1 **Running page heading:** Mycorrhized wheat, high CO₂ and drought

2

3 **Title:** Responsiveness of durum wheat to mycorrhizal inoculation under different
4 environmental scenarios

5

6 Idoia Garmendia¹, Yolanda Gogorcena², Iker Aranjuelo³ and Nieves Goicoechea^{4*}

7

8 ¹ Departamento Ciencias de la Tierra y del Medio Ambiente, Facultad de Ciencias,
9 Universidad de Alicante, Ctra. San Vicente del Raspeig, s/n. Apdo. Correos 99, E-
10 03080 Alicante, Spain.

11 ² Departamento de Pomología. Estación Experimental de Aula Dei, Consejo Superior
12 de Investigaciones Científicas (CSIC), P.O. Box 13034, 50080 Zaragoza, Spain.

13 ³ Instituto de Agrobiotecnología (IdAB), Universidad Pública de Navarra-CSIC-
14 Gobierno de Navarra, Campus de Arrosadía, 31192 Mutilva Baja, Spain.

15 ⁴ Departamento de Biología Ambiental. Grupo de Fisiología del Estrés en Plantas
16 (Unidad Asociada al CSIC, EEAD, Zaragoza e ICVV, Logroño). Facultades de Ciencias y
17 de Farmacia y Nutrición, Universidad de Navarra, Irunlarrea 1, 31008, Pamplona,
18 Spain.

19

20 * **Corresponding author:** Nieves Goicoechea: Telephone +34 948 425600, ext.
21 806489; Fax + 34 948 425619; e-mail: niegoi@unav.es; Dpto. Biología Ambiental,
22 Grupo de Fisiología del Estrés en Plantas (Unidad Asociada al CSIC, EEAD, Zaragoza e
23 ICVV, Logroño). Facultades de Ciencias y de Farmacia y Nutrición, Universidad de
24 Navarra, Irunlarrea 1, E-31008 Pamplona, Spain.

25 **Abstract**

26 A greater understanding of how climate change will affect crop photosynthetic
27 performance has been described as a target goal to improve yield potential. Other
28 concomitant stressors can reduce the positive effect of elevated atmospheric CO₂
29 on wheat yield. Arbuscular mycorrhizal fungi (AMF) are symbiotic fungi predicted to
30 be important in defining plant responses to rising atmospheric CO₂, but their role in
31 response to global climatic change is still poorly understood. This study aimed to
32 assess if increased atmospheric CO₂ interacting with drought can modify the effects
33 of mycorrhizal symbiosis on flag leaf physiology in winter wheat. The study was
34 performed in climate-controlled greenhouses with ambient (400 ppm, ACO₂) or
35 elevated (700 ppm, ECO₂) CO₂ concentrations in the air. Within each greenhouse
36 half of the plants were inoculated with *Rhizophagus intraradices*. When ear
37 emergence began, half of the plants from each mycorrhizal and CO₂ treatment were
38 subjected to terminal drought. At ACO₂ AMF improved the photochemistry
39 efficiency of PSII compared with non-mycorrhizal plants, irrespective of irrigation
40 regime. Mycorrhizal wheat accumulated more fructan than non-mycorrhizal plants
41 under optimal irrigation. The level of proline in the flag leaf increased only in
42 mycorrhizal wheat after applying drought. Mycorrhizal association avoided
43 photosynthetic acclimation under ECO₂. However, nitrogen availability to flag leaves
44 in mycorrhizal plants was lower under ECO₂ than at ACO₂. Results suggest that the
45 mechanisms underlying the interactions between mycorrhizal association and
46 atmospheric CO₂ concentration can be crucial for the benefits that this symbiosis
47 can provide to wheat plants undergoing water deficit.

48 **Keywords:** arbuscular mycorrhizal fungi, carbon dioxide, drought, flag leaf,
49 photosynthesis, *Triticum durum*.

50

51 **Abbreviations Used:** ACO₂, ambient CO₂; AMF, arbuscular mycorrhizal fungi; C_i,
52 intercellular CO₂ concentration; CER, CO₂ exchange rate; D, drought; DM, dry
53 matter; ECO₂, elevated CO₂; Fv/Fm, maximum quantum yield of photosystem II; g_w,
54 total leaf conductance to water vapour; -M, non-mycorrhizal plants; +M,
55 mycorrhizal plants; PSII, photosystem II; W, water; WW, well-watered; δ¹⁵N,
56 nitrogen isotopic composition.

