, VN31 and 1084) and four in the GCG experiment (RJ43, CL306, VN31 and 1084). Main characteristics of the clones in terms of reproductive cycle length and yield, as well as the plant material providers are presented on Table 1. RJ43, CL306 and VN31 are commercialised Tempranillo accessions, RJ43 being the widest distributed Spanish certified clone of Tempranillo during the previous decade, whereas CL306 is currently the most used certified clone in Spain [47]. Selected

dormant cuttings were used in order to obtain fruit-bearing cuttings according to an adapted protocol from Mullins and Rajasekaran [48], described in detail in Arrizabalaga et al. and Morales et al. [49,50]. Plant growth was manipulated so each plant developed a single berry bunch. Plants were irrigated with the nutritive solution described by Ollat et al. [51,52].

### 2.1.1. TGG Experiment

At fruit set, plants of the five clones studied (RJ43, CL306, T3, VN31 and 1084) that had similar characteristics in terms of bunch size and phenological stage were transplanted to pots of 13 L containing a mix of 2:1 peat:perlite (v/v). Then, they were transferred to TGGs sited in the campus of the University of Navarra (42°48'N, 1°40'W; Pamplona, Navarra, Spain), a structure for plant growth under semi-controlled conditions. Each TGG contains three temperature modules, where a gradient of temperature is created along them (from ambient temperature in module 1 to ambient temperature + 4 °C in module 3), and CO<sub>2</sub> can be injected inside increasing the concentration of CO<sub>2</sub> in the air. The design and performance of TGGs are described in detail in Morales et al. [53]. The four treatments applied in this case were a combination of two temperature regimes: ambient (T) vs. ambient + 4 °C (T+4); and two CO<sub>2</sub> levels: ambient (ACO<sub>2</sub>), ca. 400 ppm, vs. elevated (ECO<sub>2</sub>), 700 ppm. As a result, the following treatments were applied: (i) T/ACO<sub>2</sub>, (ii) T/ECO<sub>2</sub>, (iii) T+4/ACO<sub>2</sub> and (iv) T+4/ECO<sub>2</sub>. CO<sub>2</sub> was provided by Carbueros Metálicos, Spain. The number of plants of each clone per treatment was 7-8. Plants grew under these conditions from fruit set to maturity (determined by total soluble sugar solid content, TSS, of ca. 22 °Brix).

### 2.1.2. GCG Experiment

At fruit set, plants of the four clones studied (RJ43, CL306, VN31 and 1084) that had similar phenology and bunch characteristics were transplanted to 7 L pots filled with a mix of peat:sand (v/v). They were transferred to two growth chamber-greenhouses (GCGs) also sited at the University of Navarra [50]. Temperature, CO<sub>2</sub> concentration and relative humidity (RH) (T/CO<sub>2</sub>/RH) of one of the greenhouses was set at current situation conditions ("CS" treatment; 24 °C/14 °C, day/night, 400 ppm of air CO<sub>2</sub> and a relative humidity of 45 %/63 %), meanwhile the other one was used to simulate climate change conditions ("CC" treatment; 28 °C/18 °C, 700 ppm of air CO<sub>2</sub> and a relative humidity of 35 %/53 %) [40,54]. Within each greenhouse, plants were subjected to two irrigation regimes: well-watered (WW) or water deficit (WD), where WD plants received 60 % of the irrigation received by those WW, according to the prediction of the Max Planck Institute predictions [54]. Therefore, the following treatments were applied: (i) CS/WW, (ii) CS/WD, (iii) CC/WW and (iv) CC/WD. The number of plants of each clone per treatment was 7-8.

Plants were monitored with soil moisture sensors (EC-5 Soil Moisture Sensors, Decagon Devices Inc., Pullman, WA, USA) settled in the correspondent pots. Soil moisture in WW plants was kept at ca. 80-90 % of the substrate field capacity (sensor value between 30-40 %,  $\text{m}^3 \text{H}_2\text{O} \times 100 \text{m}^{-3}$  substrate), whereas WD plants were irrigated when the sensor reached humidity value of 10 % -or less-  $\text{m}^3 \text{H}_2\text{O} \text{m}^{-3}$  soil  $\times 100$  [55]. At that moment, irrigation was done with nutritive solution, adding 60 % of the irrigation volume received by the WW plants during the time corresponding to the specific drought period. Pre-dawn leaf water potential was measured at mid-veraison and 2 weeks after mid-veraison using a pressure chamber SKYE SKPM 1400, Skye Instruments Ltd, Llandrindod, Wales, UK) and according to the methodology described by Scholander et al. [56]. Average values were  $-0.67 \pm 0.02$  MPa and  $-1.40 \pm 0.04$  MPa in WW and WD plants, respectively. Watering of WW and WD was done in such a way that, independently on the irrigation regime, all plants received the same amount of nutrients.

## *2.2. Sampling process*

Three berries per bunch were sampled at three stages: mid-veraison (considered when half of the berries in the bunch had turned colour; sampled berries having the same proportion of coloured skin surface, ca. 50 %), 1 week after mid-veraison and 2 weeks after mid-veraison. At maturity (TSS of ca. 22 °Brix, the average actual values being of 19.63 °Brix in the TGG experiment and 21.10 °Brix in the GCG experiment), ten berries were sampled. Berries were frozen and stored at -80 °C. Pools of 2 samples of plants under the same treatment and belonging to the same clone were made for carrying out the analysis. Skin was separated from the rest of the frozen berry and freeze dried in a Alph1-4 lyophilizer (CHRIST, Osterode, Germany) before being ground using an MM200 ball grinder (Retsch, Haan, Germany).

## *2.3. Anthocyanin determination*

The analyses of skin anthocyanins were done according to Torres et al. and following, with minor changes, the protocol that is detailed in Acevedo De la Cruz et al. and Hilbert et al. [36,57,58]. Briefly, pigments were extracted adding methanol with 0.1 % HCl (v/v) to 20 mg of dried skin powder. Once the solution was filtered with a polypropylene syringe filter of 0.45  $\mu\text{m}$  (Pall Gelman Corp., Ann Arbor, USA), the elution passed through a Synchronis C18, 2.1  $\times$  100 mm, 1.7  $\mu\text{m}$  Column (Thermo Fisher Scientific, Waltham, MA USA), so the compounds were separated using an UltiMate 3000 UHPLC system (Thermo Electron SAS, Waltham, MA USA). The standard sample used during the analysis was malvidin-3-O-glucoside (Extrasynthese, Genay, France) and the detection was done with a DAD-3000 diode array detector (Thermo Electron SAS, Waltham, MA USA), settling the absorbance wavelength

at 520 nm. The concentration of each anthocyanin was determined calculating the peak area of the resulting chromatograms using the Chromeleon software (version 7.1) (Thermo Electron SAS, Waltham, MA USA) and was expressed as milligrams per gram ( $\text{mg g}^{-1}$ ) of dry skin weight (DW).

#### *2.4. Statistical analysis*

Data were analysed statistically with a three-way ANOVA (clone, temperature regime and  $\text{CO}_2$  concentration, in the case of the TGG experiment; and clone, T/ $\text{CO}_2$ /RH regime and irrigation regime, in the GCG experiment). The Fisher's least significant difference (LSD) test was used as a post-hoc test. These analyses were carried out with the software R (3.5.3).

### **3. RESULTS**

#### *3.1. Total anthocyanins*

In the TGG experiment and considering the clones altogether, elevated temperature and high  $\text{CO}_2$ , both acting independently or in combination, had little effect on total anthocyanin concentration during berry ripening, except 2 weeks after mid-veraison, when T+4 increased significantly total anthocyanin concentration and  $\text{ECO}_2$  reduced anthocyanin levels (Table 2). In the GCG experiment, the combination of elevated temperature, high  $\text{CO}_2$  and low relative humidity (CC treatment) reduced total anthocyanin concentration at mid-veraison and increased it 1 week after mid-veraison compared with CS. At maturity, a significant interaction between T/ $\text{CO}_2$ /RH and irrigation regimes was observed, CC significantly reducing total anthocyanins when combined with WD.

In both TGG and GCG experiments, clones showed significant differences in their total anthocyanins concentration at maturity, the clone 1084 having the lowest values consistently (Table 2). When analysing the impact of combined elevated temperature and high  $\text{CO}_2$  at maturity for each clone individually, the levels of RJ43 tended to decrease in the TGG experiment by the T+4/ $\text{ECO}_2$  compared with T/ $\text{ACO}_2$  treatment ( $P < 0.05$ ) and were hardly modified by CC in the GCG experiment ( $P > 0.05$ ). In contrast, in CL306, anthocyanins tended to increase by 24 % and 19 % under combined high temperature and elevated  $\text{CO}_2$  in the TGG ( $P < 0.05$ ) and GCG ( $P > 0.05$ ) experiments, respectively. In VN31 anthocyanins tended to decrease by 18 % and 7 %, TGG and GCG experiments respectively ( $P > 0.05$  in both cases), whereas in 1084 anthocyanins tended to increase by 15 % and 34 %, TGG and GCG experiments respectively ( $P > 0.05$  in both cases). When CC was combined with drought (CC/WD), total anthocyanin concentration decreased in all the clones compared with CS/WW, RJ43 being the most affected clone (decreasing a 31 %,  $P < 0.05$ ) and VN31 the least affected one (decreasing a 17 %,  $P > 0.05$ ).

### 3.2. Families

Malvidin was the most abundant family and cyanidin based anthocyanins the least abundant in both experiments, regardless of the clone and treatment applied (Figure 1). During ripening, environmental conditions affected the relationship among anthocyanin families. In the TGG experiment, T+4 increased significantly the abundance of cyanidin derivatives at the expense of malvidin derivatives, compared with T, both at mid-veraison and 1 week after mid-veraison ( $P_T < 0.05$  in all the cases), but these differences disappeared 2 weeks after mid-veraison and at maturity (Figure 1A, Table 3). Conversely, ECO<sub>2</sub> decreased cyanidin derivatives in favour of those anthocyanins derived from malvidin 2 weeks after mid-veraison and at maturity ( $P_{CO_2} < 0.05$  in all the cases; Table 3). Moreover, petunidin relative content was punctually affected by ECO<sub>2</sub> increasing at mid-veraison, while peonidin relative content was reduced ( $P_{T/CO_2/RH} < 0.05$  in all the cases, Table 3). In the GCG experiment, 2 weeks after mid-veraison and at maturity, CC increased cyanidin and peonidin relative content at mid-veraison, followed by a decrease in cyanidin, delphinidin and peonidin, in favour of malvidin derivatives ( $P_{T/CO_2/RH} < 0.05$  in all the cases; Figure 1A, Table 3). In relation to water scarcity, WD affected the relative content of all the families, strongly increasing the proportion of malvidin and reducing the relative content of the rest of the families at all the ripening stages studied (Figure 1A, Table 3). In general, CC and WD showed additive effects on the anthocyanin family profile, although a significant interaction between T/CO<sub>2</sub>/RH and irrigation regimes was observed for cyanidin and petunidin at maturity. The reduction of the relative abundance of cyanidin derivatives induced by WD was greater under CS than under CC, while in the case of petunidin, this decrease was greater under CC than under CS.

