

1 **Carbon balance, partitioning and photosynthetic acclimation in fruit-**
2 **bearing grapevine (*Vitis vinifera* L. cv. Tempranillo) grown under**
3 **simulated climate change (elevated CO₂, elevated temperature and**
4 **moderate drought) scenarios in temperature gradient greenhouses**

5
6 Carolina Salazar-Parra^{1,#}, Iker Aranjuelo², Inmaculada Pascual¹, Gorka Erice^{1,&}, Álvaro Sanz-
7 Sáez^{1,†}, Jone Aguirreolea¹, Manuel Sánchez-Díaz¹, Juan José Irigoyen¹, José Luis Araus³, and
8 Fermín Morales^{4,1*}

9
10 ¹Grupo de Fisiología del Estrés en Plantas (Dpto. de Biología Ambiental), Unidad Asociada al
11 CSIC, EEAD, Zaragoza e ICVV, Logroño. Facultades de Ciencias y Farmacia, Universidad
12 de Navarra, Irunlarrea 1, 31008, Pamplona, Spain.

13 ²Instituto de Agrobiotecnología (IdAB), Universidad Pública de Navarra-CSIC-Gobierno de
14 Navarra, Campus de Arrosadía, 31192-Mutilva Baja, Spain.

15 ³Unidad de Fisiología Vegetal, Universidad de Barcelona, Av. Diagonal 645, 08028, Spain.

16 ⁴Dpto. de Nutrición Vegetal, Estación Experimental de Aula Dei (EEAD). Consejo Superior
17 de Investigaciones Científicas (CSIC). Apdo. 13034, 50080 Zaragoza, Spain.

18 [#]Current address: Instituto de Investigaciones Agropecuarias, INIA La Platina, Santa Rosa
19 11610, Santiago, Chile.

20 [&]Current address: Institute for Genomic Biology, University of Illinois at Urbana Champaign,
21 1206 W. Gregory Drive, Urbana IL, 61801, USA.

22 [†]Current address: Plant Biology Department, University of Illinois at Urbana Champaign, 147
23 Edward R. Madigan Laboratory 1201 West Gregory Drive, Urbana IL, 61801, USA.

24
25 *** Correspondence**

26 **Corresponding autor:** Fermín Morales

27 Grupo de Fisiología del Estrés en Plantas (Dpto. de Biología Ambiental), Unidad Asociada al CSIC,
28 EEAD, Zaragoza e ICVV, Logroño. Facultades de Ciencias y Farmacia, Universidad de Navarra,
29 Irunlarrea 1, 31008, Pamplona, Spain.

30 email: fmorales@eead.csic.es

31 FAX: 34 948 425649; Phone: +34 948 425600 Ext: 806608

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1 **Abstract**

2 Although plant performance under elevated CO₂ has been extensively studied in the
3 past little is known about photosynthetic performance changing simultaneously CO₂,
4 water availability and temperature conditions. Moreover, despite of its relevancy in crop
5 responsiveness to elevated CO₂ conditions, plant level C balance is a topic that,
6 comparatively, has received little attention. In order to test responsiveness of grapevine
7 photosynthetic apparatus to predicted climate change conditions, grapevine (*Vitis*
8 *vinifera* L. cv. Tempranillo) fruit-bearing cuttings were exposed to different CO₂
9 (elevated, 700 ppm *versus* ambient, ca. 400 ppm), temperature (ambient *versus* elevated,
10 ambient +4 °C) and irrigation levels (partial *versus* full irrigation). Carbon balance was
11 followed monitoring net photosynthesis (A_N, C gain), respiration (R_D) and
12 photorespiration (R_L) (C losses). Modification of environment ¹³C isotopic composition
13 (δ¹³C) under elevated CO₂ (from -10.30 to -24.93 ‰) enabled the further
14 characterization of C partitioning into roots, cuttings, shoots, petioles, leaves, rachides
15 and berries. Irrespective of irrigation level and temperature, exposure to elevated CO₂
16 induced photosynthetic acclimation of plants. C/N imbalance reflected the inability of
17 plants grown at 700 ppm CO₂ to develop strong C sinks. Partitioning of labeled C to
18 storage organs (main stem and roots) did not avoid accumulation of labeled
19 photoassimilates in leaves, affecting negatively Rubisco carboxylation activity. The
20 study also revealed that, after 20 days of treatment, no oxidative damage to chlorophylls
21 or carotenoids was observed, suggesting a protective role of CO₂ either at current or
22 elevated temperatures against the adverse effect of water stress.

23

24 **Running title:** Grapevine C balance within climate change context

25 **Keywords:** Carbon balance; Climate change; Grapevine; Photosynthesis.

1

2 **Abbreviations:** A_N , photosynthesis; Chl, chlorophyll; C_i , sub-stomatal CO_2
3 concentration; DW, dry weight; E, transpiration; EEA, European Environmental
4 Agency; ETR, electron transport rate; FW, fresh weight; g_s , stomatal conductance; HI,
5 harvest index; J_{max} , maximum electron transport rate contributing to RuBP regeneration;
6 PAR, photosynthetically active radiation; PI, partially irrigated; PPFD, photosynthetic
7 photon flux density; PSI, photosystem I; PSII, photosystem II; qP, photochemical
8 quenching; R_D , dark respiration; R_L , photorespiration; ROS, reactive oxygen species;
9 RWC, relative water content; RuBP, ribulose-1,5-bisphosphate; T_{amb} , ambient
10 temperature; TGG, temperature gradient greenhouse; TOM, total organic matter; TW,
11 turgid weight; T_{+4} , elevated temperature; $V_{c_{max}}$, maximum carboxylation velocity of
12 Rubisco; VPDB, vienna pee dee celemnite calcium carbonate; WI, well irrigated; WSC,
13 water-soluble compound; $\delta^{13}C$, C isotopic composition; Φ_{PSII} , actual photosystem II
14 efficiency; $\Phi_{exc.}$, intrinsic photosystem II efficiency.

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1 **Introduction**

2 Carbon dioxide (CO₂) concentration has increased since pre-industrial period from 280
3 to 401.85 μmol mol⁻¹ (ppm) currently (NOAA-ESRL, 2014). It is expected that this
4 value could increase to an atmospheric concentration of between 750 and 1300 ppm for
5 the end of the century, if no corrective measures are taken to constrain emissions (IPCC,
6 2014). Emissions of greenhouse gases caused by human activities have augmented 70%
7 from 1970 to 2004. If greenhouse gases emissions continue at high levels, temperature
8 is predicted to increase between 1.8 and 6.0 °C (IPCC, 2014). In fact, annual average
9 minimum temperatures in Spain have increased over the last century by around 1.5 °C.
10 The expected warming is going to be greatest in summer in South-western Europe.
11 More specifically, according to the European Environmental Agency (EEA), an average
12 rise of 4 °C is predicted by 2080 and extreme summers like the 2003 are likely to
13 become four times as common in Spain (and Southern Europe). In the other hand,
14 precipitation is projected to decrease, in average, by 22 % for the same period.
15 Therefore, global agricultural production will be profoundly impacted (IPCC, 2007).

16 Since plants with C₃ photosynthetic metabolism are CO₂ limited, it was expected
17 that any CO₂ increase would lead to higher photosynthetic rates (Long, 1991). In a
18 previous study conducted by Drake et al. (1997) exposure to elevated CO₂ increased
19 photosynthetic rates (A_N) up to 58%. However, in long-term experiments, it has been
20 reported that the initial stimulation of photosynthesis following CO₂ application often
21 does not persist and A_N declines below its maximum potential in an acclimation process
22 described as photosynthetic acclimation or photosynthetic down-regulation (Jifon and
23 Wolfe, 2002; Long et al., 2004; Erice et al., 2006b). Photosynthetic down-regulation
24 can be induced by limitations in stomatal and non-stomatal processes. Limitations in
25 stomatal opening have been described to limit A_N as a consequence of stomatal closure

1 and the corresponding decreased sub-stomatal CO₂ concentration (C_i) (Sánchez-Díaz et
2 al., 2004). Both stomatal (g_s) and mesophyll conductance reductions mediated by
3 drought decrease the Rubisco CO₂ availability, decreasing the CO₂ concentration in the
4 chloroplasts (Flexas et al., 2002; 2009), and are the major cause for the decreased
5 photosynthesis observed in grapevines under water scarcity (Flexas et al., 2010). When
6 drought progresses, the photochemistry and biochemistry of the photosynthesis can be
7 affected, reducing the grapevine photosynthetic capacity (Flexas and Medrano, 2002;
8 Morales et al., 2006). In fact, non-stomatal limitations may also explain decreases in A_N
9 being the result of reduced light capture (PSII activity) and/or decreased carboxylation
10 of RuBP catalyzed by Rubisco. According to Kalina et al. (1997), inhibition is caused
11 by decreased PSII efficiency as a result of the accumulation of inactive PSII reaction
12 centers and the decrease in light harvesting complexes. Other authors suggest that non-
13 stomatal limitation of photosynthesis is attributable to reduced carboxylation efficiency
14 (Long et al., 2004), or to reduced amount/activity of Rubisco. There are two basic
15 mechanisms by which Rubisco down-regulation occurs. The first mechanism
16 hypothesizes that the reduction in Rubisco content occurs as a consequence of the leaf C
17 build-up (Moore et al., 1999; Aranjuelo et al., 2008; 2009; 2011). According to this
18 theory, when plants exposed to elevated CO₂ have limitations for increasing C sink
19 strength, plants decrease their photosynthetic rates to balance C source activity and sink
20 capacity (Aranjuelo et al., 2008; 2009). From this point of view, the reduction in
21 photosynthetic rates would be conditioned by a plant's ability to develop new sinks (e.g.
22 new vegetative or reproductive structures, enhanced respiratory rates), or to expand the
23 storage capacity or growth rate of existing sinks. According to the second mechanism,
24 decreases in Rubisco content may reflect a general decrease of leaf protein due to the
25 relocation of N within the plant (Aranjuelo et al., 2011).

1 Carbon isotope tracers have proved to be an essential tool to study carbon
2 partitioning in plants exposed to elevated CO₂ (Aranjuelo et al., 2008; 2009). After
3 feeding the plants with the stable isotopes (pulse), isotopes are distributed over the
4 different plant organ network that can be followed by the later elemental analyzer-
5 isotope ratio mass spectrometry (EA-IRMS) analyses. Labeling with ¹³C/¹²C as tracers
6 and characterization of the distribution of labeled compounds into the different plant
7 organs has provided novel and relevant information in studies determining the flow of C
8 through the plants under elevated CO₂ (Kolb and Evans, 2003; Aranjuelo et al., 2009;
9 Molero et al., 2011). In contrast to gas exchange techniques that provide measurements
10 of photosynthetic rates at a single time, when analyzed in leaf dry matter, C isotopic
11 composition ($\delta^{13}\text{C}$) integrates photosynthetic activity throughout the period the leaf
12 tissue was synthesized. Moreover, leaf $\delta^{13}\text{C}$ values reflect the interplay among all
13 aspects of plant carbon and water relations and are thereby more useful than plant gas
14 exchange measurements as integrators of whole plant function (Aranjuelo et al., 2009).