57

58 **1. Introduction**

59

60 Wheat is one of the major crops in temperate regions. Its production in the world
61 reached nearly 730 million tonnes (Tm) in 2014/2015 and the estimated production
62 for 2015/2016 is 733 million Tm. The European Union (EU), together with Argentina,
63 Australia, Canada, Kazakhstan, the Russian Federation and Ukraine, is one of the
64 major wheat exporters (FAO 2016). Despite an expected increase in cereal
65 production from the projected increase in CO₂ (Manderscheid and Weigel 1995;
66 Amthor 2001), the interaction of CO₂ with other limiting environmental factors
67 (mainly temperature and low water and/or nitrogen availability) might decrease or
68 eliminate the positive effect of elevated CO₂ (ECO₂) on plant production (Aranjuelo
69 and others 2011, 2013). The phenophases in which wheat plants are exposed to
70 drought strongly influence the effect of elevated CO₂ concentrations in the
71 atmosphere on the yield (Varga and others 2016). Indeed, as observed by Oury and

72 others (2012), the beneficial effects expected from the increase in [CO₂] in
73 European crop production during recent decades have been constrained by the
74 effects of temperature increases and extended drought. There is increasing
75 evidence that to achieve a quantum boost to cereal crop yield potential, a major
76 improvement in photosynthetic capacity and/or efficiency will be required
77 (Reynolds and others 2011). There is also evidence that historic gains in wheat yield
78 potential have been associated with increased photosynthesis. Furthermore, basic
79 research in photosynthesis has confirmed that substantial improvements are
80 theoretically possible (Parry and others 2013).

81 Arbuscular mycorrhizal fungi (AMF) are soil inhabitants belonging to the phylum
82 Glomeromycota, with a presumed origin at least 460 million years ago (Schüßler
83 and others 2001). These fungi colonize the roots of over 80% of plant species,
84 including wheat (see Singh and others 2012 for more detailed information), mostly
85 to the mutual benefit of both the plant host and the fungus. In the context of rising
86 atmospheric CO₂, AMF are predicted to be important in defining plant responses to
87 ECO₂ concentrations. According to Cavagnaro and others (2011), lower contents of
88 phosphorus (P) in tissues of plants when grown under ECO₂ can be alleviated by the
89 formation of AMF and improvements in plant nitrogen (N) nutrition resulting from
90 the formation of mycorrhizal symbiosis may be also important in determining plant
91 responses to atmospheric CO₂ enrichment. However, results from assays performed
92 under controlled conditions do not always support the hypothesis that the benefits
93 of mycorrhizal association for host plants will be greater under [CO₂] conditions
94 higher than those currently existing in air. Although AMF improved carbon (C)
95 accumulation and N uptake in wheat under ECO₂ (Zhu and others 2016), the

96 accumulation of some mineral nutrients (P, Cu, Fe) and antioxidant compounds
97 induced by AMF in leaves of lettuces cultivated at current [CO₂] levels decreased or
98 even disappeared under ECO₂ (Baslam and others 2012a). Moreover,
99 photosynthetic acclimation was increased in alfalfa associated with AMF under
100 ECO₂ (Goicoechea and others 2014). In addition, the effects of AMF on host plants
101 may depend on other limiting environmental factors interacting with ECO₂. While
102 AMF improved vegetative growth of *Triticum aestivum* subjected to salinity stress
103 under ECO₂ (Zhu and others 2016), the confluence of water deficit and ECO₂ caused
104 a general depletion of micro/macro nutrients and gliadins in grains of *T. durum*
105 (Goicoechea and others 2016). All these findings indicate that the role that AMF
106 may play in response to global climatic change is still poorly understood.

107 The process of grain filling in cereals, which is crucial for grain yield, is closely
108 linked to flag leaf functionalities. Some recent studies have focused on the primary
109 C metabolism, N assimilation and transpiration in the flag leaf of wheat plants
110 cultivated under ECO₂ at ambient (Aranjuelo and others 2015) or increased
111 (Jauregui and others 2015) air temperature. However, a reasonable understanding
112 of the function of flag leaves under drought conditions is lacking for any
113 mycorrhized cereal (Biswal and Kohli 2013). To our knowledge no information is
114 available on the metabolism and physiology of flag leaves in cereals subjected to
115 the interaction between ECO₂, drought and mycorrhizal symbiosis. We have only
116 found one study focusing on the contribution of AMF for wheat plants grown under
117 ECO₂ and undergoing salinity stress (Zhu and others 2016).