Comparing anthocyanin families among clones at maturity, 1084 showed consistently in both experiments the lowest relative content of cyanidin, delphinidin and petunidin derivatives, and the highest of malvidin (Figure 1B and 1C, Table 3).

### 3.3. Hydroxylation

Tri-hydroxylated forms were more abundant than those di-hydroxylated at the end of the ripening period, regardless of the clone and treatment applied (Figure 2). In the TGG experiment, T+4 did not have any effect on the relative abundance of tri-hydroxylated forms, whereas ECO<sub>2</sub> increased it independently of the temperature regime (Figure 2A, Table 4). Similarly, in the GCG experiment, CC treatment increased the relative abundance of tri-hydroxylated forms, especially under WW treatment (Figure 2C, Table 4). In this experiment, tri-hydroxylated anthocyanins were also increased significantly by WD, CC and WD treatments having additive effects.

Regarding the significant differences observed among clones (Table 4), T3 had the lowest proportion of tri-hydroxylated anthocyanins in the TGG experiment (Figure 2B), whereas in the GCG experiment, VN31 showed the lowest relative abundance of tri-hydroxylated forms and 1084 the highest (Figure 2D). Even though, in general terms, combined high temperature and elevated CO<sub>2</sub> (T+4/ECO<sub>2</sub> and CC/WW treatments, in TGT and GCG experiments, respectively) tended to increase the relative abundance of tri-hydroxylated anthocyanins in all the clones studied, this effect being significant and repeatedly observed in RJ43 in both experiments (Figure 2B and 2D).

### 3.4. Methylation

Di-methylated derivatives were the most abundant anthocyanin forms at maturity in both TGG and GCG experiments, regardless of the clone and treatment applied (Figure 3). Non-methylated and mono-methylated forms were reduced by ECO<sub>2</sub> in favour of di-methylated forms in the TGG experiment ( $P_{CO_2} < 0.05$  in all the cases; Figure 3A, Table 4). Similarly, in the GCG experiment, CC and WD reduced non-methylated and mono-methylated anthocyanins, in favour of those di-methylated (all the  $P_{T/CO_2/RH} < 0.05$  and  $P_I < 0.05$ ; Figure 3C, Table 4). The methylation degree was not significantly affected by T+4 at maturity.

The clones studied showed significant differences in the relative abundance of anthocyanins according to their level of methylation, 1084 having the lowest values of non-methylated and mono-methylated forms, and the highest of di-methylated (Figure 3B and 3D, Table 4). In addition, in the GCG experiment, there was a significant interaction among clone, T/CO<sub>2</sub>/RH regime and irrigation regime affecting the relative abundance of non-methylated and di-methylated anthocyanins ( $P_{CL \times T/CO_2/RH \times I} = 0.04$  and  $P_{CL \times T/CO_2/RH \times I} = 0.018$ , respectively; Table 4). Such interaction was particularly noticeable in the following cases: i) CC reduced the relative abundance of non-methylated forms regardless of the irrigation regime in RJ43, only under WW conditions in VN31, whereas in CL306 and 1084, non-methylated forms were not significantly modified by CC; ii) in RJ43 and VN31, the relative abundance of di-methylated forms increased significantly due to CC only when it was combined with WW conditions, and in 1084 only when CC was combined with WD, meanwhile in CL306, the relative abundance of di-methylated anthocyanins did not change in CC conditions compared with CS regardless of the water regime; iii) drought increased the proportion of di-methylated anthocyanins regardless of the T/CO<sub>2</sub>/RH regime in RJ43, CL306 and VN31, but only when combined with CC condition in 1084 (Figure 3D, Table 4).

### 3.5. Acylation

At maturity, non-acylated 3-monoglucosides were the most abundant anthocyanin forms, according to the acylation pattern, and acetylated forms the least abundant (Figure 4). Taking into account all the clones as a whole, in the TGG experiment, both T+4 and ECO<sub>2</sub> slightly, but significantly, reduced the relative content of non-acylated 3-monoglucosides ( $P_T = 0.023$  and  $P_{CO_2} < 0.001$ ; Figure 4A, Table 4). In consequence, it was observed a significant increase in the relative content of 3-acetyl-glucosides ( $P_T = 0.041$  and  $P_{CO_2} = 0.002$ ) and of 3-*p*-coumaroyl-glucosides ( $P_T = 0.035$  and  $P_{CO_2} < 0.001$ ; Figure 4A, Table 4). The combination of elevated temperature and high CO<sub>2</sub> increased the relative abundance of 3-acetyl-glucosides and 3-*p*-coumaroyl-glucosides in the TGG experiment, (T+4/ECO<sub>2</sub> vs. T/ACO<sub>2</sub>), as well as in the GCG experiment (CC/WW vs. CS/WW), this reduction being significant in the first case (Figure 4A and 4C, Table 4). WD reduced significantly the relative content of non-acylated 3-monoglucosides and increased that of 3-acetyl-glucosides and 3-*p*-coumaroyl-glucosides, irrespective of the T/CO<sub>2</sub>/RH regime (Figure 4C, Table 4). WD and CC conditions showed additive effects on the acylation profile.

The clones studied also showed differences in their acylation profile at maturity, 1084 showing consistently the lowest relative content of non-acylated glycosylated forms and the highest relative abundance of 3-acetyl-glucosides and 3-*p*-coumaroyl-glucosides in both TGG and GCG experiments (Figure 4B and 4D). Regarding the impact of the environmental factors applied simultaneously, the relative abundance of the 3-coumaroyl-glucosides was more affected by CC/WD, compared to CS/WW, in RJ43 (increase of 97 %) than in the other clones studied.

## 4. DISCUSSION

### 4.1. Diversity of anthocyanin profiles among Tempranillo clones

Total anthocyanin concentration at maturity varied significantly among clones in both TGG and GCG experiments. In the TGG experiment, RJ43 and T3 had the higher concentration of anthocyanins, whereas 1084 showed consistently the lowest anthocyanin levels in both experiments, thus revealing variability within the Tempranillo cultivar in relation to the anthocyanin content.

Malvidin was the most abundant family as it has been reported for Tempranillo [3,36] and other grapevine cultivars as Cabernet Sauvignon [59], Shiraz [13] and Merlot [27]. Also, the higher abundance of 3-monoglucosides forms in comparison to the acylated ones, malvidin 3-*O*-glucoside being the most abundant anthocyanin, agrees with previous studies [2,3,15,27,36,60]. The high abundance of tri-hydroxylated and di-methylated forms observed in both TGG and GCG experiments is coherent with

the high abundance of malvidin. The clones studied showed differences in their anthocyanin profiles at maturity, 1084 having the highest abundance of di-methylated forms (malvidin derivatives), 3-acetyl-glucosides and 3-*p*-coumaroyl-glucosides. These results may suggest, a preference in the biosynthetic pathway of the F3'5'H enzyme, thus leading to the higher abundance of tri-hydroxylated forms, as well as a higher activity of the OMT enzyme in this clone. Also, the increased abundance of acylated anthocyanins with respect to those non-acylated may be associated to a higher anthocyanin degradation activity in 1084, as acylated forms have been pointed out as more stable than the 3-monoglucosides [59] and di-methylated forms [61].

#### *4.2. Effect of climate change on the concentration and profile of anthocyanins*

Both elevated temperature and high CO<sub>2</sub>, acting independently or in combination, did not have a significant effect on total anthocyanins at maturity, this result being consistent in both TGG and GCG experiments. However, these results differ from other studies that have reported a decrease in anthocyanins concentration with high temperature (either applied as a single factor or combined with elevated CO<sub>2</sub>) [2,14,29,30,32]. This observation has been associated with a down-regulation of the genes involved in their biosynthesis [62,63] or with an increased expression of anthocyanin degradation linked-genes [59]. In the case of elevated CO<sub>2</sub>, our data confirm the results obtained by Gonçalves et al. [64], who did not observed a marked impact of CO<sub>2</sub> on anthocyanin levels. However, other authors have reported a negative effect of high CO<sub>2</sub> on anthocyanin concentration in table grapes or strawberry fruits, concomitant with lower transcription levels of the genes involved in the flavonoid biosynthetic pathway [62,63] or associated with a reduction in anthocyanin stability [65]. Regarding the combined effect of high temperature, elevated CO<sub>2</sub> and water deficit, the results show a negative impact of such environmental condition on anthocyanins. Studies about the impact of water stress as a single factor on different grapevine cultivars reported an increase in anthocyanin accumulation after mid-veraison [66–68]. However, similar to the present study, Salazar-Parra et al. (2010) showed different effects of water deficit depending on the temperature and CO<sub>2</sub> levels, reducing anthocyanin concentration only when combined with high temperature and high CO<sub>2</sub> [32].

High temperature has been reported to cause concomitant changes in total anthocyanin concentrations and anthocyanin profiles [2,69]. For instance, relative abundance of tri-hydroxylated anthocyanins was increased under heat-stress in Cabernet Sauvignon [66,70]. Also, Mori et al. and Tarara et al. referred to a decrease in the relative content of all the families except malvidin when high temperatures were applied in Cabernet Sauvignon and Merlot respectively [2,59]. Studies have underlined the activation or inhibition of enzyme activities, or the up- and down-regulation of genes involved in phenylpropanoid pathway under high temperature. In this sense, contrasted results have



been reported on the regulation of *F3'H* and *F3'5'H* gene expression levels by heat stress, and differences in the proportions of di- and tri-hydroxylated anthocyanins have been explained by *F3'5'H* expression being less down-regulated than that of *F3'H* [31]. However, when the heat treatment was applied after mid-veraison, no effect or a slight up-regulation of *F3'5'H* expression with an immediate up-regulation of *F3'H* was observed by Lecourieux et al. [70]. In the present study, high temperature did not have marked effects on the anthocyanin profile at maturity, in terms of families, hydroxylation and methylation patterns. Only a significant increase in the abundance of acylated forms, at the expense of 3-monoglucosides, was observed. These results agree with Mori et al. and Tarara et al. [2,59]. The latter explained such enrichment in acylated forms as a consequence of higher anthocyanin degradation of non-acylated anthocyanins, which have lower stability [59]. Also, Lecourieux et al. have reported a significant up-regulation of *Vv3AT*, involved in the acylation process during berry ripening, thus leading to a higher proportion of acylated anthocyanins [70].

Regarding the combined effect of elevated temperature and high CO<sub>2</sub> (T+4/ECO<sub>2</sub> vs. T/ACO<sub>2</sub> in the TGG experiment and CC/WW vs. CS/WW in the GCG experiment), it reduced the relative content of cyanidin and peonidin and increased the relative abundance of tri-hydroxylated and acylated forms. Whereas temperature and CO<sub>2</sub> showed additive effects on acylated anthocyanins, their impact on the hydroxylation level seemed to be most likely associated with CO<sub>2</sub> rather than to temperature. The results suggest that, under our experimental conditions, high CO<sub>2</sub> was the main factor operating in the T/CO<sub>2</sub>/RH regime.