15 Many authors have investigated the effects of CO₂, temperature and water stress
16 independently. Interactive effects of elevated CO₂, water stress and temperature have
17 been rarely examined in the past (Lloyd and Farquhar, 2008). Elevated CO₂ decreases
18 stomatal conductance and transpiration rates (Drake et al., 1997; Del Pozo et al., 2005).
19 This CO₂-mediated behavior may influence plant responses to water stress. Different
20 research groups reported decreased photosynthetic rates due to water stress delayed
21 under elevated CO₂ conditions, and an enhanced drought tolerance under elevated CO₂
22 (Robredo et al., 2007; 2010). Some studies reported increased photosynthetic rates in
23 response to elevated CO₂ under elevated temperature, but not others (Logan et al., 2010,
24 and references therein). However, the analyses of the CO₂ effect and its interaction with
25 other environmental conditions are of great relevance because the responsiveness of

1 plants to enhanced CO₂ has been shown to differ with temperature, soil water
2 availability, etc. (Aranjuelo et al., 2006; Erice et al., 2006a). Moreover, different
3 stresses often occur simultaneously in the field, such as high temperatures and drought
4 periods, especially in semi-arid or drought-stricken areas. Investigations, performed on
5 field crops as well as on model plants subjected to combined heat and drought stress,
6 have shown that the combination of these two stresses has a stronger detrimental effect
7 on plant growth and productivity compared to each single stress. Furthermore, some
8 reports indicate that it is not possible to extrapolate plant responses to combined stresses
9 starting from the response derived from a single stress (Rampino et al., 2012).

10 Carbon balance and photosynthetic acclimation remain largely unexplored in
11 grapevine growing under climatic change conditions. Furthermore, the experimental
12 design of the present work tried to emulate natural environment and also to avoid the
13 effects of other external factors (such as radiation, temperature changes, etc.). This was
14 achieved by using temperature gradient greenhouses (TGG), which allowed us to
15 provide a semi-controlled environment where simulate climate change conditions.
16 Therefore, the aim of this work was to investigate the effects of interacting CO₂,
17 temperature and water availability in photosynthetic responsiveness and C partitioning
18 of *Vitis vinifera* (cv. Tempranillo) plants grown in near field conditions. For this
19 purpose, we proceeded to the characterization of physiological parameters, C/N and
20 $\delta^{13}\text{C}$ of roots, cuttings, shoots, petioles, leaves, rachides and berries.

21 **Material and Methods**

22 *Plant material and growth conditions*

23 Dormant cuttings of *Vitis vinifera* L. cv. Tempranillo were obtained from an
24 experimental vineyard of the Station of Viticulture and Enology of Navarra (Olite,

1 Navarra, Spain). Cuttings were selected to get fruit-bearing cuttings according to
2 Mullins (1966) and modified by Ollat et al. (1998) and Santa María (2004). Rooting
3 was made in a heat-bed (27 °C) kept in a cool room (5 °C). One month later, the
4 cuttings were transplanted to 7.5-L plastic pots. Cuttings were planted in plastic pots
5 containing a mixture of peat and perlite (2:1: v/v) and transferred to a greenhouse.

6 Only a single flowering stem was allowed to develop on each plant during growth.
7 Growth conditions in the greenhouse were 26/15 °C and 40/80 % relative humidity
8 (RH) (day/night) and a photoperiod of 15 h with natural daylight supplemented with
9 high-pressure sodium lamps (SON-T Agro Phillips, Eindhoven, Netherlands), providing
10 a minimum photosynthetic photon flux density (PPFD) of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at
11 inflorescence level. Plants were irrigated until veraison with the nutrient solution
12 proposed by Ollat et al. (1998).

13 *Experimental design and temperature gradient greenhouses (TGG)*

14 When plants reached veraison stage, they were transferred to the TGG where they were
15 divided according to the different combinations of CO₂ concentration, temperature and
16 water availability to which they were subjected until grapes complete maturity (reaching
17 21-23 °Brix). The design of the TGG was based on those described previously
18 (Aranjuelo et al., 2005a; Erice et al., 2006b; Morales et al., 2014). Two greenhouses
19 were maintained at an ambient CO₂ concentration level (approximately 400 ppm) and
20 the other two were maintained at an elevated CO₂ level (approximately 700 ppm) (Fig.
21 1). Each greenhouse was divided into three modules, thereby providing different
22 temperature values. The central module was regarded as a transition module and no
23 experimental plants were included in it. In each greenhouse, the inlet module was
24 maintained at ambient temperature and the outlet module was maintained at this

1 ambient temperature +4 °C (T_{+4}) (Fig. 1). Inside the greenhouses, the pots were placed
2 in holes made in the soil to ensure natural temperature fluctuations at root zone, thus
3 approximating field conditions (Morales et al., 2014). Well-irrigated plants (WI) were
4 watered until maximum soil volumetric water content. Soil water sensors (Watermark
5 soil moisture sensor, Spectrum Technologies Inc., Illinois) were placed into the pots,
6 and were used to control irrigation. Partially irrigated plants (PI) were watered at 40%
7 of pot capacity. Plants were irrigated with half-strength Hoagland nutrient solution
8 (Hoagland and Arnon, 1950) or distilled water in order to provide all the treated plants
9 the same amount of nutrients. The pots were rotated daily, within the corresponding
10 greenhouse compartment, to avoid edge effects.

11 *C labeling and sampling*

12 Plant C labeling was conducted parallel with exposure to elevated CO₂ conditions.
13 During this period, the plants exposed to elevated CO₂ conditions were grown in an
14 environment where the isotopic composition of the air ¹³C ($\delta^{13}\text{C}$) of the greenhouses
15 was deliberately modified (-24.93 ‰) to distinguish it from the $\delta^{13}\text{C}$ of ambient CO₂ (-
16 10.30 ‰). The CO₂ was provided by Air Liquide (Pamplona, Spain). Air isotopic
17 composition inside the corresponding TGG was collected daily using 50 mL syringes
18 (SGE International Pty Ltd, Ringwood, Vic., Australia) and kept in 10 mL vacutainers
19 (BD Vacutainers, Plymouth, UK). To avoid contamination with the air present in the
20 syringe and the needle, both were purged with nitrogen prior to each sampling. The
21 vacutainers were also over-pressurised with the same nitrogen gas so that the pressure
22 inside the vacutainer was above ambient. The labeling period lasted until the end of the
23 experiment.

24 *Water status*

1 Leaf discs were cut with a calibrated cork borer and the fresh (FW), turgid (TW) and
2 dry (DW) weights were determined. Relative water content (RWC) was calculated as:
3 $RWC (\%) = [(FW-DW)/(TW-DW)] \times 100$.

4 *Gas exchange and chlorophyll fluorescence measurements*

5 Gas exchange and chlorophyll (Chl) fluorescence measurements (n = 5-6 plants, 2
6 measurements each) were made on young, fully expanded leaves at the same
7 physiological stage (from 8th to 10th node from the top) inside each greenhouse at the
8 respective growth conditions of CO₂ (i.e., plants grown at current CO₂ were measured at
9 375 ppm CO₂, whereas those grown at elevated CO₂ were measured at 700 ppm). In
10 some experiments, gas exchange parameters were measured at atmospheric CO₂
11 concentrations of 375 or 700 ppm in all plants, either grown at current or elevated CO₂
12 concentrations. Also in other cases, CO₂ response curves were made measuring
13 photosynthesis at different atmospheric CO₂ concentrations, ranging from very low to
14 saturating ones. Measurements were made first at 375 ppm CO₂, and then the
15 atmospheric CO₂ concentration was subsequently lowered in a stepwise manner, set
16 again at 375 ppm CO₂ (used as reference) and finally increased stepwise (Larbi et al.,
17 2006). In all these measurements, temperature was 25 °C. The measurements were
18 performed using a portable photosynthesis system (GFS-3000, Walz, Germany) with a
19 3-cm² cuvette. Gas exchange parameters (net photosynthesis, A_N; maximum
20 carboxylation velocity of Rubisco, V_{cmax}; maximum electron transport rate contributing
21 to RuBP regeneration, J_{max}; transpiration, E; stomatal conductance, g_s; and sub-stomatal
22 CO₂ concentration, C_i) were measured in early morning under a photon flux density of
23 1200 μmol m⁻² s⁻¹. Calculations were made according to Von Caemmerer and Farquhar
24 (1981) and Harley et al. (1992). Gas exchange was also measured in dark-adapted
25 leaves after one night in darkness to obtain dark respiration (R_D).

1 Chlorophyll fluorescence parameters were measured immediately after the
2 photosynthesis measurements with a fluorescence module (PAM-Fluorometer 3055-FL,
3 Walz, Germany) attached to the photosynthesis equipment. The experimental protocol
4 for analysis of Chl fluorescence quenching was performed according to Morales et al.
5 (2000). Parameters monitored were actual ($\Phi_{\text{PSII}}=(F'_m-F_s)/F'_m$) and intrinsic ($\Phi_{\text{exc.}}=(F'_m-$
6 $F'_0)/F'_m$) PSII efficiencies, and photochemical quenching ($qP=(F'_m-F_s)/(F'_m-F'_0)$). The
7 electron transport rate (ETR) was calculated according to Krall and Edwards (1992) as
8 $\Phi_{\text{PSII}} \times \text{PPFD} \times 0.84 \times 0.5$, where PPFD is the photosynthetic photon flux density
9 incident on the leaf, 0.5 was used as the fraction of excitation energy distributed to PSII
10 (Ogren and Evans, 1993) and 0.84 as the fractional light absorbance (Morales et al.,
11 1991). Multiplying 0.84 x 0.5 gives a value of 0.42, a value very similar to the α term
12 used by other researchers to calculate ETR, which includes the product of leaf
13 absorbance and the partitioning of absorbed quanta between PSI and PSII and
14 determined as the slope of the relationship between Φ_{PSII} and Φ_{CO_2} (i.e., the quantum
15 efficiency of gross CO₂ fixation), obtained by varying light intensity under non-
16 photorespiratory conditions in an atmosphere containing <1 % O₂ (Valentini et al.,
17 1995). For grapevine cv. Tempranillo, α was reported to be 0.425 (Perez-Martin et al.,
18 2009). Photorespiration (R_L) was estimated as $1/12(\text{ETR} - 4 \times (A_N + R_D))$, according to
19 Valentini et al. (1995).