118 All these findings lead us to pose the following hypotheses: (1) the association
119 of wheat with AMF might improve the water and nutritional status of host plants

120 and consequently (2) this symbiotic association could reduce the negative effect of
121 water deficit on the physiology of flag leaves; (3) in contrast, the elevated CO₂ in the
122 atmosphere may decrease the expected beneficial effect of AMF on flag leaf
123 physiology under drought conditions.

124 In order to test such hypotheses we performed measurements related to
125 growth parameters directly affected by drought, mycorrhizal symbiosis and/or the
126 CO₂ concentration in the atmosphere, such as biomass and spike production.
127 Moreover, we determined certain physiological parameters (carbon exchange rate,
128 photochemistry efficiency of PSII or the accumulation of osmolites) that can
129 highlight the response of plants to water deficit and these measurements were
130 carried out in the flag leaf because of its direct connection with the grain filling. In
131 this way, we have more exhaustively analyzed the sugar profile and nitrogen
132 assimilation in the flag leaves of wheat plants undergoing different growth
133 conditions.

134

135 **2. Materials and methods**

136

137 *2.1. Plant material and experimental design*

138 High yielding durum wheat (*Triticum durum*, Def. cv. Amilcar) seeds were vernalized
139 during three weeks in a cold chamber at 4°C. After vernalization, seedlings were
140 transplanted, in a controlled environmental chamber (Convion PGV 36, Winnipeg,
141 Canada), into 13 L pots (two plants per pot) containing a mixture of 2.5:2.5:1 (v/v/v)
142 vermiculite/sand/peat. The peat had a pH of 5.2-6.0, 70-150 mg L⁻¹ of nitrogen, 80-
143 180 mg L⁻¹ of total P₂O₅ and 140-220 mg L⁻¹ K₂O and it was previously sterilized at

144 100°C for 1 h on three consecutive days. Growth conditions within the chamber
145 were fixed at 25/15°C (day/night), 45% relative humidity (RH) values, with a
146 photoperiod of 14 h fluorescent and photosynthetic photon flux density (PPDF) of
147 about 300–400 $\mu\text{molm}^{-2} \text{s}^{-1}$ provided by lamps (SylvaniaDECOR183, Professional-
148 58W, Germany).

149 After 7 days of growth in the growth chambers (on 18th March), half of the
150 plants (64 plants in 32 pots) (growth stage 12 according to the Zadoks scale, 1974)
151 were inoculated with the mycorrhizal inoculum 'Glomygel Intensivo' (Mycovitro
152 S.L., Pinos Puente, Granada, Spain) (+M plants). The concentrated commercial
153 inoculum was derived from an *in vitro* culture of the AMF *Rhizophagus intraradices*
154 (Schenck and Smith) Walker & Schüßler comb. nov. (Krüger and others 2012) and
155 contained around 2,000 mycorrhizal propagules (inert pieces of roots colonized by
156 AMF, spores and vegetative mycelium) per mL of inoculum. In order to facilitate its
157 application, the concentrated commercial inoculum was diluted with distilled water
158 until a resultant mycorrhizal inoculum with around 250 propagules per mL was
159 obtained. Each +M plant received 8 mL of the diluted mycorrhizal inoculum close to
160 the roots thus making a total of 2,000 propagules. A filtrate was added to plants
161 that did not receive the mycorrhizal inoculum (-M plants, 64 plants in 32 pots) in an
162 attempt to restore other soil free-living microorganisms accompanying AMF (Jansa
163 and others 2013). The filtrate was obtained by passing the diluted mycorrhizal
164 inoculum through a layer of 15-20 μm filter papers (Whatman, GE Healthcare, UK)
165 and each -M plant received 8 mL of filtrate close to the roots.