The higher the relative content of di-methylated forms in the ECO<sub>2</sub> (TGG experiment) and CC/WW (GCG experiment) treatments suggest an up-regulation of the methylation process under these conditions. Unfortunately, the studies on the impact of ECO<sub>2</sub> on anthocyanin profile are scarce. However, increases in the relative abundance of di-methylated forms, induced by either environmental (water scarcity) or genetic factors, have been associated with higher *OMT1* expression [71,72]. Considering the clones altogether, ECO<sub>2</sub> showed additive effects to T+4, increasing the relative abundance of acylated forms. Also, in the GCG experiment, grapes developed under CC conditions had a higher proportion of acylated forms. The increase in the proportion of acylated anthocyanins in the must may have as a result the increase in the stability of the wines produced in the future, as acylation confers more stability to anthocyanins, thus reducing their reactivity [17–20].

The effect of drought increasing the proportion of malvidin derivatives throughout most part of the ripening period is in agreement with previous studies [67,68,73–75]. These results might be explained by the fact that drought may act differently on the enzymes involved in the synthesis of different forms of anthocyanins or may affect the activation or inhibition of the catabolism of anthocyanins [72]. In

this way, Castellarin et al. reported the up-regulation of the *O*-methyl transferase (OMT) in plants with water scarcity [72], thus increasing the proportion of malvidin derivatives in relation to other non-methylated forms such as cyanidin and delphinidin. Drought also reduced the relative abundance of 3-monoglucosides in our study, which is in agreement with the results of Torres et al. [36] in cv. Tempranillo. The increased relative abundance of tri-hydroxylated forms in the grapes of plants subjected to WD agrees with the up-regulation of *F3'5'H*, in comparison to *F3'H*, observed by Castellarin et al., Berdeja and Movahed et al. under water scarcity [66,72,76,77]. Moreover, drought increased the acylated anthocyanins as previously reported by Hilbert in Merlot [67], although in our case, the relative abundance of *p*-coumaroyled anthocyanins was higher than that of the *p*-acetylated forms, contrary to the results showed by this author. As anthocyanins, mainly those acylated with aromatic acids, participate in intramolecular co-pigmentations that intensify and stabilize colour [78], this aspect could be an interesting point for the subsequent vinification process. Regarding the combination of CC and WD, the results reveal additive effects on the anthocyanin profile of grapes, WD reinforcing the enrichment in acylated forms induced by CC.

#### *4.3. Response of different Tempranillo clones to climate change*

Clones characterised by their providers as long-reproductive cycle accessions VN31 and 1084 [49,79,80] showed the lowest total anthocyanin concentration under T+4/ECO<sub>2</sub>, meanwhile CL306, a short-reproductive cycle accession (ITACyL, unpublished data), was the only one in which anthocyanins increased under T+4/ECO<sub>2</sub>, reaching the highest values of all the clones studied. In the GCG experiment, VN31 and 1084, showed the highest and lowest anthocyanin levels, respectively, under simulated 2100 environmental conditions (CC/WD treatment). These data suggest that the long-reproductive cycle clones studied in these experiments (VN31 and 1084) not always maintained higher anthocyanin concentrations under future environmental conditions.

Regarding the impact of the conditions projected for the end of the present century on anthocyanin profile, the results indicate that the response may differ among clones. When considering the anthocyanin methylation level, the response of clones to the combination of climate change conditions and water deficit (CC/WD treatment) varied, the 1084 accession having the smallest changes when compared to current conditions (CS/WW), probably because the methylation level was already high under CS/WW conditions. Even though anthocyanin profile of 1084 was the less affected by climate change conditions and this accession presented a higher relative abundance of more stable anthocyanin forms [20], the lower phenolic potential of this accession, regarding total anthocyanin concentration, may detract from its potential use in a future climate change scenario. Conversely, RJ43, one of the widest distributed Spanish certified clones of Tempranillo [47], was one of the most affected

clones by simulated 2100-environmental conditions, with marked decreases in non-methylated forms, in favour of those mono- and di-methylated, and increases in the hydroxylation and acylation levels. However, as these changes were towards more stable anthocyanins, they could be interpreted as a positive result of the expected environmental conditions in the future, which may contribute to higher colour stability in grapes and wines. The double methylation confers high stability to anthocyanins [19,23,25], as well as it intensifies their red coloration [17,59,81–83], thus guaranteeing wine preservation. Also, the marked increase in malvidin derivatives in RJ43 under future conditions may involve the improvement of the health promoting effects of the wine produced from this clone, due to the anti-inflammatory activity described for these compounds [84].

## 5. CONCLUSION

Considering the results obtained, malvidin regulation worked in an opposite manner than in the other families as CC conditions and WD conditions increased its relative abundance but reduced the proportion of cyanidin, delphinidin and peonidin. Besides, the results indicate that elevated temperature shifted towards the production of acylated forms of anthocyanins, while elevated CO<sub>2</sub>, both individually and combined with high temperature, modified the relative abundance of anthocyanin families, acylation, hydroxylation and methylation, in a similar way as water deficit did. In general terms, climate change conditions (combined high temperature, elevated CO<sub>2</sub> and low relative humidity) and water deficit showed additive effects increasing the relative abundance of malvidin derivatives (and therefore di-methylated forms), acylated and tri-hydroxylated anthocyanins. Although the changes in anthocyanin profile observed followed a common pattern among clones, RJ43 was the most responsive to the expected environmental conditions. Also, these changes in anthocyanin profile were towards anthocyanin forms, which may contribute to higher colour stability in grapes and wines produced in the future. Still, further research on the expression of genes coding enzymes involved in the anthocyanins biosynthesis and their activity is needed, especially to understand the effect of rise of CO<sub>2</sub> on them.

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## TABLES

Table 1. Tempranillo clones used for the experiments.

| Clone | Reproductive-cycle length | Yield characterisation | Plant material provider | References                  |
|-------|---------------------------|------------------------|-------------------------|-----------------------------|
| RJ43  | Intermediate              | High                   | EVENA                   | [85]                        |
| CL306 | Short                     | Low                    | EVENA                   | (ITACyL , unpublished data) |
| T3    | Short                     | Intermediate           | EVENA                   | [86]                        |
| VN31  | Long                      | Intermediate           | Vitis Navarra S.A.      | [79]                        |
| 1084  | Long                      | Intermediate-high      | ICVV                    | ICVV (unpublished data)     |

EVENA: Estación de Viticultura y Enología de Navarra (Olite, Spain)

ICVV: Instituto de Ciencias de la Vid y del Vino (Logroño, Spain)

ITACyL: Instituto Tecnológico Agrario de Castilla y León (Valladolid, Spain)

Table 2. Total anthocyanins concentration in *V. vinifera* cv. Tempranillo clones grown in TGG and in GCG experiments, throughout ripening. Conditions TGG: five Tempranillo clones (RJ43, CL306, T3, VN31 and 1084) grown under four T/CO<sub>2</sub> conditions: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). Conditions in GCG: four Tempranillo clones (RJ43, CL306, VN31 and 1084) grown under two T/CO<sub>2</sub>/RH conditions: current situation ("CS": 400 ppm, 24 °C/14 °C, and 45 %/65 % RH) and climate change ("CC": 700 ppm, 28 °C/18 °C, and 33 %/53 % RH), combined with two irrigation regimes: well-watered (WW) and water deficit (WD).

|     |                                   | Total anthocyanin concentration (mg·g DW skin <sup>-1</sup> ) |                           |                            |                |  |  |
|-----|-----------------------------------|---|---------------------------|----------------------------|----------------|--|--|
|     |                                   | Mid-veraison  | 1 week after mid-veraison | 2 weeks after mid-veraison | Maturity       |  |  |
| TGG | RJ43                              | 1,02 ± 0,10 a   | 4,36 ± 0,38 a             | 19,66 ± 1,17 ab            | 21,58 ± 0,71 a |  |  |
|     | CL306                             | 1,10 ± 0,11 a   | 5,00 ± 0,47 a             | 20,13 ± 0,87 a             | 18,59 ± 0,83 b |  |  |
|     | T3                                | 1,08 ± 0,10 a   | 4,69 ± 0,73 a             | 16,54 ± 1,33 c             | 21,31 ± 0,84 a |  |  |
|     | VN31                              | 0,94 ± 0,07 a   | 3,70 ± 0,37 a             | 17,10 ± 1,03 bc            | 18,30 ± 0,80 b |  |  |
|     | 1084                              | 1,11 ± 0,13 a   | 5,34 ± 0,83 a             | 15,95 ± 0,86 c             | 14,62 ± 0,64 c |  |  |
| GCG | RJ43                              | 0,69 ± 0,04 a   | 6,82 ± 0,62 a             | 20,41 ± 1,25 ab            | 22,96 ± 1,42 a |  |  |
|     | CL306                             | 0,81 ± 0,09 a   | 8,26 ± 0,93 a             | 18,26 ± 1,40 b             | 21,40 ± 1,36 a |  |  |
|     | VN31                              | 0,77 ± 0,05 a   | 7,20 ± 0,81 a             | 21,60 ± 1,30 a             | 22,82 ± 1,21 a |  |  |
|     | 1084                              | 0,79 ± 0,06 a   | 6,74 ± 0,95 a             | 18,57 ± 1,42 ab            | 15,21 ± 0,96 b |  |  |
| TGG | T ACO <sub>2</sub>                | 0,84 ± 0,08 b   | 4,62 ± 0,76 a             | 18,05 ± 1,08 ab            | 19,86 ± 1,01 a |  |  |
|     | ECO <sub>2</sub>                  | 1,09 ± 0,09 ab  | 4,82 ± 0,39 a             | 15,34 ± 0,79 b             | 18,37 ± 0,81 a |  |  |
|     | T+4 ACO <sub>2</sub>              | 1,07 ± 0,10 ab  | 5,03 ± 0,47 a             | 20,36 ± 1,05 a             | 18,24 ± 0,93 a |  |  |
|     | ECO <sub>2</sub>                  | 1,20 ± 0,09 a   | 4,01 ± 0,42 a             | 17,56 ± 0,82 b             | 19,03 ± 0,77 a |  |  |
| GCG | CS WW                             | 0,85 ± 0,06 a   | 5,61 ± 0,38 b             | 20,70 ± 1,07 b             | 21,73 ± 1,53 a |  |  |
|     | WD                                | 0,86 ± 0,07 a   | 6,68 ± 1,05 b             | 17,46 ± 0,97 c             | 20,86 ± 1,55 a |  |  |
|     | CC WW                             | 0,59 ± 0,03 b   | 9,16 ± 0,74 a             | 24,38 ± 1,44 a             | 23,66 ± 1,12 a |  |  |
|     | WD                                | 0,74 ± 0,05 ab  | 7,30 ± 0,77 ab            | 16,59 ± 0,94 c             | 16,26 ± 1,14 b |  |  |
| TGG | P(CL)                             | ns  | ns                        | *                          | ***            |  |  |
|     | P(T)                              | ns  | ns                        | *                          | ns             |  |  |
|     | P(CO <sub>2</sub> )               | *   | ns                        | **                         | ns             |  |  |
|     | P(CL x T)                         | ns  | ns                        | ns                         | ns             |  |  |
|     | P(CL x CO <sub>2</sub> )          | ns  | ns                        | ns                         | ns             |  |  |
|     | P(T x CO <sub>2</sub> )           | ns  | ns                        | ns                         | ns             |  |  |
|     | P(CL x T x CO <sub>2</sub> )      | ns  | ns                        | ns                         | ns             |  |  |
| GCG | P(CL)                             | ns  | ns                        | ns                         | ***            |  |  |
|     | P(T/CO <sub>2</sub> /RH)          | ***   | *                         | ns                         | ns             |  |  |
|     | P(I)                              | ns  | ns                        | ***                        | ***            |  |  |
|     | P(CL x T/CO <sub>2</sub> /RH)     | ns  | ns                        | ns                         | ns             |  |  |
|     | P(CL x I)                         | ns  | ns                        | ns                         | ns             |  |  |
|     | P(T/CO <sub>2</sub> /RH x I)      | ns  | ns                        | ns                         | **             |  |  |
|     | P(CL x T/CO <sub>2</sub> /RH x I) | **  | ns                        | ns                         | ns             |  |  |