20 *Photosynthetic pigments determination*

21 One cm² leaf disc was cut with a calibrated cork borer, immersed in liquid N₂ and then
22 stored at -80 °C until use for photosynthetic pigments determinations. Chlorophyll (Chl)
23 *a*, Chl *b* and total carotenoids were measured. Leaf pigments were extracted with

1 acetone in presence of sodium ascorbate, filtered through a 0.45 µm filter, and analyzed
2 spectrophotometrically according to Morales et al. (2000).

3 *Total organic matter (TOM) and water-soluble compound (WSC) C isotope composition*
4 *($\delta^{13}C$)*

5 Berry, rachis, leaf, petiole, shoot, root and cutting samples were collected the last day of
6 labeling (i.e., last day of the experiment), dried at 60 °C for 48 h, and analyzed for the C
7 isotopic composition ($\delta^{13}C$) of TOM. One mg of ground sample was used for each
8 determination.

9 To extract the water-soluble compounds (WSC), leaf samples were lyophilized and
10 then ground to a fine powder. About 50 mg of the fine powder were suspended in 1 mL
11 of distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany),
12 mixed, and then centrifuged at 12,000 g for 5 min at 5 °C. After centrifugation, the
13 supernatant was heated for 3 min at 100 °C and afterward the solution was put on ice for
14 3 min. The supernatant containing the WSC fraction was centrifuged at 12,000 g for 5
15 min at 5 °C (Nogués et al., 2004). Supernatant fraction was transferred to tin capsules
16 for isotope analysis.

17 The $^{13}C/^{12}C$ ratios (R) of plant material were determined using an elemental analyzer
18 (EA1108, Series 1, Carlo Erba Instrumentazione, Milan, Italy) coupled to an isotope
19 ratio mass spectrometer (Delta C, Finnigan, Mat., Bremen, Germany) operating in
20 continuous flow mode.

21 The $^{13}C/^{12}C$ ratios (R) of air samples were determined by Gas Chromatography-
22 Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS). Briefly, water vapor and
23 oxygen from gas samples were removed and the carbon dioxide, argon, and nitrogen

1 gases were separated by gas chromatography (Agilent 6890 Gas Chromatograph)
2 coupled to an isotope ratio mass spectrometer Delta^{plus} via a GC-C Combustion III
3 interphase (ThermoFinnigan). The column used was a 30 cm x 0.32 mm i.d. GS-
4 GASPRO (J. and W. Scientific, USA). The carrier gas was helium at a flow rate of 1.2
5 mL min⁻¹. The injection port temperature was 220 °C. The oven temperature was kept at
6 60 °C during the entire run. Injection was conducted in the split mode (injected volume
7 0.3 mL, split flow 20 mL min⁻¹).

8 The ¹³C/¹²C ratios (*R*) of plant material and air samples were expressed as δ¹³C
9 values using international secondary standards of known ¹³C/¹²C ratios (IAEA CH7
10 polyethylene foil, IAEA CH6 sucrose and USGS 40 L-glutamic acid) calibrated against
11 Vienna Pee Dee Belemnite calcium carbonate (VPDB) with an analytical precision of
12 0.1‰: $\delta^{13}\text{C} = (R_{\text{sample}}/R_{\text{standard}}) - 1$.

13 *C and N content*

14 Carbon and nitrogen content were determined in dry samples previously ground to
15 powder. One mg samples were stored in tin capsules for TOM analyses. Carbon and N
16 contents were determined at the Serveis Científico-Técnicos of the University of
17 Barcelona (Barcelona, Spain) using an elemental analyzer (EA1108, Series 1; Carbo
18 Erba Instrumentazione, Milan, Italy).

19 *Statistical analysis*

20 Four TGG were used, two set at current CO₂ concentration and the other two at elevated
21 CO₂ concentration. The design of the experiment was split-split-plot, with three factors
22 (CO₂, temperature and water availability). The experiment was repeated once (two
23 biological replicates, comparing grapevine plants growing at current or elevated CO₂

1 concentrations in the TGG 1 and 2 (Experiment 1) and in the TGG 3 and 4 (Experiment
2 2)). The number of plants per treatment was 8, but we used only 5-6 of them as
3 experimental replicates (5-6 plants) and then 2 measurements were made per plant
4 (technical replicates). For statistical purposes, we used the 5-6 (gas exchange and Chl
5 fluorescence) or 3 (other determinations) experimental replicates. Data were first tested
6 using a three-way ANOVA (three factors: (i) CO₂ concentration (ii) temperature, and
7 (iii) water availability; and two levels, (i) 700 ppm CO₂ vs. Amb, (ii) T_{amb} vs. T₊₄ and
8 (iii) WI vs. PI, in order to determine the effects of the treatments and their possible
9 interactions. Differences among groups were tested with the Least Significant
10 Differences (*LSD*) post-hoc test. This test was especially useful when effects of
11 treatments were statistically significant or when interaction between factors was
12 detected (not allowing to conclude about the main effects). Results were considered
13 statistically significant at p<0.05. Data are presented as means ± standard error (SE). All
14 these statistical analyses were carried out with the SPSS 15.0 statistical package for
15 windows (SPSS inc., Chicago). Each harvest or sampling date was treated as
16 independent data when statistics were carried out, and therefore comparisons were
17 always made among treatments for a given date.

18

19 **Results**

20 *Gas exchange*

21 Figure 2 shows photosynthetic rates measured in plants grown under current or elevated
22 CO₂ concentrations at the prevailing CO₂ concentration of their respective TGG. After
23 10 and 20 days of treatment, plants grown under water stress conditions had lower
24 (p<0.001) photosynthetic rates regardless of CO₂ concentration and temperature (Fig.

1 2A and 2B). Differences were not significant, however, in plants exposed to elevated
2 temperature after 10 days of treatment (at both CO₂ levels), and those exposed to
3 current CO₂ and temperature after 20 days of treatment (Fig. 2A and 2B). The impaired
4 photosynthetic rates of water stressed plants were mediated by decreases in stomatal
5 conductance, lowering as a consequence transpiration rates (Table 1). Elevated CO₂
6 increased (p<0.001) photosynthetic rates irrespective of water availability and
7 temperature after 10 days of treatment, except in plants grown under ambient
8 temperature and partial irrigation in which increases were not statistically significant
9 (Fig. 2A). Regardless of temperature and irrigation conditions, no CO₂ effects on
10 photosynthetic rates were observed after 20 days of treatment (Fig. 2B). Except in well-
11 irrigated plants grown under elevated temperature and those well irrigated but grown
12 under current temperature and at the sampling date of 10 days, elevated CO₂ closed
13 stomata and reduced transpiration rates at both sampling dates (Table 1). Obviously, all
14 plants grown under elevated CO₂ had higher sub-stomatal CO₂ concentrations (C_i) than
15 their respective controls (Table 1), indicating that the excess CO₂ had entered the leaf.
16 At 20 days, the ANOVA analysis revealed CO₂ x water availability (g_s and C_i, p<0.01),
17 CO₂ x temperature (g_s, p<0.05) and water availability x temperature (transpiration,
18 p<0.05) interactions (Table 1).

19 No differences in photorespiration (R_L) were found among treatments after 10 days
20 of imposing the different experimental conditions (Fig. 2C). After 20 days of treatment,
21 plants grown under elevated temperature, elevated CO₂ and partial irrigation had the
22 highest photorespiration rates, significantly (p<0.01) different from the rest of
23 treatments (Fig. 2D). There was CO₂ x water availability interaction (p<0.01), and thus
24 leaves from plants grown under elevated CO₂ and partial irrigation had increased their
25 photorespiration both under ambient or elevated temperature conditions (Fig. 2D).

1 Leaves from plants grown under ambient temperature, regardless of water status,
2 respired more under elevated than under current CO₂ concentrations either after 10 or 20
3 days of treatment ($p < 0.001$ under well watered conditions, and $p < 0.01$ under partial
4 irrigation at both sampling dates) (Fig. 2E and 2F). When grown under elevated
5 temperature, all leaves respired similarly to control (ambient CO₂, ambient temperature
6 and well irrigated) leaves at both sampling dates (Fig. 2E and 2F). These respiration
7 changes reflect an interaction between CO₂ and temperature ($p < 0.001$) at both sampling
8 dates (Fig. 2E and 2F).

9 The increase in atmospheric CO₂ concentration may compensate the decrease in
10 Rubisco activity experienced by plants growing at elevated CO₂. Therefore, under
11 elevated CO₂ there is usually no decrease of photosynthetic activity, but rather a
12 decrease in photosynthetic capacity (Irigoyen et al., 2014). A general reduction of
13 photosynthetic capacity in plants grown under elevated CO₂ suggests photosynthetic
14 acclimation. This is generally evidenced in comparisons of plants grown at ambient and
15 elevated CO₂ and measured at the same CO₂ concentration, either current or elevated.
16 Therefore, all plants were also measured at either 375 or 700 ppm CO₂ irrespective of
17 the CO₂ concentration they were growing (Fig. 3).

18 Despite some of the effects of water stress already described from Fig. 2 data that
19 were also observable when measurements were made in all plants at the same CO₂
20 concentration (Fig. 3), a main difference between Fig. 2 and 3 data is that (i) after 10
21 days of treatment no CO₂ effects on photosynthetic rates were seen either when
22 measured at 375 or 700 ppm (Fig. 3A and 3C), and (ii) after 20 days of treatment plants
23 grown under elevated CO₂ regardless of water availability and temperature had lower (p
24 ranged from < 0.01 to < 0.001) photosynthetic rates than those grown under current CO₂
25 both when measurements were made setting CO₂ concentration at 375 or 700 ppm

1 (decreases were not significant only in the case of plants grown under ambient
2 temperature and well irrigated after 20 days of treatment and measured at 700 ppm CO₂)
3 (Fig. 3B and 3D). The ANOVA analyses revealed only significant interaction ($p < 0.05$)
4 between water availability and temperature after 20 days of treatment when
5 measurements were made at 375 ppm (Fig. 3B).