166 The plants were then transferred (also on 18th March) to four [CO₂] controlled
167 greenhouses located on the Universidad de Navarra campus (42.80 N, 1.66 W;

168 Pamplona, Spain) (two ambient CO₂ (ACO₂) and two elevated CO₂ (ECO₂)
169 greenhouses). The design of the greenhouses was similar to that described by
170 Morales and others (2014). Sixteen -M pots (32 plants) and sixteen +M pots (32
171 plants) were placed in ACO₂ greenhouses (eight -M and eight +M pots in each ACO₂
172 greenhouse). Sixteen -M pots (32 plants) and sixteen +M pots (32 plants) were
173 placed in ECO₂ greenhouses (eight -M and eight +M pots in each ECO₂ greenhouse).
174 In order to prevent the CO₂ effect being confounded with greenhouse effects (De
175 Luis and others 1999), we used two ACO₂ greenhouses and two ECO₂ greenhouses
176 and the sixteen pots belonging to the same treatment were divided between the
177 two greenhouses with equal atmospheric CO₂ concentrations. Data obtained for the
178 same treatment from the two equivalent greenhouses were then pooled for
179 statistical analyses. In the two ACO₂ greenhouses, no CO₂ was added and the [CO₂]
180 in the atmosphere was approximately 392 $\mu\text{mol mol}^{-1}$. In the other two
181 greenhouses (ECO₂), [CO₂] was fixed at $\sim 700 \mu\text{mol mol}^{-1}$ by injecting pure CO₂
182 (purity up to 99.99%) from cylinder-gases (34 L of CO₂ per cylinder) through the two
183 inlet fans during the light hours. Injection of CO₂ to greenhouses began when light
184 intensity was equal or greater than 5 watts m⁻² as measured by a Silicon
185 Pyranometer PYR-S (APOGEE Instruments, Inc., Logan, UT, USA) making a total of
186 13-15 h of high CO₂ a day from March to June. The CO₂ was provided by Air Liquide
187 (Bilbao, Spain). The [CO₂] was continuously monitored using a Guardian Plus gas
188 monitor (Edinburgh Instruments Ltd, Livingston, UK). The monitor's signal was fed
189 into a proportional integrative differential controller that regulated the opening
190 time (within a 10 s cycle) of a solenoid valve that injected CO₂ into both inlet fans.
191 Inside the greenhouses, the pots were placed in holes made in the soil in order to

192 provide natural temperature fluctuations, thus simulating the temperature
193 differences observed between shoots and roots under field conditions (Rawson and
194 others 1995).

195 Until ear emergence (growth stage 50 according to Zadoks scale 1974) all plants
196 were watered with a complete Hoagland solution (Arnon and Hoagland 1939) twice
197 a week and with water once a week to avoid excessive salt accumulation. At that
198 moment, the plants were randomly assigned to two water treatments. Within each
199 greenhouse, half of the plants were labelled as fully-watered plants (irrigated until
200 pot capacity, WW) and the other half as water stressed plants (drought, D). The
201 maximum soil volumetric water content (θ_v), corresponding to the well-irrigated
202 treatments, was around $0.44 \text{ cm}^3 \text{ cm}^{-3}$. The applied drought level corresponded to
203 50% θ_v of well-watered plants (around $0.22 \text{ cm}^3 \text{ cm}^{-3}$). Terminal drought was
204 selected because it is characteristic of regions with Mediterranean-type climates
205 where rainfall decreases and evaporation and temperature increase in spring, when
206 wheat enters its reproductive stage (Fitzpatrick 1970). Therefore we had a total of
207 eight different treatments: -M WW ACO₂; -M D ACO₂; +M WW ACO₂; +M D ACO₂; -
208 M WW ECO₂; -M D ECO₂; +M WW ECO₂; +M D ECO₂, and the experiment, with 4
209 repetitions, was replicated in 2 greenhouse pairs thus making a total of 8 pots per
210 treatment with 2 plants per pot. One plant per pot was used to determine growth
211 parameters as well as nitrogen and carbon isotopic composition; the other plant
212 was used for physiological (CO₂ exchange rate, leaf conductance, intercellular CO₂
213 concentration and maximum quantum yield of PSII) measurements, biochemical
214 (sugars, proteins and proline) determinations and for the analysis of mycorrhizal
215 colonization.