Probability values (P) in GCG for the main effects of clone, P(CL); temperature regime P(T); CO<sub>2</sub> regime P(CO<sub>2</sub>); and their interactions, P(CL x T), P(CL x CO<sub>2</sub>), P(T x CO<sub>2</sub>) and P(CL x T x CO<sub>2</sub>). Probability values (P) in TGG for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I), P(T/CO<sub>2</sub>/RH x I) and P(CL x T/CO<sub>2</sub>/RH x I). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant.

Table 3. ANOVA analysis of the relative abundance of anthocyanin families at maturity in *V. vinifera* cv. Tempranillo clones grown in the TGG and the GCG experiments. TGG experiment: five clones (RJ43, CL306, T3, VN31 and 1084) grown under four temperature/CO<sub>2</sub> conditions: ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). GCG experiment: four clones (RJ43, CL306, VN31 and 1084) grown under two T/CO<sub>2</sub>/RH conditions: current situation ("CS": 400 ppm, 24 °C/14 °C, and 45 %/65 % RH) or climate change ("CC": 700 ppm, 28 °C/18°C, and 33 %/53 % RH), combined with two irrigation regimes: well-watered (WW) or water deficit (WD).

|             |                              | TGG          |                     |                     |          | GCG                               |                     |                     |          |
|-------------|------------------------------|--------------|---------------------|---------------------|----------|-----------------------------------|---------------------|---------------------|----------|
|             |                              | Mid-veraison | Mid-veraison<br>+1w | Mid-veraison<br>+2w | Maturity | Mid-veraison                      | Mid-veraison<br>+1w | Mid-veraison<br>+2w | Maturity |
| Cyanidin    | P(CL)                        | ns           | ns                  | ns                  | *        | P(CL)                             | **                  | **                  | ***      |
|             | P(T)                         | *            | **                  | ns                  | ns       | P(T/CO <sub>2</sub> /RH)          | *                   | ns                  | **       |
|             | P(CO <sub>2</sub> )          | ns           | ns                  | *                   | **       | P(I)                              | ***                 | ***                 | ***      |
|             | P(CL x T)                    | ns           | ns                  | ns                  | ns       | P(CL x T/CO <sub>2</sub> /RH)     | ns                  | ns                  | ns       |
|             | P(CL x CO <sub>2</sub> )     | ns           | ns                  | ns                  | ns       | P(CL x I)                         | ns                  | ns                  | ns       |
|             | P(T x CO <sub>2</sub> )      | ns           | ns                  | ns                  | ns       | P(T/CO <sub>2</sub> /RH x I)      | ns                  | ns                  | ns       |
|             | P(CL x T x CO <sub>2</sub> ) | ns           | ns                  | ns                  | ns       | P(CL x T/CO <sub>2</sub> /RH x I) | ns                  | ns                  | ns       |
| Delphinidin | P(CL)                        | ns           | ns                  | ***                 | ***      | P(CL)                             | ns                  | *                   | ***      |
|             | P(T)                         | *            | ns                  | ns                  | ns       | P(T/CO <sub>2</sub> /RH)          | ns                  | ns                  | **       |
|             | P(CO <sub>2</sub> )          | ns           | ***                 | **                  | ns       | P(I)                              | ***                 | ***                 | ***      |
|             | P(CL x T)                    | ns           | ns                  | ns                  | ns       | P(CL x T/CO <sub>2</sub> /RH)     | ns                  | ns                  | ns       |
|             | P(CL x CO <sub>2</sub> )     | ns           | ns                  | ns                  | ns       | P(CL x I)                         | ns                  | ns                  | ns       |
|             | P(T x CO <sub>2</sub> )      | *            | ns                  | ns                  | ns       | P(T/CO <sub>2</sub> /RH x I)      | ns                  | ns                  | ns       |
|             | P(CL x T x CO <sub>2</sub> ) | ns           | ns                  | ns                  | ns       | P(CL x T/CO <sub>2</sub> /RH x I) | ns                  | ns                  | ns       |
| Malvidin    | P(CL)                        | ns           | ns                  | **                  | ***      | P(CL)                             | **                  | **                  | ***      |
|             | P(T)                         | **           | *                   | ns                  | ns       | P(T/CO <sub>2</sub> /RH)          | *                   | ns                  | **       |
|             | P(CO <sub>2</sub> )          | ns           | ns                  | *                   | ***      | P(I)                              | ***                 | ***                 | ***      |
|             | P(CL x T)                    | ns           | ns                  | ns                  | ns       | P(CL x T/CO <sub>2</sub> /RH)     | ns                  | ns                  | ns       |
|             | P(CL x CO <sub>2</sub> )     | ns           | ns                  | ns                  | ns       | P(CL x I)                         | ns                  | ns                  | ns       |
|             | P(T x CO <sub>2</sub> )      | ns           | ns                  | ns                  | ns       | P(T/CO <sub>2</sub> /RH x I)      | ns                  | ns                  | ns       |
|             | P(CL x T x CO <sub>2</sub> ) | ns           | ns                  | ns                  | ns       | P(CL x T/CO <sub>2</sub> /RH x I) | ns                  | ns                  | ns       |
| Peonidin    | P(CL)                        | ns           | **                  | *                   | **       | P(CL)                             | **                  | *                   | ns       |
|             | P(T)                         | ns           | ns                  | ns                  | ns       | P(T/CO <sub>2</sub> /RH)          | ***                 | ns                  | ***      |
|             | P(CO <sub>2</sub> )          | **           | **                  | ns                  | ***      | P(I)                              | ***                 | ***                 | ***      |
|             | P(CL x T)                    | ns           | ns                  | ns                  | ns       | P(CL x T/CO <sub>2</sub> /RH)     | ns                  | ns                  | ns       |
|             | P(CL x CO <sub>2</sub> )     | ns           | ns                  | ns                  | ns       | P(CL x I)                         | ns                  | ns                  | ns       |
|             | P(T x CO <sub>2</sub> )      | ns           | ns                  | ns                  | ns       | P(T/CO <sub>2</sub> /RH x I)      | ns                  | ns                  | ns       |
|             | P(CL x T x CO <sub>2</sub> ) | ns           | ns                  | ns                  | ns       | P(CL x T/CO <sub>2</sub> /RH x I) | ns                  | ns                  | ns       |
| Petunidin   | P(CL)                        | ns           | ns                  | ***                 | ***      | P(CL)                             | ns                  | ns                  | ***      |
|             | P(T)                         | ns           | ns                  | ns                  | ns       | P(T/CO <sub>2</sub> /RH)          | ns                  | ns                  | ns       |
|             | P(CO <sub>2</sub> )          | ***          | ns                  | ns                  | ns       | P(I)                              | ***                 | ***                 | ***      |
|             | P(CL x T)                    | ns           | ns                  | ns                  | ns       | P(CL x T/CO <sub>2</sub> /RH)     | ns                  | ns                  | ns       |
|             | P(CL x CO <sub>2</sub> )     | ns           | ns                  | ns                  | ns       | P(CL x I)                         | ns                  | ns                  | ns       |
|             | P(T x CO <sub>2</sub> )      | ns           | ns                  | ns                  | ns       | P(T/CO <sub>2</sub> /RH x I)      | ns                  | ns                  | *        |
|             | P(CL x T x CO <sub>2</sub> ) | ns           | ns                  | ns                  | ns       | P(CL x T/CO <sub>2</sub> /RH x I) | ns                  | ns                  | ns       |

Probability values (P) in GCG for the main effects of clone, P(CL); temperature regime P(T); CO<sub>2</sub> regime P(CO<sub>2</sub>); and their interactions, P(CL x T), P(CL x CO<sub>2</sub>), P(T x CO<sub>2</sub>) and P(CL x T x CO<sub>2</sub>). Probability values (P) in TGG for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I), P(T/CO<sub>2</sub>/RH x I) and P(CL x T/CO<sub>2</sub>/RH x I). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant.