6 Maximum carboxylation velocity of Rubisco ($V_{c_{max}}$) analyses showed that while
7 after 10 days of elevated CO₂ exposure no significant differences derived from CO₂ and
8 water availability treatment were detected, 10 days later, 20 days treated plants grown
9 under 700 ppm and/or low irrigation conditions showed lower ($p < 0.001$) $V_{c_{max}}$ values
10 (Fig. 4A and 4B). Similarly, maximum electron transport rate contributing to RuBP
11 regeneration (J_{max}) showed that CO₂ exposure derived decreases were only detected
12 after 20 days of exposure to elevated CO₂ conditions (Fig. 4C and 4D). There were CO₂
13 x water availability interactions ($p < 0.01$) after 20 days of treatments with respect to
14 both $V_{c_{max}}$ and J_{max} (Fig. 4B and 4D).

15 *Leaf relative water content (RWC)*

16 Leaf RWC values found at harvest time ranged between 85 and 91% with no significant
17 differences among treatments (Table 2).

18 *Chlorophyll fluorescence*

19 Despite changes observed in gas exchange properties, no remarkable effects of
20 treatments were found ($p < 0.001$) on photosynthetic electron transport rates (ETR) at
21 any sampling date (Table 1). This result was confirmed by measuring Φ_{PSII} , $\Phi_{exc.}$ and qP
22 that in most cases did not change in response to the treatments (Table 1). Φ_{PSII} (10 days,
23 $p < 0.05$; 20 days, $p < 0.01$), $\Phi_{exc.}$ (20 days, $p < 0.05$) and qP (20 days, $p < 0.01$) showed CO₂
24 x water availability interactions (Table 1).

1 After growing plants 10 days under treatments, the $ETR/A_N+R_D+R_L$ ratio only
2 increased in partially irrigated plants grown under ambient temperature and current CO_2
3 concentration (Table 1). Elevated CO_2 decreased this ratio in all plants, except in those
4 well irrigated grown under ambient temperature (Table 1). After 20 days of treatment,
5 this ratio increased due to water stress in plants grown under elevated temperature and
6 elevated CO_2 (Table 1). Elevated CO_2 increased the $ETR/A_N+R_D+R_L$ ratio only in plants
7 grown under partial irrigation and elevated temperature (Table 1). All these changes
8 were significant at $p<0.05$.

9 *Photosynthetic pigments*

10 No effects of water stress were observed at any sampling date on Chl *a* and Chl *b*
11 concentrations and on the Chl *a*/Chl *b* ratio (Table 2). Elevated CO_2 decreased
12 transiently (after 10 days of treatment) Chl *a* or Chl *b* concentration in some treatments,
13 but effects disappeared with longer exposures to CO_2 (after 20 days of treatment) (Table
14 2). No effects of CO_2 were found on the Chl *a*/Chl *b* ratio (Table 2). Water stress
15 reduced total carotenoids concentration in plants grown under ambient temperature and
16 current CO_2 concentration after 10 days of treatment but not after 20 (Table 2). Also,
17 elevated CO_2 decreased total carotenoids concentration in well-irrigated plants grown
18 under ambient temperature at both sampling dates (Table 2). Changes in photosynthetic
19 pigment and CO_2 x water availability interactions, when occurred, were significant at
20 $p<0.05$ (Table 2).

21 *C isotope composition ($\delta^{13}C$)*

22 As a consequence of the C labeling, the $\delta^{13}C$ of TOM of plants exposed to elevated CO_2
23 conditions was ^{13}C -depleted when compared with the respective ambient CO_2 treatment
24 plants (Table 3). More specific analyses revealed that main shoot, roots, leaves and

1 berries were the organs where more labeled C was detected. In the other hand, in the
2 rachis, and especially in the cutting, the availability of labeled C was the lowest.
3 However, when considering this, it should also be observed that in plants exposed to
4 elevated temperature and partial irrigation such differences between organs were less
5 marked than in fully watered plants grown under ambient temperature. Isotopic analyses
6 also confirmed the fact that, regardless of the organ analyzed, both elevated temperature
7 and partial irrigation diminished the presence of labeled C in TOM. The ANOVA
8 analyses revealed significant interactions between factors (Table 3). In particular, there
9 were interactions between CO₂ and water availability in the δ¹³C of TOM in 4 out of 7
10 organs tested (i.e., root, main shoot, petiole and leaf; p ranged from <0.01 to <0.001).
11 The interaction between CO₂ and temperature was only observed in roots (p<0.01).

12 Although leaf WSC δ¹³C values were similar to the ones corresponding to TOM,
13 they were a little bit less ¹³C depleted. Interestingly, leaf WSC δ¹³C values in all
14 treatments were similar to that of berries. As observed with TOM, elevated temperature
15 and partial irrigation diminished the presence of labeled C (Table 3). In the case of leaf
16 WSC δ¹³C values, significant interactions were observed between CO₂ and water
17 availability (p<0.05) and between CO₂ and temperature (p<0.001) (Table 3).

18 *C and N content*

19 Carbon content analyses showed that the main shoot, followed by the leaves, were the
20 organs with the larger C content, whereas the petiole was the one with the lowest one
21 (Table 3). In general terms, irrespective of the organ analyzed, no CO₂, temperature nor
22 water availability significant effects were detected in C content (Table 3).

23 On the other hand, leaves, followed by rachis and roots, were the organs with more N
24 content, while the cuttings, berries and petioles showed the lowest N content values

1 (Table 3). While water treatment and growth temperature did not significantly affect N
2 content of different organs (with the exception of main shoot, organ where in addition a
3 significant ($p<0.05$) interaction between these two factors was observed), plants
4 exposed to 700 ppm CO₂ were the ones with the lowest N content (Table 3). Partial
5 irrigation diminished main shoot N content under elevated CO₂ (Table 3). The only
6 organ in which interaction between CO₂ and water availability was detected was the
7 rachis ($p<0.05$; Table 3).

8 Regardless of the organ analyzed, exposure to elevated CO₂ increased C/N (Table 4).
9 No clear effects in the C/N ratio were observed with water availability or temperature
10 treatments (Table 4). Half of the organs analyzed (root, leaf and rachis) showed
11 significant interactions between CO₂ and water availability ($p<0.05$), whereas main
12 shoot was the organ showing more type of interactions (i.e., CO₂ x temperature
13 ($p<0.05$), and water availability x temperature ($p<0.01$)) (Table 4).

14

15 **Discussion**

16 Climate change could influence grapevine physiology. The objective of the present
17 research project focuses on evaluating the effect of climate change (elevated CO₂,
18 elevated temperature and water stress) on grapevine physiology and carbon balance.
19 Carbon balance was followed monitoring carbon gains (i.e., net photosynthesis) and
20 losses (i.e., respiration and photorespiration). All these physiological processes and
21 grape quality are sensitive to some extent to one or several stress factors related to
22 climate change. Grapevine photosynthesis, as in other C₃ species, is limited by CO₂
23 (Mullins et al., 1992; Bindi et al., 1996). Any CO₂ increase therefore may enhance CO₂
24 fixation rates (Long, 1991). However, plants may experience photosynthetic acclimation

1 when exposed to elevated CO₂ in long-term experiments, which decreases
2 photosynthesis capacity below its maximum potential (Jifon and Wolfe, 2002; Long et
3 al., 2004; Erice et al., 2006b).

4 Although 10 days after the beginning of CO₂ treatment, plants exposed to elevated
5 CO₂ conditions showed higher photosynthetic rates, 20 days after beginning, exposure
6 to 700 ppm CO₂ had no significant effect in photosynthesis measured at growth CO₂
7 concentration (Fig. 2) and decreased photosynthesis measured either at 375 or 700 ppm
8 CO₂ (Fig. 3), in line with previous reports (Aranjuelo et al., 2005b; Del Pozo et al.,
9 2005; Seneweera et al., 2011) and evidencing photosynthetic acclimation. Causes for
10 such photosynthetic acclimation are under debate (Sanz-Sáez et al., 2010). One
11 hypothesis proposes that it comes from stomatal limitations derived from a leaf
12 conductance reduction in plants grown under elevated CO₂ (Sánchez-Díaz et al., 2004).
13 Alternatively, it may come from metabolic limitations, overall ascribed to diminished
14 Rubisco carboxylation activity (Aranjuelo et al., 2005b; Erice et al., 2006a) and/or
15 reduced levels of this enzyme in elevated CO₂-grown plants (Aranjuelo et al., 2005b).
16 Irrespective of water and temperature treatment, the larger sub-stomatal CO₂
17 concentrations (C_i) of plants exposed to 700 ppm CO₂ discarded limitations in CO₂
18 availability as a factor involved in photosynthetic down-regulation. After 20 days of
19 treatment, *in vivo* maximum rates of Rubisco carboxylation (V_{cmax}) were markedly
20 decreased in plants grown under elevated CO₂ and partial irrigation (but not in those
21 well watered) regardless of temperature (Fig. 4), supporting the hypothesis of impaired
22 Rubisco carboxylation activity as origin of acclimation. J_{max}, the *in vivo* maximum rate
23 of electron transport driving regeneration of RuBP, also decreased significantly in all
24 plants exposed to elevated CO₂ after 20 days of treatment (Fig. 4). Although in this
25 study Rubisco content was not determined, the 30-50 % decrease in leaf N content

1 (Table 3) revealed that the inhibition of $V_{c_{max}}$ and J_{max} was linked to the lower Rubisco
2 protein content. However, it should not be discarded that, as observed by Pérez et al.
3 (2011), depleted Rubisco activity could have also been affected by the enhancement of
4 Rubisco binding inhibitors. More specific CO_2 , temperature and irrigation treatment
5 analyses revealed that while photosynthetic responsiveness to elevated CO_2 was not
6 conditioned by growth temperature, partial watering strongly increased the depletion of
7 $V_{c_{max}}$. Interestingly, gas exchange analyses also remarked that the lower photosynthetic
8 rates of water-stressed plants were caused by the lower Rubisco activity and C_i of
9 plants. The fact that Rubisco catalyzes CO_2 and O_2 fixation implies that
10 photorespiration (R_L) diminishes the potential photosynthetic activity of plants.
11 According to Andrews and Lorimer (1987), an increase in atmospheric CO_2 increases
12 the leaf internal CO_2 concentration and the CO_2/O_2 ratio at the Rubisco site, which
13 should favor carboxylation rather than oxygenation of ribulose-1,5-bisphosphate
14 (RuBP) with the consequent increase in photosynthetic rates. The fact that in partially
15 watered plants exposed to 700 ppm CO_2 , R_L increased suggests that, opposite to what
16 expected, in those plants R_L enhancement (Fig. 2D) contributed to the photosynthetic
17 down-regulation. Moreover, the fact that elevated temperature did not affect $V_{c_{max}}$, J_{max}
18 and C_i confirmed that temperature increase did not alter photosynthetic machinery.