216 In order to determine N absorption, ^{15}N labelling was conducted for one week
217 in four pots of each of the above-mentioned treatments (two per greenhouse),
218 starting one week after anthesis (time at which >50% of spikes showed protruding
219 anthers) (growth stage 65 according to the Zadoks scale 1974). This labelling time
220 was selected since it falls within the critical timeframe for grain yield (Aranjuelo and
221 others 2013), with the onset of protein remobilization (Hirel and Gallais 2006). The
222 N isotope composition ($\delta^{15}\text{N}$) of the non-labelled natural Hoagland solution was -
223 1.53‰ (i.e. 0.37% ^{15}N). N labelling was carried out by replacing 1% of the KNO_3 of
224 the nutrient solution (1.22 g L^{-1}) with the same concentration of ^{15}N -enriched
225 K^{15}NO_3 (enriched at 98 %) for five days. After this exposure period, plants were
226 harvested for later analysis of total organic matter (TOM) ^{15}N isotopic composition
227 ($\delta^{15}\text{N}$) (see below). All determinations were conducted in flag leaf samples collected
228 immediately after the end of the labelling period.

229

230 2.2. Mycorrhizal analyses

231 In order to verify if AMF was established in the roots of wheat before applying
232 drought conditions, five fragments of roots were taken from each pot (thus making
233 a total of 40 root fragments per treatment) when the first spikelet of inflorescence
234 was visible (growth stage 50, Zadoks 1974), and then cleared and stained according
235 to Phillips and Hayman (1970). Fragments of roots were carefully collected to avoid
236 major disturbance of the whole root system. Root fragments were also taken from -
237 M plants in order to apply disturbance comparable to that applied to +M plants. The
238 percentage of mycorrhizal colonization in roots was calculated as the ratio between

239 the number of roots showing fungal structures (hyphae, arbuscules and/or vesicles)
240 and the total number of roots examined. Results were expressed as percentages.

241

242 2.3. *Plant growth*

243 Growth analyses were conducted in plants harvested two weeks after anthesis (on
244 21st June 2013) (growth stage 69, Zadoks 1974). Shoot, root and spike samples were
245 dried at 60°C in an oven for 48 h to determine the dry mass (DM).

246

247 2.4. *Carbon (C) and nitrogen (N) isotope and content analysis*

248 Flag leaves (harvested on 21st June 2013) were dried at 60°C for 48 h, and analysed
249 for C and N isotope composition ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) in total organic matter (TOM), and
250 elemental C and N content (%C and %N). One milligram mg of ground sample was
251 used for each determination. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and C and N content were determined
252 using an isotope ratio mass spectrometer (IsoPrime, Elementar France,
253 Villeurbanne) coupled to an elemental analyser (EA 3000, EuroVector, Milan, Italy).

254 The $^{15}\text{N}/^{14}\text{N}$ ratio (R) in plant material was expressed in δ notation ($\delta^{15}\text{N}$) with
255 respect to atmospheric N_2 , and measured with an analytical precision of 0.2‰:

$$256 \quad \delta^{15}\text{N} = (\text{R}_{\text{sample}} - \text{R}_{\text{standard}}) / \text{R}_{\text{standard}} \times 1000$$

257 $\delta^{15}\text{N}$ accuracy was monitored using international secondary standards of known
258 $^{15}\text{N}/^{14}\text{N}$ ratios (IAEA-N₁ and IAEA-N₂ ammonium sulphate and IAEA-NO₃ potassium
259 nitrate, IAEA, Austria). The nitrogen isotope composition was then converted to a
260 percentage: $\%^{15}\text{N} = \text{R}_{\text{sample}} / (1 + \text{R}_{\text{sample}})$ where R_{sample} was obtained with
261 $\text{R}_{\text{standard}} \times (\delta^{15}\text{N} + 1)$, with $\text{R}_{\text{standard}} = 0.003667$.

262 The $^{13}\text{C}/^{12}\text{C}$ ratio (R) in plant material was expressed in δ notation ($\delta^{13}\text{C}$) with
263 respect to Vienna Pee Dee Belemnite calcium carbonate (V-PDB), and measured
264 with an analytical precision of 0.1‰:

$$265 \quad \delta^{13}\text{C} = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1$$

266 $\delta^{13}\text{C}$ accuracy was monitored using international secondary standards of known
267 $^{13}\text{C}/^{12}\text{C}$ ratios (IAEA-CH7 polyethylene foil, IAEA-CH6 sucrose and USGS-40 glutamic
268 acid, IAEA, Austria).