Table 4. ANOVA analysis of the relative abundance of anthocyanins according to their hydroxylation (A), methylation (B) and acylation (C), at maturity in *V. vinifera* cv. Tempranillo clones grown in the TGG and the GCG experiments.

| <b>A</b>         |                              | TGG |                                   | GCG |  |
|------------------|------------------------------|-----|-----------------------------------|-----|--|
| Di-hydroxylated  | P(CL)                        | **  | P(CL)                             | **  |  |
|                  | P(T)                         | ns  | P(T/CO <sub>2</sub> /RH)          | **  |  |
|                  | P(CO <sub>2</sub> )          | *** | P(I)                              | *** |  |
|                  | P(CL x T)                    | ns  | P(CL x T/CO <sub>2</sub> /RH)     | ns  |  |
|                  | P(CL x CO <sub>2</sub> )     | ns  | P(CL x I)                         | ns  |  |
|                  | P(T x CO <sub>2</sub> )      | ns  | P(T/CO <sub>2</sub> /RH x I)      | ns  |  |
|                  | P(CL x T x CO <sub>2</sub> ) | ns  | P(CL x T/CO <sub>2</sub> /RH x I) | ns  |  |
| Tri-hydroxylated | P(CL)                        | **  | P(CL)                             | **  |  |
|                  | P(T)                         | ns  | P(T/CO <sub>2</sub> /RH)          | **  |  |
|                  | P(CO <sub>2</sub> )          | *** | P(I)                              | *** |  |
|                  | P(CL x T)                    | ns  | P(CL x T/CO <sub>2</sub> /RH)     | ns  |  |
|                  | P(CL x CO <sub>2</sub> )     | ns  | P(CL x I)                         | ns  |  |
|                  | P(T x CO <sub>2</sub> )      | ns  | P(T/CO <sub>2</sub> /RH x I)      | ns  |  |
|                  | P(CL x T x CO <sub>2</sub> ) | ns  | P(CL x T/CO <sub>2</sub> /RH x I) | ns  |  |

| <b>C</b>     |                              | TGG |                                   | GCG |  |
|--------------|------------------------------|-----|-----------------------------------|-----|--|
| Non-acylated | P(CL)                        | *** | P(CL)                             | *** |  |
|              | P(T)                         | *   | P(T/CO <sub>2</sub> /RH)          | *** |  |
|              | P(CO <sub>2</sub> )          | *** | P(I)                              | *** |  |
|              | P(CL x T)                    | ns  | P(CL x T/CO <sub>2</sub> /RH)     | ns  |  |
|              | P(CL x CO <sub>2</sub> )     | ns  | P(CL x I)                         | ns  |  |
|              | P(T x CO <sub>2</sub> )      | ns  | P(T/CO <sub>2</sub> /RH x I)      | ns  |  |
|              | P(CL x T x CO <sub>2</sub> ) | ns  | P(CL x T/CO <sub>2</sub> /RH x I) | ns  |  |
| Acetylated   | P(CL)                        | *** | P(CL)                             | ns  |  |
|              | P(T)                         | *   | P(T/CO <sub>2</sub> /RH)          | *** |  |
|              | P(CO <sub>2</sub> )          | **  | P(I)                              | *** |  |
|              | P(CL x T)                    | ns  | P(CL x T/CO <sub>2</sub> /RH)     | ns  |  |
|              | P(CL x CO <sub>2</sub> )     | ns  | P(CL x I)                         | ns  |  |
|              | P(T x CO <sub>2</sub> )      | ns  | P(T/CO <sub>2</sub> /RH x I)      | ns  |  |
|              | P(CL x T x CO <sub>2</sub> ) | ns  | P(CL x T/CO <sub>2</sub> /RH x I) | ns  |  |
| Coumaroyled  | P(CL)                        | *** | P(CL)                             | *** |  |
|              | P(T)                         | *   | P(T/CO <sub>2</sub> /RH)          | *** |  |
|              | P(CO <sub>2</sub> )          | *** | P(I)                              | *** |  |
|              | P(CL x T)                    | ns  | P(CL x T/CO <sub>2</sub> /RH)     | ns  |  |
|              | P(CL x CO <sub>2</sub> )     | ns  | P(CL x I)                         | ns  |  |
|              | P(T x CO <sub>2</sub> )      | ns  | P(T/CO <sub>2</sub> /RH x I)      | ns  |  |
|              | P(CL x T x CO <sub>2</sub> ) | ns  | P(CL x T/CO <sub>2</sub> /RH x I) | ns  |  |

| <b>B</b>        |                              | TGG |                                   | GCG |  |
|-----------------|------------------------------|-----|-----------------------------------|-----|--|
| Non-methylated  | P(CL)                        | *** | P(CL)                             | *** |  |
|                 | P(T)                         | ns  | P(T/CO <sub>2</sub> /RH)          | *** |  |
|                 | P(CO <sub>2</sub> )          | *   | P(I)                              | *** |  |
|                 | P(CL x T)                    | ns  | P(CL x T/CO <sub>2</sub> /RH)     | ns  |  |
|                 | P(CL x CO <sub>2</sub> )     | *   | P(CL x I)                         | *   |  |
|                 | P(T x CO <sub>2</sub> )      | ns  | P(T/CO <sub>2</sub> /RH x I)      | ns  |  |
|                 | P(CL x T x CO <sub>2</sub> ) | ns  | P(CL x T/CO <sub>2</sub> /RH x I) | *   |  |
| Mono-methylated | P(CL)                        | **  | P(CL)                             | *** |  |
|                 | P(T)                         | ns  | P(T/CO <sub>2</sub> /RH)          | **  |  |
|                 | P(CO <sub>2</sub> )          | *** | P(I)                              | *** |  |
|                 | P(CL x T)                    | ns  | P(CL x T/CO <sub>2</sub> /RH)     | ns  |  |
|                 | P(CL x CO <sub>2</sub> )     | ns  | P(CL x I)                         | ns  |  |
|                 | P(T x CO <sub>2</sub> )      | ns  | P(T/CO <sub>2</sub> /RH x I)      | ns  |  |
|                 | P(CL x T x CO <sub>2</sub> ) | ns  | P(CL x T/CO <sub>2</sub> /RH x I) | ns  |  |
| Di-methylated   | P(CL)                        | *** | P(CL)                             | *** |  |
|                 | P(T)                         | ns  | P(T/CO <sub>2</sub> /RH)          | *** |  |
|                 | P(CO <sub>2</sub> )          | *** | P(I)                              | *** |  |
|                 | P(CL x T)                    | ns  | P(CL x T/CO <sub>2</sub> /RH)     | ns  |  |
|                 | P(CL x CO <sub>2</sub> )     | ns  | P(CL x I)                         | ns  |  |
|                 | P(T x CO <sub>2</sub> )      | ns  | P(T/CO <sub>2</sub> /RH x I)      | ns  |  |
|                 | P(CL x T x CO <sub>2</sub> ) | ns  | P(CL x T/CO <sub>2</sub> /RH x I) | *   |  |

Probability values (P) in GCG for the main effects of clone, P(CL); temperature regime P(T); CO<sub>2</sub> regime P(CO<sub>2</sub>); and their interactions, P(CL x T), P(CL x CO<sub>2</sub>), P(T x CO<sub>2</sub>) and P(CL x T x CO<sub>2</sub>). Probability values (P) in TGG for the main effects of clone, P(CL); T/CO<sub>2</sub>/RH regime P(T/CO<sub>2</sub>/RH); irrigation regime, P(I); and their interactions, P(CL x T/CO<sub>2</sub>/RH), P(CL x I), P(T/CO<sub>2</sub>/RH x I) and P(CL x T/CO<sub>2</sub>/RH x I). \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, not significant.

# FIGURES

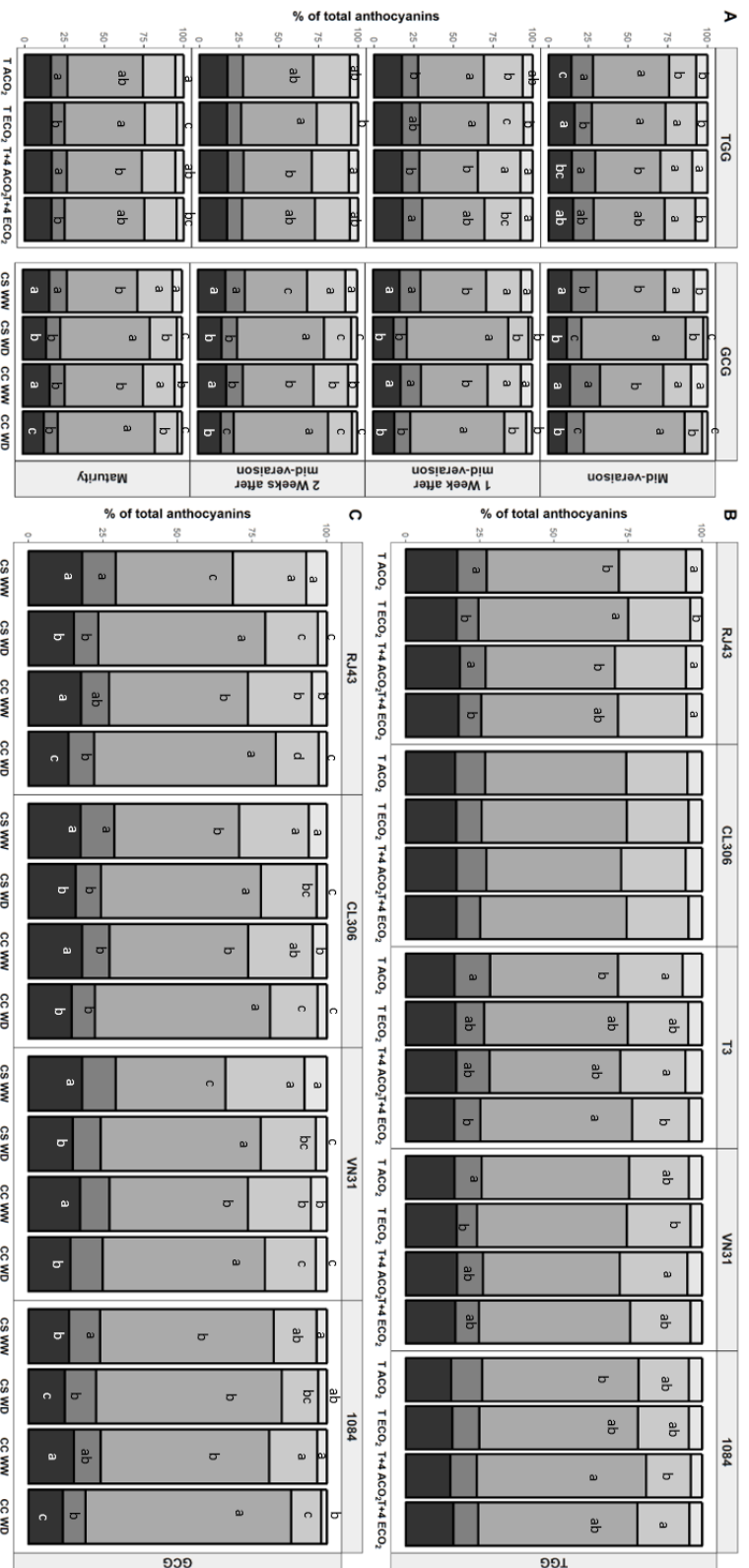


Figure 1. Relative abundance of anthocyanin families (%) in *V. vinifera* cv. Tempranillo clones grown in TGG and GCG experiments. TGG experiment: five clones (RJ43, CL306, T3, VN31 and 1084) grown under ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). GCG experiment: four clones (RJ43, CL306, VN31 and 1084) grown under two current situation (“CS”, 400 ppm, 24 °C/14 °C, and 45%/65% RH) or climate change conditions (“CC”, 700 ppm, 28 °C/18 °C, and 33%/53% RH), combined with two irrigation regimes: well-watered (WW) or water deficit (WD). Data are presented (values are means ± SE): (A) throughout ripening and according to the growing conditions considering the clones altogether (n<sub>TGG</sub> = 3-4; n<sub>GCG</sub> = 3-4). Letters in common within the same experiment, stage and anthocyanin form (in A) or within the same clone and anthocyanin form (in B and C) are not significantly different according to LSD test (P > 0.05). Figures with no letters showed not significant differences.