19 The maintenance of equilibrium between light capture and photochemistry
20 requirements is a key point for the avoidance of reactive oxygen species (ROS;
21 Niinemets and Kull, 2001). Our study showed that photochemical changes were
22 accompanied by similar changes in CO_2 fixation in all treatments, with few exceptions.
23 Only plants treated 20 days with elevated CO_2 , elevated temperature and drought had
24 marked and physiologically relevant increases of the electron transport rate (ETR) to
25 photosynthesis (A_N) + respiration (R_D) + photorespiration (R_L) ratio (Table 1). Other

1 researchers have reported similar results of increased ETR/A_N ratios in grapevines
2 grown under water stress and in the field (Flexas et al., 1999; Flexas and Medrano,
3 2002). These data indicate that the generation (ETR) and the electrons consumption
4 ($A_N+R_D+R_L$) could be unbalanced in that case. Excess of electrons over those used in
5 photosynthesis could react with O_2 generating ROS and ultimately could lead to damage
6 to cell constituents. Chl and carotenoids (Table 2), main targets of ROS, were not
7 affected in that treatment. If any oxidative damage was present, as suggested by the
8 increased $ETR/(A_N+R_D+R_L)$ ratio of droughted plants grown under elevated
9 temperature and CO_2 , this damage was not strong enough to affect photosynthetic
10 pigments. Only Chl and carotenoids decreases were observed in plants treated 10 days
11 but they recovered with time in 20 days-treated plants.

12 Although studies conducted in ambient CO_2 concentration conditions highlight the
13 relevance of C sink strength as a key factor limiting plant yield, very little attention has
14 been given to this topic in elevated CO_2 concentration studies. This is a matter of major
15 concern because low C sink strength is a key factor conditioning photosynthetic
16 acclimation and therefore plant yield under elevated CO_2 concentration. When plants
17 exposed to elevated CO_2 concentration have limitations in increasing C sink strength,
18 photosynthetic rates are decreased to balance C source activity and sink capacity
19 (Aranjuelo et al., 2009; 2013). A parameter that may indicate the source/sink balance is
20 the C/N ratio, which increases when plant sink capacity is not strong enough to
21 consume or mobilize carbohydrates. Jifon and Wolfe (2002) observed that the effect of
22 N on photosynthetic performance to elevated CO_2 depends on the balance between the
23 availability and demand for N due to its relationship with biomass allocation and
24 source-sink carbon balance. Low N availability may affect plant growth, and thus the
25 capacity to develop new sinks (Erice et al., 2006b). The 183 and 150 % increase in leaf

1 C/N detected in leaves exposed to elevated CO₂ (under control and partial irrigation
2 conditions respectively) (Table 4) confirmed that they had problems to adjust C
3 sink/source balance. As mentioned above, development of strong C sinks is essential to
4 avoid leaf carbohydrate build-up.

5 In a recent study where two wheat genotypes with contrasting harvest index (HI)
6 were exposed to elevated CO₂ concentration, Aranjuelo et al. (2013) confirmed that
7 plants with high grain C demand (high HI) were capable of overcoming photosynthetic
8 acclimation with a consequent increase in yield. However, in the case of plants with low
9 grain C demand (low HI), leaf carbohydrate together with depleted N assimilation
10 induced photosynthetic acclimation and the absence of a CO₂ concentration-derived
11 increase in plant biomass. The ¹²CO₂ labeling conducted during the experiment enabled
12 the characterization of C assimilation and partitioning toward all the plant organs until
13 grape maturity. Concerning the veraison-maturity period, obtained ¹³C isotopic
14 composition δ¹³C data highlighted that after 30 days of C-labeling, the main shoot,
15 berries, leaves and root were the more C-labeled organs (Table 3). Carbon partitioning
16 among sinks is regulated by the sink themselves and their ability to import
17 photoassimilates (Patric, 1997; Botas, 2004; Zapata et al., 2004). Although a large C-
18 labeling was expected in leaves and berries, our results revealed that an important
19 fraction of photoassimilates remained in leaves and the rest was partitioned toward
20 storage organs like the stem and roots (Botas, 2004; Zapata et al., 2004). Such results
21 highlight the fact that, as mentioned above, those plants had problems to avoid leaf
22 carbohydrate accumulation. According the C/N ratio (Table 4), the C/N increases under
23 elevated CO₂ were mainly due to N reduction (Table 3).

24 On the other hand, the low labeling observed in the rachis and especially in the
25 cutting, showed that, at this phenological stage, those plant organs did not have any

1 recently fixed C sink activity. The low-labeled C sink strength of the rachis was
2 remarkable, especially taking into account that it was recently formed. Such results
3 highlighted that the main C source required for the synthesis of the rachis proceeded
4 from remobilization of pre-labeling C. Interestingly, more specific analyses of water
5 and temperature effect on C management highlighted that, at the leaf, stem and in a
6 lesser extent in petioles, water availability and elevated temperature decreased C-
7 labeling, being the plants exposed to drought and elevated temperature the ones with the
8 lowest labeled C. Such differences could have been explained by the deleterious effect
9 of drought and temperature on CO₂ fixation (Fig. 2 and 3). However, when analyzing
10 those data it should be considered that temperature effect on respiration activity could
11 also be involved. Plants use up to 50% of recently formed C in respiration processes,
12 which means that an important fraction of photoassimilates could have been wasted
13 through respiration processes. This point is especially important in heterotrophic organs
14 such as stem with large respiratory activity. It is also remarkable the fact that
15 partitioning of recently assimilated C toward roots was similar in all the treatments.
16 Such results revealed that under stressful growth conditions, compared with the rest of
17 the organs, those plants invested more C on root development.

18 20-30 days of growth at elevated CO₂ may result short for investigating the
19 acclimation process in grapevine. However, our study showed that even if in an initial
20 step (10 days), exposure to elevated CO₂ increased A_N, after 20 days, and regardless of
21 temperature and irrigation treatment, exposure to 700 ppm CO₂ induced photosynthetic
22 acclimation. The larger C/N ratio detected in leaves exposed to elevated CO₂ affected
23 negatively V_{c,max} and J_{max} with the consequent inhibition of photosynthetic capacity.
24 Such down-regulation was especially marked under drought conditions. ¹²C labeling
25 highlighted that although storage organs such as main stem and the root represented

1 important labeled C sink, the large amount of leaf labeled C confirmed that plants
2 exposed to elevated CO₂ were not capable to develop strong C sinks that would enable
3 the avoidance of leaf carbohydrate build-up. Decreases in photosynthetic pigments were
4 observed only in plants grown 10 days under partial irrigation, elevated temperature and
5 CO₂, but they recovered afterwards. In fact, under most experimental conditions, no
6 oxidative damage to Chls and carotenoids was observed, suggesting a protective role of
7 CO₂ either at current or elevated temperatures against the adverse effects of water stress.

8 In conclusion, irrespective of water availability and temperature, growing under
9 elevated CO₂ concentration induced photosynthetic acclimation in grapevine. Evidence
10 comes from decreases in photosynthetic capacity (measuring photosynthesis at the same
11 CO₂ concentration either 375 ppm or 750 ppm after 20 days of treatment), decreases in
12 photosynthetic parameters such as V_{cmax} and J_{max} in 20 days-treated plants and increases
13 in the leaf C/N ratio at grape ripeness stage (i.e., after 30 days of treatment). Measuring
14 photosynthetic rates or CO₂ response curves at grape ripeness stage is not recommended
15 because photosynthesis and stomatal conductance are low, due to a veraison-ripeness
16 developmental-related decreasing trend (Salazar-Parra et al., 2012). All these changes
17 can be interpreted as symptoms of photosynthetic acclimation in grapevine.

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Figure legends

Figure 1. Temperature (left panels) and air CO₂ concentration (right panels) data recorded in the temperature gradient greenhouses (TGG) along the experimental period (1 month) where CO₂ concentration was set at 400 (ambient) or 700 (elevated) ppm. Each TGG has one module at ambient temperature and another at ambient temperature +4 °C (T₊₄).

Figure 2. Photosynthetic rates (A_N) (A and B), photorespiration (R_L) (C and D) and dark respiration (R_D) (E and F) measured at the CO₂ concentration prevailing in the greenhouse at 10 (A, C and E) and 20 (B, D and F) days of treatment in leaves of *V. vinifera* cv. Tempranillo grown under different CO₂ concentrations (ambient or 700 ppm CO₂), two temperature regimes (T_{amb} or T₊₄) and water availability (WI, well irrigated or PI, partially irrigated). Determinations were conducted at 10 and 20 days after treatments imposition. Data represent the average value of 5-6 analyses ± S.E. Different letter indicates significant differences among treatments (P<0.05) based on *LSD* test. When significant, interactions between CO₂ concentration (CO₂), water availability (WA) and temperature (T) are also shown.

Figure 3. Photosynthetic rates (A_N) measured at 375 ppm CO₂ at 10 (A) and 20 (B) days of treatment and measured at 700 ppm CO₂ at 10 (C) and 20 (D) days of treatment in leaves of *V. vinifera* cv. Tempranillo grown under different CO₂ concentrations (ambient or 700 ppm CO₂), two temperature regimes (T_{amb} or T₊₄) and water availability (WI, well irrigated or PI, partially irrigated). Sampling was made at 10 and 20 days after treatments imposition. Data represent the average value of 5-6 analyses ± S.E. Different letter indicates significant differences among treatments (P<0.05) based on *LSD* test. When significant, interactions between CO₂ concentration (CO₂), water availability (WA) and temperature (T) are also shown.

Figure 4. Rubisco maximum carboxylation efficiency (V_{c_{max}}) (A and B) and the maximum electron transport rate contributing to RuBP regeneration (J_{max}) (C and D) at 10 (A and C) and 20 (B and D) days of treatment in leaves of *V. vinifera* cv. Tempranillo grown under different CO₂ concentrations (ambient or 700 ppm CO₂), two temperature regimes (T_{amb} or T₊₄) and water availability (WI, well irrigated or PI, partially irrigated). Sampling was made at 10 and 20 days after treatments imposition. Data represent the average value of 5-6 analyses ± S.E. Different letter indicates significant differences among treatments (P<0.05) based on *LSD* test. When significant, interactions between CO₂ concentration (CO₂), water availability (WA) and temperature (T) are also shown.