269

270 *2.5. Gas exchange and chlorophyll fluorescence determinations*

271 Well-developed and healthy flag leaves were selected to conduct gas exchange
272 analyses two weeks after anthesis (growth stage 69, Zadoks 1974), using a Li-Cor
273 6400 XT portable photosynthesis system (Li-Cor, Lincoln, NE, USA). Photosynthetic
274 parameters were obtained using the equations of von Caemmerer and Farquhar
275 (1981). CO_2 exchange rate (CER) and stomatal conductance (g_w) were measured
276 either at $400 \mu\text{mol mol}^{-1} \text{CO}_2$ for plants grown at ACO_2 or $700 \mu\text{mol mol}^{-1} \text{CO}_2$ for
277 plants grown at ECO_2 . The gas exchange system was maintained at 25°C and 1200
278 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) was provided by LED light.

279 To estimate the acclimation to the elevated CO_2 of stomatal conductance (g)
280 and photosynthesis (A) Bunce's formulas were used (Bunce 2001). The acclimatory
281 response of g_w and CER was quantified as the ratio of g_w or CER for plants grown at
282 ECO_2 to those of plants grown at ACO_2 , when both were measured at $700 \mu\text{mol mol}^{-1}$
283 CO_2 . When the ratio reached values lower than 1, an acclimation process was
284 deemed to have taken place.

285 Measurements of chlorophyll fluorescence emission were made two weeks
286 after anthesis (growth stage 69) with the LiCor 6400XP gas exchange analyzer on
287 dark-adapted flag leaves. Minimal (F_o) and maximal (F_m) fluorescence emission were
288 recorded. Then the maximum quantum yield of photosystem II (F_v/F_m , where F_v is F_m
289 $- F_o$) were calculated (Genty and others 1989).

290

291 2.6. Biochemical analyses

292 Samples (0.1 g DM of leaves) for soluble carbohydrate analyses were collected at
293 final harvest (21st June 2013, growth stage 69 according to Zadoks 1974) and freeze
294 crushed. Polar compounds were extracted into 1 mL aqueous 80% ethanol at 80°C,
295 in three steps, each lasting 20 min (Jiménez and others 2011). The mixture of each
296 step was centrifuged for 5 min at 4,800 x g and slurries were pooled. Ethanol was
297 evaporated under vacuum in a speed vac system (Thermo Fisher Scientific Inc.,
298 Waltham, MA, USA) and dry extracts were solubilized in 500 μ L double-distilled
299 water. The soluble carbohydrates of the samples were purified using about 3.5 g g^{-1}
300 plant material ion exchange resins (Bio-Rad AG 50 W-X8 Resin 200-400 mesh
301 hydrogen form, Bio-Rad AG 1-X4 Resin 200-400 chloride form). The samples were
302 concentrated to 400 μ L, filtered through a 0.22 μ m filter and 20 μ L were used for
303 analysis by high-performance liquid chromatography (HPLC), using a Ca-column
304 (Aminex HPX-87C 300 mm x 7.8 mm column Bio-Rad) flushed with 0.6 mL min^{-1}
305 double distilled water at 85°C with a refractive index detector (Waters 2410,
306 Milford, MA, USA). Concentrations of the main carbohydrates, nystose, 1-kestose,
307 sucrose, glucose, xylose, fructose and sorbitol were calculated for each sample
308 using mannitol as an internal standard since it is not present in wheat samples.

309 Carbohydrate quantification was performed with the Empower Login software,
310 Waters (Millford, Mass, USA) using standards of analytical grade from Panreac
311 Química S.A. (Barcelona, Spain) and Sigma-Aldrich (Schnelldorf, Germany).
312 Concentrations of carbohydrates were expressed as mg g⁻¹ DM.

313 Starch, proline and total soluble proteins were quantified in potassium
314 phosphate buffer (KPB) (50 mM, pH = 7.5) extracts of leaves (1 g FW of leaves).
315 These extracts were filtered through four cheese cloth layers and centrifuged at
316 38,720 x *g* for 10 min at 4°C. The pellet was used for starch determination (Jarvis
317 and Walker 1993). The supernatant was collected and stored at 4°C for protein and
318 proline determinations. Total soluble proteins were measured by the protein dye-
319 binding method of Bradford (1976) using bovine serum albumin (BSA) as a standard.
320 Free proline was estimated by spectrophotometric analysis at 515 nm of the
321 ninhydrine reaction (Irigoyen and others 1992). Results were expressed as mg of
322 starch or total soluble proteins per g of DM and µmol of proline per g of DM.