Figure 2. Relative abundance of anthocyanins according to their hydroxylation (%) at maturity in *V. vinifera* cv. Tempranillo clones grown in the TGG (A and B) and the GCG experiments (C and D). TGG experiment: five clones (RJ43, CL306, T3, VN31 and 1084) grown under ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). GCG experiment: four clones (RJ43, CL306, VN31 and 1084) grown under current situation ("CS", 400 ppm, 24 °C/14 °C, and 45 %/65 % RH) or climate change conditions ("CC", 700 ppm, 28 °C/18 °C, and 33 %/53 % RH), combined with two irrigation regimes: well-watered (WW) or water deficit (WD). Data are presented (values are means ± SE), according to the growing conditions in TGG (A) and GCG (C) (n<sub>TGG</sub> = 19-20; n<sub>GCG</sub> = 14-15) and according to the clone and environmental conditions in TGG (B) and GCG (D) (n<sub>TGG</sub> = 3-4; n<sub>GCG</sub> = 3-4). Means with letters in common within the same experiment and anthocyanin form (in A and C) or within the same experiment, clone and anthocyanin form (in B and D) are not significantly different according to LSD test (P > 0.05). Figures with no letters showed not significant differences.



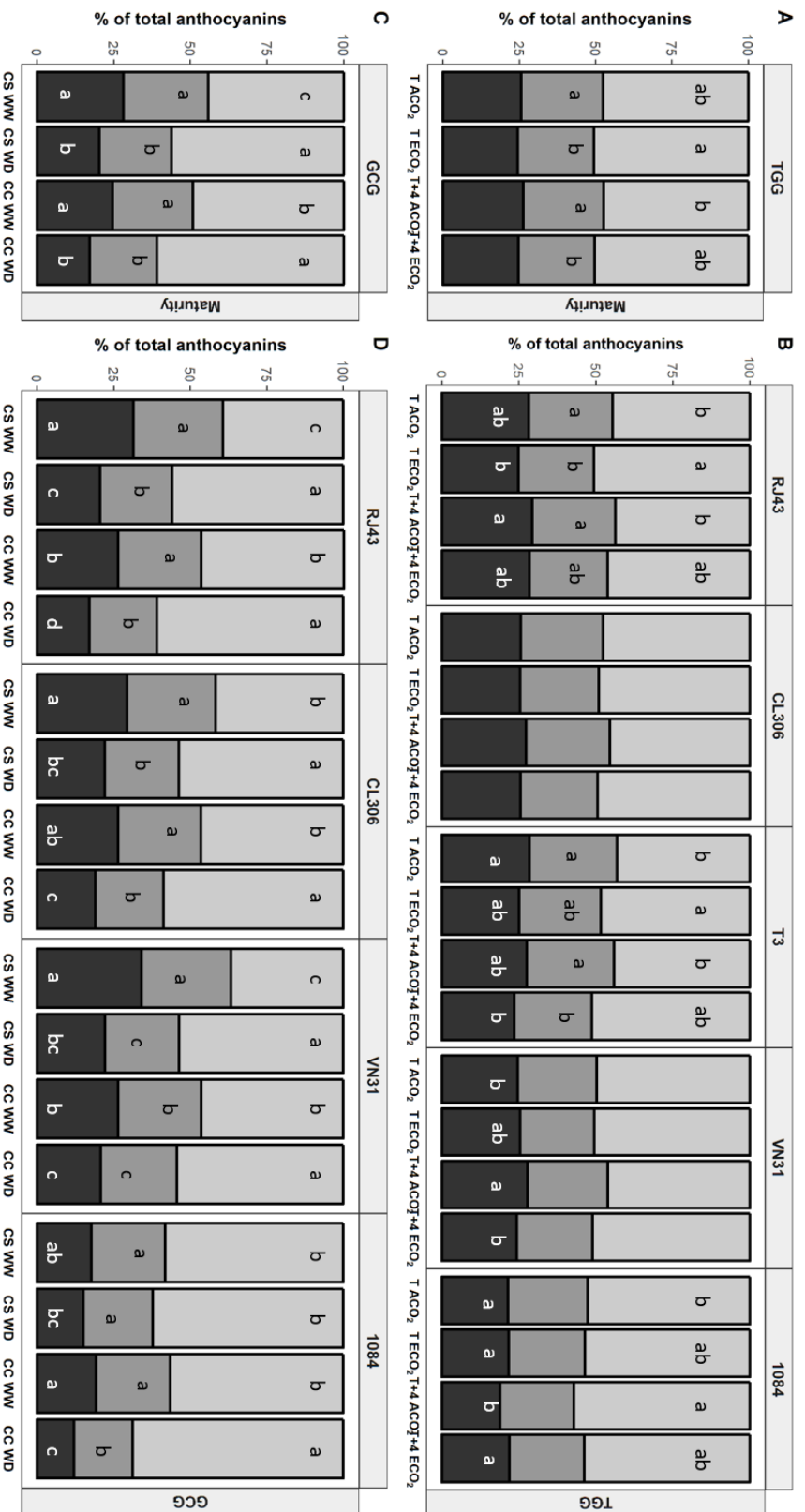


Figure 3. Relative abundance of anthocyanins according to their methylation (%) at maturity in *V. vinifera* cv. Tempranillo clones grown in the TGG (A and B) and the GCG experiments (C and D). TGG experiment: five clones (RJ43, CL306, T3, VN31 and 1084) grown under ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). GCG experiment: four clones (RJ43, CL306, VN31 and 1084) grown under current situation (“CS”, 400 ppm, 24 °C/14 °C, and 45 %/65 % RH) or climate change conditions (“CC”, 700 ppm, 28 °C/18 °C, and 33 %/53 % RH), combined with two irrigation regimes: well-watered (WW) or water deficit (WD). Data are presented according to the growing conditions in TGG (A) and GCG (C) (n<sub>TGG</sub> = 19-20; n<sub>GCG</sub> = 14-15) and according to the clone and environmental conditions in TGG (B) and GCG (D) (n<sub>TGG</sub> = 3-4; n<sub>GCG</sub> = 3-4). Means with letters in common within the same experiment and anthocyanin form (in A and C) or within the same experiment, clone and anthocyanin form (in B and D) are not significantly different according to LSD test (P > 0.05). Figures with no letters showed not significant differences.

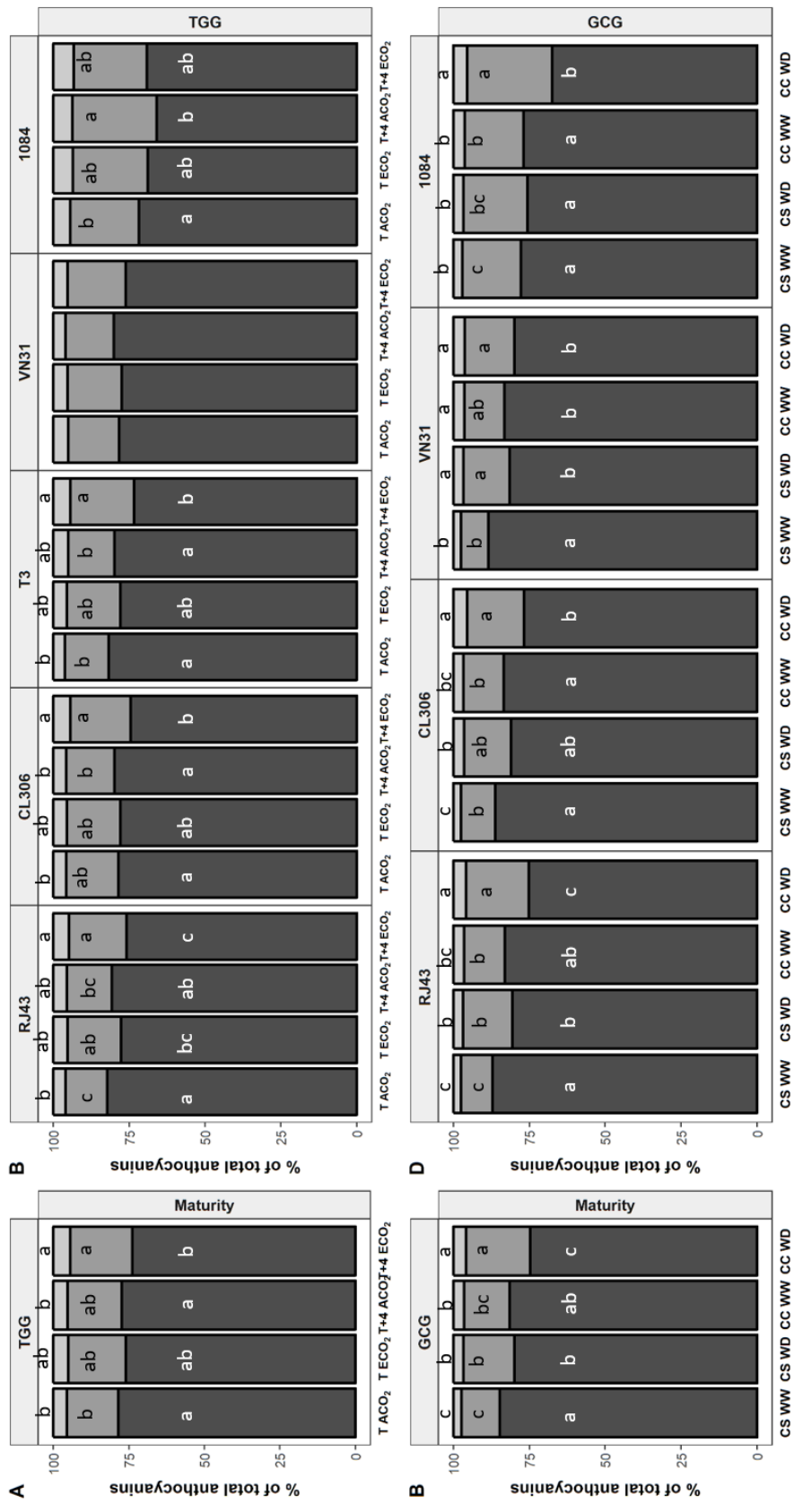


Figure 4. Relative abundance of anthocyanins according to their acylation (%) at maturity in *V. vinifera* cv. Tempranillo clones grown in the TGG and the GCG experiments. TGG experiment: five clones (RJ43, CL306, T3, VN31 and 1084) grown under ambient temperature (T) or ambient temperature + 4 °C (T+4), combined with ambient CO<sub>2</sub> (ca. 400 ppm, ACO<sub>2</sub>) or elevated CO<sub>2</sub> (700 ppm, ECO<sub>2</sub>). GCG experiment: four clones (RJ43, CL306, VN31 and 1084) grown under current situation ("CS", 400 ppm, 24 °C/14 °C, and 45%/65% RH) or climate change conditions ("CC", 700 ppm, 28 °C/18 °C, and 33%/53% RH), combined with two irrigation regimes: well-watered (WW) or water deficit (WD). Data are presented according to the growing conditions in TGG (A) and GCG (C) (n<sub>TGG</sub> = 19-20; n<sub>GCG</sub> = 14-15) and according to the clone and environmental conditions in TGG (B) and GCG (D) (n<sub>TGG</sub> = 3-4; n<sub>GCG</sub> = 3-4). Means with letters in common within the same experiment and anthocyanin form (in A and C) or within the same experiment, clone and anthocyanin form (in B and D) are not significantly different according to LSD test (P > 0.05). Figures with no letters showed not significant differences.