Table 1

Table 1. Stomatal conductance (g_s), transpiration (E), sub-stomatal CO_2 concentration (C_i), electron transport rate (ETR), actual and intrinsic photosystem II (PSII) efficiencies (Φ_{PSII} and Φ_{exc} , respectively), photochemical quenching (qP) and $ETR/A_N+R_D+R_L$ ratios at 10 and 20 days of treatments in leaves of *V. vinifera* cv. Tempranillo grown under ambient (A) or elevated (E, 700 ppm) CO_2 concentrations, ambient (T_{amb}) or elevated (T_{+4}) temperature regimes and well (WI) or partially (PI) irrigated. Data represent the average value of 5-6 analyses \pm S.E. Different letter indicates significant differences between treatments ($p < 0.05$) based on *LSD* test. Significance (p) of the ANOVA analyses for the interactions $CO_2 \times WA$, $CO_2 \times T$ and $WA \times T$ are also shown. CO_2 , WA and T refer to CO_2 concentration, water availability and temperature, respectively. ns, * and ** indicate no significant differences, and differences at $p < 0.05$ and 0.01 respectively.

Treatments									p		
	A_ T_{amb} _WI	A_ T_{amb} _PI	A_ T_{+4} _WI	A_ T_{+4} _PI	E_ T_{amb} _WI	E_ T_{amb} _PI	E_ T_{+4} _WI	E_ T_{+4} _PI	$CO_2 \times WA$	$CO_2 \times T$	$WA \times T$
g_s (mmol H_2O m^{-2} s^{-1})											
10 days	116 \pm 26 a	80 \pm 42 bc	103 \pm 35 ab	81 \pm 24 bc	77 \pm 25 bcd	44 \pm 18 d	98 \pm 36 ab	56 \pm 21 cd	ns	ns	ns
20 days	81 \pm 26 bc	58 \pm 18 cd	113 \pm 32 a	50 \pm 24 de	93 \pm 28 ab	24 \pm 12 e	116 \pm 32 a	18 \pm 8 e	**	*	ns
E (mmol H_2O m^{-2} s^{-1})											
10 days	2.1 \pm 0.4 ab	1.5 \pm 0.8 cd	2.2 \pm 0.7 a	1.9 \pm 0.5 abc	1.6 \pm 0.4 bcd	1.0 \pm 0.4 e	2.3 \pm 0.7 a	1.4 \pm 0.5 d	ns	ns	ns
20 days	1.5 \pm 0.4 bc	1.1 \pm 0.4cd	2.6 \pm 0.7 a	1.3 \pm 0.6 c	1.8 \pm 0.5 b	0.5 \pm 0.2 e	2.6 \pm 0.7 a	0.5 \pm 0.2 de	ns	ns	*
C_i (μ mol CO_2 mol^{-1} air)											
10 days	219 \pm 47 c	187 \pm 90 c	199 \pm 52 c	203 \pm 34 c	398 \pm 27 ab	334 \pm 145 b	426 \pm 65 a	319 \pm 112 b	ns	ns	ns
20 days	175 \pm 83 d	179 \pm 46 d	221 \pm 77 d	173 \pm 53 d	491 \pm 57 ab	311 \pm 28 c	537 \pm 45 a	436 \pm 121 b	**	ns	ns
ETR (μ mol e^- m^{-2} s^{-1})											
10 days	80 \pm 15 bc	72 \pm 7 c	87 \pm 23 abc	82 \pm 16 bc	88 \pm 24 abc	78 \pm 21 bc	104 \pm 6 a	95 \pm 16 ab	ns	ns	ns
20 days	70 \pm 10 cd	62 \pm 10 d	101 \pm 11 a	75 \pm 10 bcd	75 \pm 11 bcd	65 \pm 5 d	84 \pm 24 abc	92 \pm 23 ab	ns	ns	ns
Φ_{PSII}											
10 days	0.16 \pm 0.03 bc	0.14 \pm 0.01 c	0.17 \pm 0.04 abc	0.16 \pm 0.03 bc	0.18 \pm 0.05 abc	0.16 \pm 0.04 bc	0.21 \pm 0.01 a	0.19 \pm 0.03 ab	*	ns	ns
20 days	0.13 \pm 0.01 c	0.12 \pm 0.02 c	0.20 \pm 0.02 a	0.15 \pm 0.02 bc	0.15 \pm 0.02 bc	0.13 \pm 0.01 c	0.17 \pm 0.05 abc	0.18 \pm 0.05 ab	**	ns	ns
Φ_{exc}											
10 days	0.46 \pm 0.10 a	0.50 \pm 0.15 a	0.53 \pm 0.06 a	0.53 \pm 0.10 a	0.51 \pm 0.07 a	0.48 \pm 0.10 a	0.47 \pm 0.06 a	0.52 \pm 0.07 a	ns	ns	ns
20 days	0.53 \pm 0.12 ab	0.53 \pm 0.18 ab	0.50 \pm 0.10 abc	0.58 \pm 0.05 a	0.52 \pm 0.05 ab	0.42 \pm 0.12 bc	0.51 \pm 0.03 ab	0.36 \pm 0.04 c	*	ns	ns
qP											
10 days	0.35 \pm 0.06 ab	0.31 \pm 0.12 b	0.33 \pm 0.09 ab	0.31 \pm 0.06 b	0.35 \pm 0.12 ab	0.35 \pm 0.14 ab	0.44 \pm 0.07 a	0.37 \pm 0.09 ab	ns	ns	ns
20 days	0.26 \pm 0.06 c	0.26 \pm 0.07 c	0.41 \pm 0.08 ab	0.29 \pm 0.09 c	0.29 \pm 0.06 c	0.32 \pm 0.08 bc	0.33 \pm 0.12 bc	0.51 \pm 0.14 a	**	ns	ns
$ETR/A_N+R_D+R_L$											
10 days	8 \pm 2 bc	11 \pm 2 a	10 \pm 2 ab	12 \pm 3 a	7 \pm 2 c	8 \pm 2 bc	7 \pm 2 c	8 \pm 2 bc	ns	ns	ns
20 days	6 \pm 5 bc	8 \pm 7 bc	6 \pm 5 c	10 \pm 8 bc	7 \pm 2 bc	13 \pm 5 b	10 \pm 5 bc	27 \pm 7 a	ns	ns	ns

Table 2

Table 2. Relative water content (RWC), Chl *a* and *b*, and Chl *a*/Chl *b* ratio and total carotenoids at 10 and 20 days of treatment in leaves of *V. vinifera* cv. Tempranillo grown under ambient (A) or elevated (E, 700 ppm) CO₂ concentrations, ambient (T_{amb}) or elevated (T₊₄) temperature regimes and well (WI) or partially (PI) irrigated. Data represent the average value of 3 analyses ± S.E. Different letter indicates significant differences between treatments (P<0.05) based on *LSD* test. Significance (p) of the ANOVA analyses for the interactions CO₂xWA, CO₂xT and WAxT are also shown. CO₂, WA and T refer to CO₂ concentration, water availability and temperature, respectively. ns and * indicate no significant differences and differences at p<0.05 respectively.

Treatments	A_T _{amb} _WI	A_T _{amb} _PI	A_T ₊₄ _WI	A_T ₊₄ _PI	E_T _{amb} _WI	E_T _{amb} _PI	E_T ₊₄ _WI	E_T ₊₄ _PI	p		
									CO ₂ xWA	CO ₂ xT	WAxT
RWC (%)	89±7 a	87±6 a	89±4 a	89±4 a	91±3 a	88±4 a	90±6 a	85±10 a	ns	*	ns
Chl <i>a</i> (μmol m ⁻²)											
10 days	525±77 a	445±64 ab	397±9 b	433±77 ab	365±25 b	443±19 ab	380±31 b	334±123b	ns	ns	ns
20 days	426±69 a	343±59 a	405±71 a	329±75 a	298±55 a	400±117 a	337±90 a	387±72 a	*	ns	ns
Chl <i>b</i> (μmol m ⁻²)											
10 days	175±37 a	169±71 ab	118±8 abc	140±32 abc	101±9 c	126±9 c	114±12 bc	93±37 c	ns	ns	ns
20 days	135±30 a	96±18 ab	114±21 ab	84±46 ab	80±15 b	117±42 ab	89±33 ab	112±22 ab	*	ns	ns
Chl <i>a</i> /Chl <i>b</i>											
10 days	3.0±0.3 a	2.9±0.8 a	3.4±0.2 a	3.1±0.3 a	3.6±0.3 a	3.5±0.1 a	3.4±0.4 a	3.6±0.2 a	ns	ns	ns
20 days	3.2±0.3 b	3.6±0.1 ab	3.6±0.1 ab	4.7±2.2 a	3.7±0.1 ab	3.5±0.2 ab	3.9±0.5 ab	3.5±0.1 ab	ns	ns	ns
Total carotenoids (μg m ⁻²)											
10 days	273±49 a	196±29 b	201±11 b	215±40 b	180±9 b	217±10 ab	191±13 b	165±59b	ns	ns	ns
20 days	207±42 a	171±24 ab	201±31 ab	163±30 ab	146±27 b	193±47 ab	157±39 ab	191±25 ab	*	ns	ns

Table 3

Table 3. Carbon isotopic composition ($\delta^{13}\text{C}$, ‰) and C and N content (% of DW) at the end of the treatments (grape ripeness stage) in the different organs of *V. vinifera* cv. Tempranillo grown from veraison to maturity under ambient (A) or elevated (E, 700 ppm) CO_2 concentrations, ambient (T_{amb}) or elevated (T_{+4}) temperature regimes and well (WI) or partially (PI) irrigated. Data represent the average value of 3 analyses \pm S.E. Different letter indicates significant differences between treatments ($p < 0.05$) based on *LSD* test. WSC indicates water-soluble compounds. Significance (p) of the ANOVA analyses for the interactions $\text{CO}_2 \times \text{WA}$, $\text{CO}_2 \times \text{T}$ and $\text{WA} \times \text{T}$ are also shown. CO_2 , WA and T refer to CO_2 concentration, water availability and temperature, respectively. ns, *, ** and *** indicate no significant differences, and differences at $p < 0.05$, 0.01 and 0.001 respectively.