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## **GENERAL DISCUSSION**



## GENERAL DISCUSSION

The research carried out in the framework of this thesis aimed to understand the response of grapevine to climate change, considering different aspects of plant performance and berry composition. In order to obtain information more complete, environmental factors expected to be significantly affected by climate change (temperature, air CO<sub>2</sub> concentration and water scarcity) were considered both individually and combined. For this purpose, two different facilities were used: growth chamber greenhouses (GCGs), with a higher control of temperature and relative humidity; and temperature gradient greenhouses (TGGs), which maintain natural fluctuations in temperature, RH and radiation, and in which the impact of elevated temperature and elevated CO<sub>2</sub> can be assessed under more realistic conditions. Moreover, this research explores the intra-varietal diversity of grapevine, specifically the one originated from the difference on the length of the reproductive cycle, as a potential tool that may help to maintain the sustainability of viticulture in the future in terms of quality factors of berries in relation with wine production.

### 1. PHOTOSYNTHETIC ACTIVITY AND VEGETATIVE GROWTH

There is no consensus on the effect of elevated temperature on photosynthesis and vegetative production of grapevine, as some authors have described a decrease in vegetative growth [1], while others report no effect either on the leaf area [2] or on the photosynthetic activity [3,4]. In the present work, high temperature applied as a single factor tended to decrease leaf area in some clones, when it was applied in GCGs, which maintained a constant temperature regime (24 °C/14 °C vs. 28 °C/18 °C) throughout the experiment. In contrast, in the experiments carried out in TGGs, high temperature enhanced photosynthetic activity and vegetative growth. The modular structure of TGGs allows to study the impact of higher temperature under more “natural” conditions, since in module 1 temperature tracks as much as possible the diurnal changes in external temperature, whereas in the other extreme (module 3) temperature was ambient + 4 °C. Regarding the impact of elevated air CO<sub>2</sub> concentration, the response of grapevine plants included the increase of final vegetative dry weight without modifying leaf area, thus rising significantly the specific leaf weight, phenomenon already observed by Kizildeniz et al. [2]. Such effect of elevated CO<sub>2</sub> was also observed when this factor was combined with elevated temperature, with consistent results in both GCG and TGG experiments. The increased vegetative growth was concomitant with higher photosynthetic rates measured around mid-veraison in plants exposed to elevated CO<sub>2</sub> as well as a higher photosynthetic activity [1,5–9]. However, signs of photosynthesis acclimation to elevated CO<sub>2</sub>, such as an increased C/N ratio, were observed especially when elevated CO<sub>2</sub> was combined with high temperature. The results suggest a down-

regulation of the photosynthetic capacity of grapevine after a long-exposure to elevated CO<sub>2</sub> [10], which was intensified by elevated temperature, as described in other plant species [11]. Water deficit reduced stomatal conductance, a response to water deficit well-described in grapevine [12], decreasing photosynthetic activity and C assimilation, thus reducing leaf area and vegetative dry weight [13–15]. Under drought conditions, the positive effect of high temperature and elevated CO<sub>2</sub> on C fixation and plant growth disappeared, as observed by Leibar et al. under similar experimental conditions [16].

The clones studied showed differences in their vegetative growth at maturity, 1084 showing consistently in TGG and GCG experiments the largest leaf area and vegetative dry weight. Also, this clone showed a higher presence of labelled C in the cutting. However, all these differences were not concomitant with a higher photosynthetic activity in this clone, but they were probably associated with the longer phenological cycle observed in this accession, thus having more time to increase its vegetative growth and the C reserves in the cutting. Such reason, rather than a higher sensitivity of this clone to the environmental conditions, may also explain the stronger impact of elevated CO<sub>2</sub>, high temperature (especially their combination) and water deficit on 1084 compared with the other clones studied.

## **2. REPRODUCTIVE GROWTH**

Plants grown under elevated temperature in TGGs had a lower bunch weight, as a result of the reduction in berry number and berry size (both weight and diameter), such effect being also evident when high temperature was combined with elevated CO<sub>2</sub> in these facilities. The results agree with previous studies, which have reported a negative impact of elevated temperature on yield and yield components for different grapevine cultivars [17–21]. In contrast, in the GCG experiments, CC treatment (combined high temperature, elevated CO<sub>2</sub> and low relative humidity) did not have a deleterious effect either on bunch or berry characteristics. The heat waves experienced by plants in the treatment with high temperature in the TGGs (that mimicked ambient temperature + 4 °C) may explain the higher impact of temperature in this experiment. Elevated CO<sub>2</sub> applied as a single factor did not have a marked impact on bunch and berry size, as recently reported by Kizildeniz et al. in a three-year experiment with red and white Tempranillo fruit-bearing cuttings [6]. Conversely, longer exposure to high CO<sub>2</sub> using Free Air CO<sub>2</sub> Enrichment (FACE) in several consecutive years increased the final fruit production of Riesling, Cabernet Sauvignon and Sngiovese, especially from the second year onwards [7,22]. Wohlfahrt et al. [22] associated this result to an effect of elevated CO<sub>2</sub> on the inflorescence initiation in the previous year, rather than to a direct effect of the CO<sub>2</sub> during inflorescence and bunch development. Water deficit was the factor that most affected bunch weight,

reducing both the number of berries and their weight, as observed by other authors, especially when drought was applied before veraison [23]. Medrano et al. described a significant correlation between the reduction of photosynthetic activity and the low grape yield observed in Tempranillo under drought conditions [15]. So, the low yield under conditions mimicking 2100-situation could be caused by the reduction of photosynthetic activity observed in this treatment as well as by the reduction of cell expansion [13,23,24].

The clones studied showed clear differences in their bunch and berry characteristics, 1084 having the lower bunch weight, associated with a lower number of berries, but the highest berry size, which are not desirable traits in viticulture [25–28]. When considering a future scenario of climate change combining high temperature, high CO<sub>2</sub> concentration and drought, RJ43 and CL306 were the most affected clones in terms of bunch and berry characteristics, meanwhile VN31 and 1084 maintained similar values of bunch weight and berry size compared with current conditions.

### **3. PHENOLOGY AND RIPENING**

High temperature increased sugar accumulation in the berries, thus advancing maturity, supporting model predictions as well as previous studies [20,29–39]. Sugar accumulation is mainly dependant on the storage of translocated photoassimilates [40], although reserves from other organs as well as gluconeogenesis might also get involved in the process [41]. Although no significant differences in net photosynthesis per unit of area were observed between temperature treatments in the experiment under more realistic conditions in TGGs, integrated higher C fixation rate at the whole plant level, as a result of the increased leaf area in this treatment, may also have been behind the advanced maturity under elevated temperature. In addition, the reduction in berry size might have also contributed to sugar concentration, thus hastening the ripening period [13]. Elevated CO<sub>2</sub> slightly advanced maturity when it was applied independently, but it had a more marked effect between onset of veraison and mid-veraison, as observed by Martínez-Lüscher et al. [30]. In fact, sugar accumulation during the ripening period in the grapes grown under high CO<sub>2</sub> was not modified, thus suggesting that other organs (leaves, roots, stem or cuttings) acted as powerful sinks of photoassimilates. The combination of both high temperature and elevated CO<sub>2</sub> concentration had additive effects advancing maturity, whereas water deficit reduced berry sugar accumulation and delayed maturity, through a drastic reduction in net photosynthesis. Severe water deficit has been reported to slow-down ripening in grapevine [30,42,43]. Intrigliolo et al. concluded that reducing in cv. Tempranillo water supply post-veraison can impair sugar accumulation due to detrimental effects of water stress on leaf photosynthesis, behaviour also observed by other authors [42,44]. The present results suggest that



water scarcity may partially alleviate the hastening effect of elevated temperature in a future climate change scenario.

Among the clones studied, 1084 showed consistently a lower sugar accumulation rate, thus leading to a longer ripening period. In fact, this clone was not able to reach in some cases common maturity levels of 22 °Brix. The low sugar concentration of this clone may have negative implications for wine making, since the fermentation process may be affected, and produce wines with a low alcoholic degree [45,46]. Besides, it was observed some degree of variability among clones in their phenological response to simulated CC conditions. RJ43, one of the most widely distributed clones of Tempranillo, was more affected by elevated temperature, high CO<sub>2</sub> and water deficit, whereas in 1084 the phenological development was almost not modified by these environmental factors.

## 4. BERRY COMPOSITION

### 4.1. *Organic acids*

Total acidity in grapes decreases throughout berry ripening due mainly to the degradation of malic acid. This process is well-known to be enhanced by the increase in air temperature [18,47–49], as could also be observed in the present study. Malic acid can be degraded through different pathways, including respiration [47,50] and gluconeogenesis [51,52]. In the present study we did not observe an increase in total amino acid concentration with high temperature, did not either in those derived from  $\alpha$ -ketoglutarate, pyruvate or aspartate, which may rule out the flux of malic acid through the TCA cycle providing precursors for amino acid biosynthesis. This, together with the raise in sugar accumulation, suggests that other routes such as gluconeogenesis may have contributed to malic acid degradation under these conditions, thus promoting, in some extent, sugar accumulation. Malic acid accumulation was enhanced by high atmospheric CO<sub>2</sub> concentration in early ripening stages, but also its degradation in later stages, thus reinforcing the impact of elevated temperature. However, in this case, the rise in malic acid degradation was not translated into higher levels of sugar, while the concentration of some amino acids (as GABA and pyruvate derivatives) was increased. In his case, malic acid degradation might be boosted by an increased flux through the TCA cycle and elevated CO<sub>2</sub> may increase the anaplerotic capacity of the TCA cycle for amino acid biosynthesis[47]. Conversely, malic acid levels were not affected by water deficit, but total acidity was lower as tartaric acid concentration was reduced. The reduction in malic acid and total acidity observed at maturity when the environmental factors studied were applied simultaneously might have implications for wine making, affecting yeast activity and the presence of undesired microorganism [45] as well as wine colour and stability [18,45].

Regarding differences among clones, the lower malic acid and total acidity values repeatedly observed in 1084 at maturity might be a consequence of the long reproductive cycle of this accession. In addition, the higher relative abundance GABA and arginine, as well as pyruvate and aspartate derivatives, not accompanied by higher sugar accumulation rates, may suggest the use of malate in supplementing TCA cycle and its anaplerotic capacity for amino acid biosynthesis in this clone, rather than in gluconeogenesis. Under combined of high temperature, high CO<sub>2</sub> and drought, all the clones showed a reduced total acidity at maturity compared with current conditions. However, 1084 stood out for reaching the lowest levels, whereas CL306 was slightly less affected than the rest of the clones.