Treatments	A_	A_	A_	A_	E_	E_	E_	E_	p		
	T_amb_WI	T_amb_PI	T_+4_WI	T_+4_PI	T_amb_WI	T_amb_PI	T_+4_WI	T_+4_PI	$\text{CO}_2 \times \text{WA}$	$\text{CO}_2 \times \text{T}$	$\text{WA} \times \text{T}$
$\delta^{13}\text{C}$ (‰)											
Root	-25.5 \pm 0.6 a	-25.8 \pm 1.0 a	-26.2 \pm 1.1 a	-26.9 \pm 0.4 a	-37.0 \pm 0.7 d	-33.1 \pm 0.9 c	-34.5 \pm 0.4 c	-30.8 \pm 0.5 b	***	**	ns
Cutting	-27.0 \pm 0.8 ab	-27.0 \pm 0.4 ab	-26.5 \pm 0.2 a	-26.7 \pm 0.4 a	-29.4 \pm 0.7 b	-28.2 \pm 0.5 ab	-28.3 \pm 0.4 ab	-27.7 \pm 0.3 ab	ns	ns	ns
Main Shoot	-27.3 \pm 0.1 a	-27.4 \pm 0.5 a	-26.6 \pm 0.3 a	-28.3 \pm 0.7 a	-37.6 \pm 2.4 d	-32.2 \pm 0.4 bc	-34.8 \pm 0.7 cd	-31.6 \pm 0.2 b	***	ns	ns
Petiole	-27.0 \pm 0.4 a	-27.0 \pm 0.5 a	-27.7 \pm 0.2 a	-27.0 \pm 0.6 a	-34.5 \pm 0.5 c	-32.4 \pm 0.4 bc	-33.9 \pm 0.7 c	-30.9 \pm 0.1 b	**	ns	ns
Leaf	-28.1 \pm 0.1 a	-27.6 \pm 0.6 a	-27.7 \pm 0.4 a	-27.9 \pm 0.1 a	-36.9 \pm 0.5 d	-34.1 \pm 0.2 bc	-35.5 \pm 1.2 cd	-32.9 \pm 0.2 b	**	ns	ns
Rachis	-29.1 \pm 0.2 a	-28.8 \pm 0.7 a	-28.6 \pm 0.7 a	-28.8 \pm 0.2 a	-32.3 \pm 0.1 b	-32.1 \pm 0.5 b	-31.4 \pm 0.2 b	-30.7 \pm 0.2 ab	ns	ns	ns
Berry	-25.8 \pm 0.3 a	-25.4 \pm 0.3 a	-25.4 \pm 0.5 a	-25.3 \pm 0.6 a	-36.2 \pm 0.8 c	-33.7 \pm 0.9 b	-34.4 \pm 1.4 bc	-32.6 \pm 0.5 b	ns	ns	ns
Leaf (WSC)	-24.2 \pm 0.3 a	-23.4 \pm 0.5 a	-23.9 \pm 0.2 a	-24.0 \pm 0.3 a	-36.0 \pm 0.5 c	-33.8 \pm 0.7 b	-33.6 \pm 0.6 b	-31.6 \pm 0.2 b	*	***	ns
C (%)											
Root	42.5 \pm 0.2 ab	42.2 \pm 0.9 ab	44.1 \pm 0.3 ab	44.4 \pm 0.9 a	41.2 \pm 1.5 b	44.0 \pm 1.9 ab	44.2 \pm 0.5 ab	43.5 \pm 0.5 ab	ns	ns	ns
Cutting	44.8 \pm 0.5 a	46.3 \pm 1.5 a	45.9 \pm 0.3 a	45.6 \pm 1.7 a	45.1 \pm 0.4 a	47.4 \pm 1.2 a	46.1 \pm 0.2 a	42.1 \pm 3.8 a	ns	ns	ns
Main Shoot	48.9 \pm 0.7 a	47.2 \pm 0.6 abc	45.8 \pm 0.3 bcd	45.2 \pm 0.8 cd	44.7 \pm 1.4 d	47.7 \pm 0.4 ab	46.3 \pm 1.0 bcd	45.3 \pm 0.4 cd	ns	ns	ns
Petiole	38.1 \pm 1.9 a	38.7 \pm 1.0 a	36.4 \pm 2.1 a	37.8 \pm 0.8 a	40.7 \pm 0.8 a	40.2 \pm 1.1 a	39.3 \pm 0.7 a	38.0 \pm 1.5 a	ns	ns	ns
Leaf	46.9 \pm 1.2 a	46.3 \pm 1.2 a	46.4 \pm 0.4 a	46.8 \pm 0.4 a	41.6 \pm 3.0 a	47.7 \pm 0.8 a	47.3 \pm 1.4 a	46.4 \pm 0.4 a	ns	ns	ns
Rachis	42.9 \pm 1.2 a	42.0 \pm 0.2 a	41.6 \pm 0.6 a	42.0 \pm 0.2 a	41.5 \pm 0.4 a	41.5 \pm 0.5 a	43.0 \pm 1.2 a	42.3 \pm 0.3 a	ns	ns	ns
Berry	42.7 \pm 3.4 a	40.9 \pm 1.7 a	44.1 \pm 1.2 a	39.6 \pm 0.5 a	43.0 \pm 2.2 a	43.5 \pm 1.7 a	41.0 \pm 1.6 a	42.2 \pm 2.7 a	ns	ns	ns
N (%)											
Root	1.17 \pm 0.17 bc	1.34 \pm 0.17 ab	1.48 \pm 0.06 ab	1.64 \pm 0.02 a	0.64 \pm 0.04 d	0.93 \pm 0.13 cd	0.79 \pm 0.12 d	1.14 \pm 0.09 bc	ns	ns	ns
Cutting	0.62 \pm 0.07 a	0.56 \pm 0.04 a	0.67 \pm 0.06 a	0.66 \pm 0.09 a	0.59 \pm 0.04 a	0.57 \pm 0.11 a	0.50 \pm 0.02 a	0.60 \pm 0.10 a	ns	ns	ns
Main Shoot	0.87 \pm 0.01 bc	0.89 \pm 0.05 bc	1.06 \pm 0.07 abc	1.22 \pm 0.19 a	1.18 \pm 0.20 ab	0.78 \pm 0.02 c	0.78 \pm 0.07 c	1.06 \pm 0.05 abc	ns	ns	*
Petiole	0.76 \pm 0.04 a	1.00 \pm 0.07 a	1.16 \pm 0.21 a	1.19 \pm 0.15 a	0.68 \pm 0.08 a	0.79 \pm 0.05 a	0.65 \pm 0.09 a	0.97 \pm 0.11 a	ns	ns	ns
Leaf	3.12 \pm 0.24 ab	3.19 \pm 0.15 a	3.10 \pm 0.24 ab	3.30 \pm 0.08 a	1.50 \pm 0.05 d	2.26 \pm 0.19 cd	1.94 \pm 0.20 cd	2.35 \pm 0.03 bc	ns	ns	ns
Rachis	1.92 \pm 0.28 b	2.37 \pm 0.05 ab	2.39 \pm 0.06 ab	3.11 \pm 0.23 a	1.99 \pm 0.19 b	1.58 \pm 0.06 b	2.13 \pm 0.42 ab	2.21 \pm 0.15 ab	*	ns	ns
Berry	0.68 \pm 0.07 b	0.70 \pm 0.01 b	0.71 \pm 0.05 b	0.87 \pm 0.05 a	0.61 \pm 0.04 b	0.66 \pm 0.03 b	0.59 \pm 0.06 b	0.67 \pm 0.05 b	ns	ns	ns

Table 4

Table 4. C/N ratio at the end of the treatments (grape ripeness stage) in the different organs of *V. vinifera* cv. Tempranillo grown from veraison to maturity under ambient (A) or elevated (E, 700 ppm) CO₂ concentrations, ambient (T_{amb}) or elevated (T₊₄) temperature regimes and well (WI) or partially (PI) irrigated. Data represent the average value of 3 analyses ± S.E. Different letter indicates significant differences between treatments (P<0.05) based on *LSD* test. Significance (p) of the ANOVA analyses for the interactions CO₂xWA, CO₂xT and WAxT are also shown. CO₂, WA and T refer to CO₂ concentration, water availability and temperature, respectively. ns, * and ** indicate no significant differences, and differences at p<0.05 and 0.01 respectively.

Treatments									<u>p</u>		
	A_T _{amb} _WI	A_T _{amb} _PI	A_T ₊₄ _WI	A_T ₊₄ _PI	E_T _{amb} _WI	E_T _{amb} _PI	E_T ₊₄ _WI	E_T ₊₄ _PI	CO ₂ xWA	CO ₂ xT	WAxT
C/N											
Root	38.0±6.0 cd	32.3±3.1 d	29.9±1.2 d	27.1±0.8 d	64.2±1.7 a	48.6±4.5 bc	58.4±7.4 ab	38.6±2.5 cd	*	ns	ns
Cutting	73.9±9.6 a	83.0±5.6 a	69.4±5.7 a	71.4±9.0 a	77.1±5.6 a	89.5±16.3 a	92.0±3.1 a	72.7±7.0 a	ns	ns	ns
Main Shoot	56.3±1.4 ab	53.6±3.6 abc	43.6±2.8 bcd	39.2±6.5 d	40.2±6.9 d	61.3±1.8 a	60.6±5.9 a	42.8±1.9 cd	ns	*	**
Petiole	50.6±4.8 ab	39.0±3.5 bc	33.4±4.2 c	33.1±5.4 c	61.9±7.7 a	51.2±2.8 ab	63.2±8.8 a	40.2±4.8 bc	ns	ns	ns
Leaf	15.1±0.8 d	14.6±0.3 d	15.2±1.2 d	14.2±0.5 d	27.7±1.3 a	21.5±2.3 bc	24.8±1.7 ab	19.8±0.2 c	*	ns	ns
Rachis	23.4±3.5 ab	17.8±0.4 bc	17.4±0.5 bc	13.7±1.0 c	21.2±2.0 ab	26.4±0.7 a	21.5±3.6 ab	19.3±1.2 bc	*	ns	ns
Berry	63.6±6.8 ab	58.7±1.9 b	62.8±4.5 ab	45.8±3.0 c	70.9±3.0 a	65.9±1.7 ab	70.9±4.7 a	63.4±0.8 ab	ns	ns	ns

Figure 1

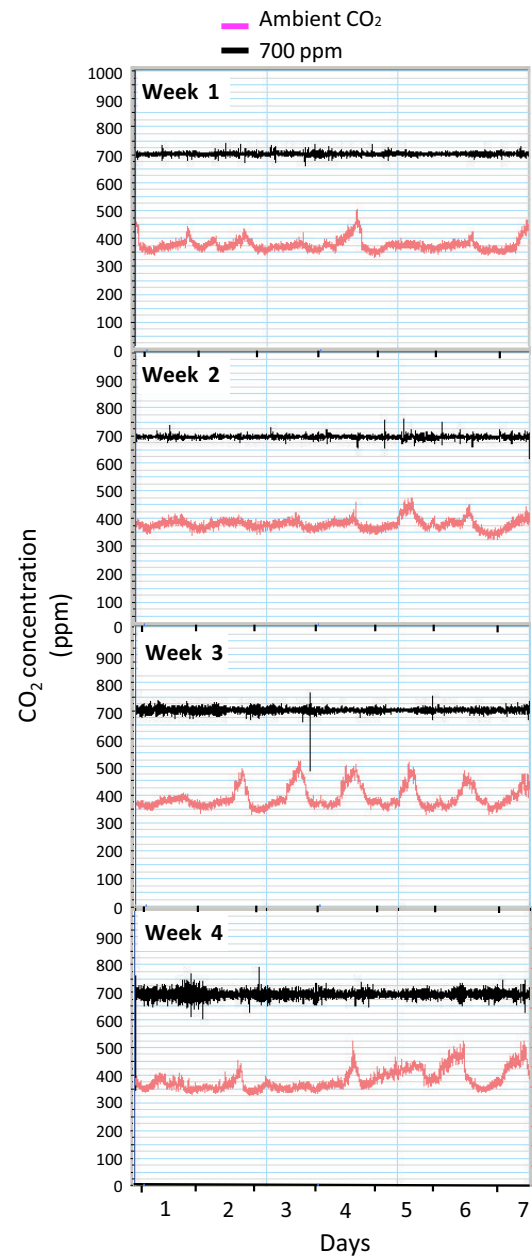
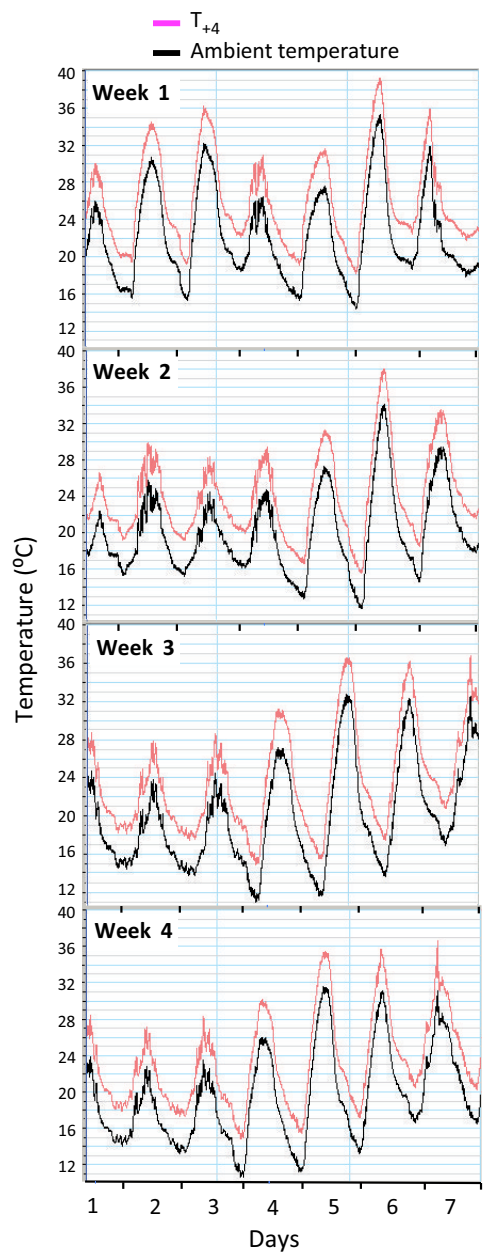


Figure 2

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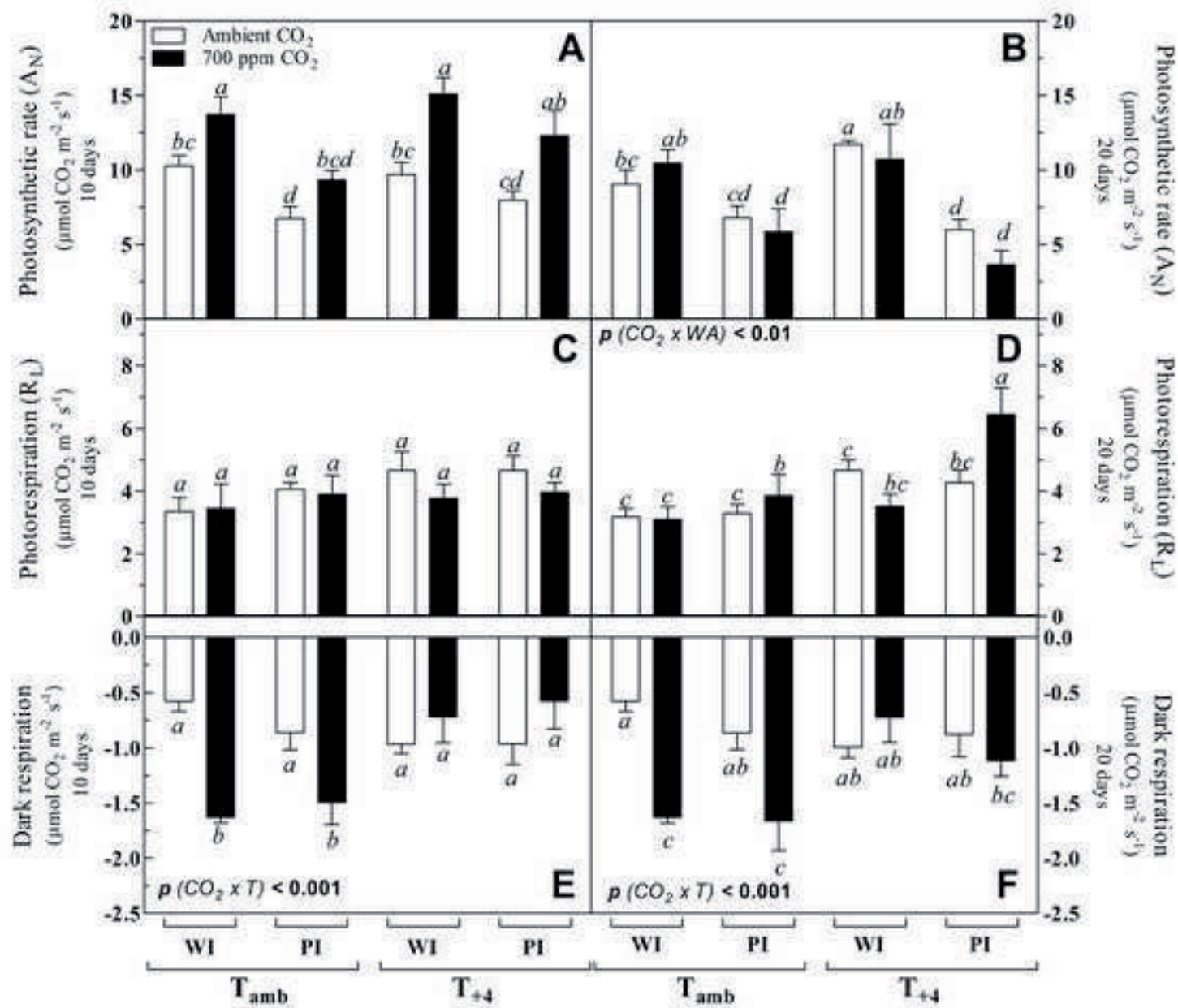


Figure 3

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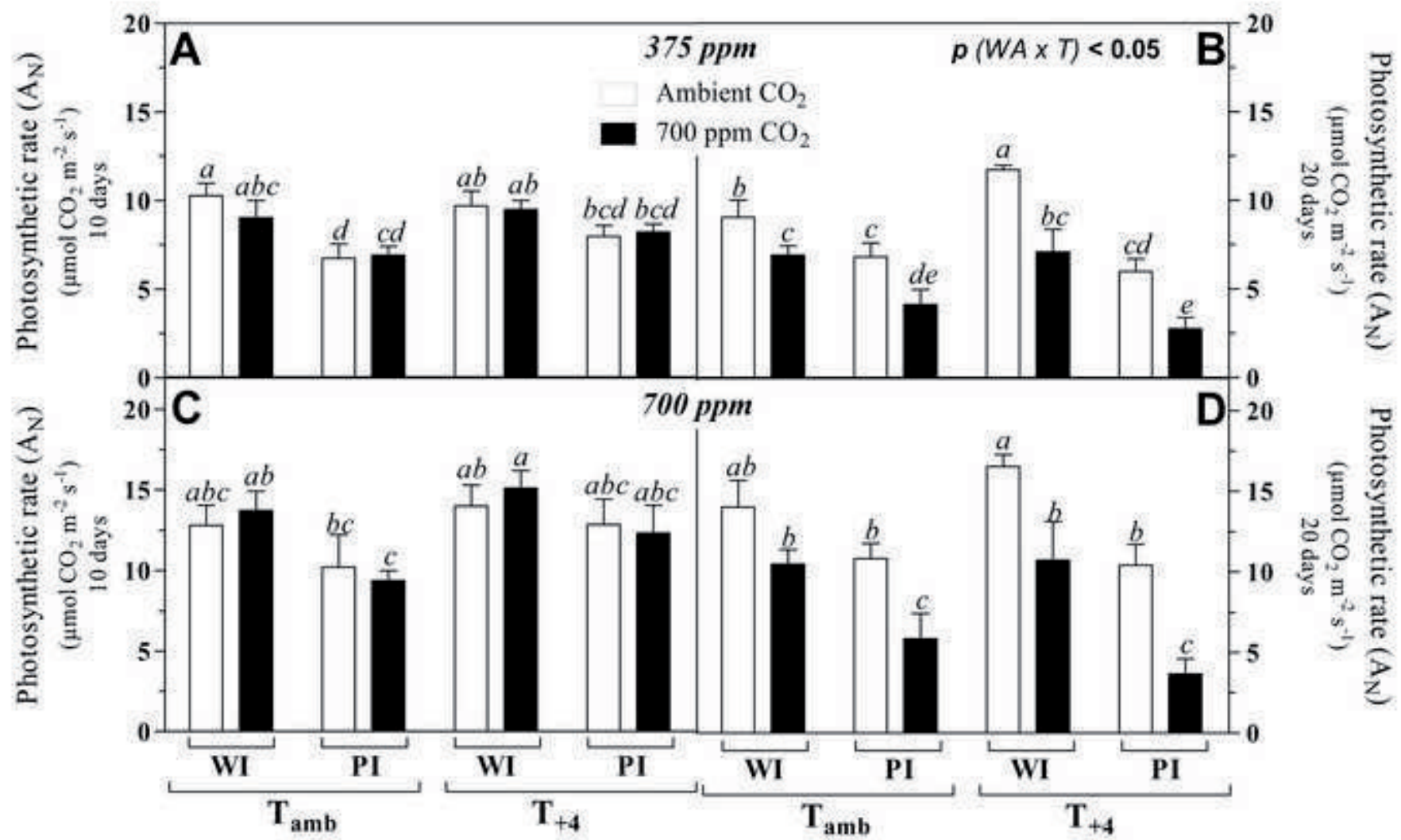


Figure 4

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