#### *4.2. Amino acid concentration and profile*

Amino acids play a major role in wine production, as they are the main source of nitrogen for yeast during must fermentation. Proline, however, is not a component of the yeast assimilable nitrogen (YAN) [53] but it is reported to be an important compound as plant protector against multiple environmental stresses [54,55]. Warm temperatures have been described to increase amino acid concentration in grapes and to increase the relative abundance of proline and arginine [46,50]. In the present study, the increase of 4 °C, both individually and combined with elevated CO<sub>2</sub>, and both under temperature controlled conditions (GCGs) and under more realistic temperature conditions (TGGs), did not modified significantly total amino acid levels at maturity, although these tended to decrease, as reported by Martínez-Lüscher et al. [56]. High temperature did not modify proline relative abundance either, but it increased the proportion of arginine in the berry, which is the most important amino acid for yeast in the must and plays an important role in grape berry nitrogen metabolism [57,58]. Consequently, the decrease of proline:arginine ratio may result in a more fermentable must for the same total amino acid concentration in the future, as proline-N is not utilized by yeast under normal conditions in the fermentation process [59].

Vine water status has been reported to affect the amino acid concentration of grapes in different ways. Decreases in the concentration of must amino acids have been described in Tempranillo plants subjected to water deficit [53,60]. In contrast, drought conditions resulted in higher amino acid levels in Grenache Noir and Chardonnay [61,62]. In the present study, water deficit, acting as a single environmental factor and combined with high CO<sub>2</sub> and elevated temperature, increased the concentration of total amino acids and modified the amino acid profile, although the degree of this effect depended on the clone, 1084 being the least affected. Although, to the best of our knowledge, there is no work in the literature that describes differences in amino acid levels and profiles among grapevine clones, diversity of responses to drought among cultivars have been reported [63,64]. Moreover, the response of the clones studied to high temperature and elevated CO<sub>2</sub> also differed

depending on the water availability, affecting total amino acid concentration and amino acid profile. In consequence, the amino acid levels increased in RJ43 and CL306 under simulated 2100 environmental conditions (high temperature, elevated CO<sub>2</sub> and water deficit), while decreasing in the case of VN31 and not changing in 1084. However, reduction of shikimate derivatives due to the mentioned conditions was stronger in RJ43 and CL306 than in the other clones, which may affect their anthocyanin production and the aromatic capacity of wines.

### *4.3. Anthocyanin concentration and profiles*

High temperature has been reported to decrease total anthocyanin concentration [50,65–68], phenomena associated with a reduction in the expression of genes coding enzymes with significant roles in the phenylpropanoid pathway, as well as an increase in anthocyanin degradation [68,69]. Such decrease in anthocyanins together with the increase in sugar accumulation usually led to a temperature induced decoupling of these traits, as described by Sadras et al. and Martínez de Toda and Balda in different grapevine cultivars [70,71].

In the present study, the impact of elevated temperature depended on the clone studied. In the experiment with 13 clones in GCG conditions, elevated temperatures reduced significantly anthocyanin levels at maturity when considering all the clones as a whole. However, analysing each clone individually these differences were significant in 5 out of the 13 clones studied, and 6 out of the 13 clones showed a significant reduction in the anthocyanin to sugar ratio. In the case of TGGs experiment, the clones studied did not showed a reduction in total anthocyanins induced by temperature, which agrees with the results obtained by Kizildeniz et al. [6]. However, it was observed a generalised decrease in the anthocyanins to TSS ratio, more marked in some accessions as in RJ43. The results suggest that the thermal disruption of the anthocyanins:TSS relationship can differ among clones. Differences in anthocyanin concentration in response to temperature changes have been reported for Merlot [72–74], Malbec [75], Pione [76], Cabernet Sauvignon [50,68,77], Muscat Hamburg [78] and Tempranillo [1]. In addition, Tempranillo clones have been reported to respond in different ways to temperature [46]. Besides genetic diversity, the berry stage at which the thermal treatment was applied, the intensity of the increase in day and night temperatures as well as the gap between them may be factors that modulate the sensitivity to elevated temperatures [50,68,79–81]. When high temperature was combined with elevated CO<sub>2</sub>, both in TGGs and GCGs, the decoupling effect induced by temperature on anthocyanin:TSS ratio was partially or totally alleviated in some clones. In contrast, the combination of those factors with drought caused a strong reduction in anthocyanin concentration similarly to the response observed by Kizildeniz et al. [6], thus leading to an imbalance between anthocyanins and sugars. Severe water deficit has been reported to have a negative impact on

anthocyanin accumulation [1,6,82–84], which has been related to an increased anthocyanin degradation at the latter stages of ripening and to a reduction of their biosynthesis [83].

Regarding the impact of climate change related factors on anthocyanin profile, both high temperature and especially elevated CO<sub>2</sub> modified the anthocyanin profile towards more stable form: acylated anthocyanins in the case of T+4, and malvidin, tri-hydroxylated, di-methylated and acylated forms in the case of ECO<sub>2</sub>. Also the combination of these factors increased the relative abundance of acylated and tri-hydroxylated anthocyanins in both TGG and GCG experiments. Climate change conditions and water deficit showed additive effects on the anthocyanin profile, since grapes ripened under CC and WD treatments exhibited the highest abundances of malvidin, tri-hydroxylated, di-methylated and acylated anthocyanins. The changes induced by water deficit agrees with previous studied [46,63,85–88]. In particular, the increase in the proportion of malvidin, and therefore, of di-methylated forms, has been associated with a higher OMT1 gene expression under water deficit conditions [89,90], but also could be related with a higher degradation of anthocyanins in this treatment. Also, Zarrouk et al. reported that anthocyanin methoxylation might represent a strategy to cope with the combined effects of water shortage and heat stress [83]. Consequently, water deficit contributed to increase the level of stability of anthocyanins under climate change conditions, which in terms of wine production, may significantly contribute to the accumulation, intensity and stability of colour [91].

A wide variability in the grape anthocyanin content and profile was observed among clones, which may have consequences in the wine colour, as this trait is mainly determined by anthocyanin content and profile [92]. Even though in 1084 anthocyanin profile did not suffer great changes due to expected future environmental conditions, this accession stood out repeatedly for having the lowest levels of total anthocyanin concentration, regardless of the experiment, suggesting wines with low colour intensity. Taking into account the relatively high concentration of phenylalanine in the 1084 accession, the difference of anthocyanin concentration might result from a lower biosynthetic activity, associated with a low sugar accumulation rate, rather than to a limitation in anthocyanin precursors [93]. In this sense, further research would be needed, including transcriptomic analyses to evaluate this hypothesis and to understand better the difference in anthocyanin concentration among clones.

Regarding the performance of the clones studied under the 2100-foreseen environmental conditions, the results suggest that the clones of Tempranillo most widely distributed and used in the last decades (RJ43 and CL306) [94] may be more affected in terms of grape colour in a future scenario. However, despite such reduction in total anthocyanins, the grapes of the RJ43 accession seemed to be enriched in more stable forms that may improve colour intensity and stability, while guaranteeing wine preservation [68,95–101]. Besides, the higher increase in malvidin content in this clone may improve

the health promoting effects of the wine produced [102]. Nevertheless, it would be interesting to study other phenolic compounds, notably tannins, in order to evaluate the ability of anthocyanin-tannin complexation and to elaborate wine through micro-vinification to determine its astringency capacity. Considering the behaviour of the clones studied in relation to their characterisation as short or long phenological development clones, results suggest that the use of intra-varietal diversity attending to this feature does not ensure the anthocyanin levels to be maintained under future environmental conditions.

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## **GENERAL CONCLUSIONS**



## GENERAL CONCLUSIONS

1. In the experimental conditions studied, the combination of elevated temperature and high CO<sub>2</sub> increased plant photosynthetic activity and, consequently, vegetative growth. Although these factors had a minor impact on the reproductive growth, reductions in bunch size cannot be ruled out in the future as a consequence of a higher frequency of heat waves, as it was observed in the experiment under more realistic temperature conditions in the TGGs. Water deficit negatively impacted C fixation, vegetative and reproductive production, annulling the positive effects of high temperature and CO<sub>2</sub> on plant growth.
2. Elevated CO<sub>2</sub> increased grapevine photosynthesis, but signs of photosynthetic acclimation were observed, especially when elevated CO<sub>2</sub> and high temperature were applied simultaneously.
3. Maturity was advanced by elevated temperature regardless of the CO<sub>2</sub> level. However, when plants under high temperature and high CO<sub>2</sub> were also subjected to water deficit, the latter alleviated the advance in maturity by slowing down sugar accumulation and, consequently, the ripening process.
4. Malic acid degradation during ripening was enhanced by elevated temperature and high CO<sub>2</sub>. The latter seeming to favour the catabolic flux through the TCA cycle over its use in gluconeogenesis. The combination of these environmental factors with drought decreased malic acid and total acidity at maturity, which may imply wine production to be negatively affected in the future.
5. The impact of elevated temperature and high CO<sub>2</sub> on total amino acid concentration in grapes was dependent on the water availability and on the clone studied. In general terms, combined elevated temperature, high CO<sub>2</sub> and water deficit increased amino acid concentration. Water deficit was the factor that most impacted amino acid profile, decreasing shikimate and phosphoglycerate derivatives.
6. Elevated temperature and CO<sub>2</sub>, both acting individually or in combination, did not markedly affect total skin anthocyanins under well-watered conditions. When these two climate change related factors were combined with water deficit, a significant reduction in anthocyanins was observed, as well as on the anthocyanin to sugar ratio.



7. The environmental factors studied induced changes in anthocyanin profile. Elevated temperature increased acylated forms, whereas both elevated CO<sub>2</sub> and water deficit increased the relative abundance of malvidin, acylated, tri-hydroxylated and methylated forms. The results suggest that, under the environmental conditions projected by the end of the 21<sup>st</sup> century, grapes could be enriched in more stable anthocyanin forms.
8. Intra-varietal diversity was observed in relation to vegetative and reproductive growth, phenological development and grape composition. 1084 was the clone that most differed from the others studied, showing the longest reproductive cycle, bigger berries, low sugar levels, acidity and anthocyanin concentration at maturity. These characteristics suggest that this clone may not be an interesting alternative for wine production in the future.
9. Variability in the response to changes in temperature, CO<sub>2</sub> and water availability was observed among the clones studied but it depended on the parameter analyzed. RJ43 was the most affected accession in terms of phenological development, grape skin anthocyanin concentration and profile when all the environmental factors were applied simultaneously. Conversely, VN31 was the clone that maintained the highest anthocyanin concentration and anthocyanin:TSS ratio under the projected environmental conditions. These results reveal the importance of testing the performance of different clones under foreseen climate conditions.
10. The length of the reproductive cycle conditioned some responses to elevated temperature, high CO<sub>2</sub> and water deficit in terms of vegetative growth and of the potential to allocate C into different organs. Regarding grape composition, the differences in the response to the expected environmental conditions observed among clones were not always associated with differences in the length of their reproductive period. In this sense, further research considering transcriptomic analyses would help to understand the mechanisms behind the observed diversity of responses.