323

324 *2.7. Statistical analysis*

325 As explained above, plants were grown in two greenhouses per [CO₂] treatment.
326 Two-factor ANOVA analyses (IBM SPSS v. 21) (between the same [CO₂]
327 greenhouses, with AMF inoculation and water regime as the main factors)
328 conducted in the above-mentioned parameters ruled out significant differences ($P \leq$
329 0.05) derived from growth in the different greenhouses. For this reason, the
330 subsequent statistical analyses were carried out with all the values (per [CO₂]
331 treatment) combined. Data were subjected to a three-factor ANOVA (factorial 2 × 2
332 × 2) (IBM SPSS v. 21). The variance was related to the main treatments (AMF

inoculation, AMF; atmospheric CO₂ concentration, CO₂ and water regime, W) and to the interaction between these parameters (AMF × CO₂, AMF × W, CO₂ × W, AMF × CO₂ × W). Means ± standard errors (SE) were calculated and, when the F ratio was significant ($P \leq 0.05$), a Duncan Multiple Range Test was applied. The acclimation of conductance and photosynthesis was tested by using single-group *t*-tests, evaluating whether the mean ratios of each treatment differed significantly from a value of one ($P \leq 0.05$). Data on mycorrhizal colonization were analysed by Chi-square (χ^2) test and were subjected to arc-sin transformation before applying χ^2 -test. Tests results were always considered significant at $P \leq 0.05$.

3. Results

3.1. Plant growth, gas exchange and chlorophyll fluorescence parameters

Neither the concentration of CO₂ in the atmosphere nor the water regime applied to plants altered the percentage of wheat roots showing mycorrhizal structures at growth stage 50 (Zadoks 1974) (Fig. 1). Fungal structures were never found in roots of plants not inoculated with AMF.

At ECO₂, shoot biomass of wheat plants was enhanced, especially when plants were well-watered (CO₂, $P \leq 0.001$; CO₂ × W, $P \leq 0.01$) (Table 1). However, the effect of growth [CO₂] was not significant in root and spike DM (CO₂, $P > 0.05$), although ECO₂ increased the number of spikes in well-watered -M plants at growth stage 69 (Zadoks, 1974) (CO₂, $P \leq 0.01$; CO₂ × W, $P \leq 0.05$). In relation to mycorrhizal symbiosis, there was no response in growth parameters, with the exception of a decreased root DM in +M plants grown with limited irrigation together with ACO₂

357 (AMF, $P \leq 0.05$). On the other hand, water limitation reduced shoot biomass under
358 ECO_2 conditions, regardless of whether plants were inoculated or not with AMF (W,
359 $P \leq 0.05$; $\text{CO}_2 \times \text{W}$, $P \leq 0.01$).

360 Photosynthetic rates in flag leaves of plants exposed to ECO_2 were significantly
361 higher and concomitant with g_w reductions compared with those of plants
362 cultivated at ACO_2 (CO_2 , $P \leq 0.001$ for CER and g_w) (Table 2). Nevertheless, this fact
363 depended on mycorrhizal symbiosis ($\text{CO}_2 \times \text{AMF}$, $P \leq 0.01$ for CER and g_w). When
364 wheat plants were inoculated with AMF, they exhibited an additional enhanced
365 photosynthetic rate at ECO_2 . Photosynthetic parameters were down-regulated in
366 wheat plants subjected to limited irrigation (W, $P \leq 0.001$ for CER, g_w , C_i and F_v/F_m),
367 with a significant interaction between the three factors that were analyzed in this
368 study related to the photochemistry efficiency of PSII ($\text{CO}_2 \times \text{AMF} \times \text{W}$, $P \leq 0.01$ for
369 F_v/F_m).

370 The acclimatory responses of stomatal conductance and photosynthesis are
371 shown in Table 3. While -M plants exhibit detectable effects on g_w and CER due to
372 ECO_2 exposure (ratios significantly lower than one ($P \leq 0.05$), especially in plants
373 undergoing water restrictions), in +M plants no acclimatory responses were
374 observed.

375

376 *3.2. C and N isotopic composition and content in flag leaves*

377 Wheat plants showed a triple interaction on non-labeled N isotopic composition of
378 flag leaves between the factors analyzed in the study ($\text{CO}_2 \times \text{AMF} \times \text{W}$, $P \leq 0.05$)
379 (Table 4). Thus, while -M well-watered plants increased $\delta^{15}\text{N}_{\text{non-lab}}$ under ECO_2
380 conditions, +M plants with limited irrigation levels were decreased. In addition,

381 wheat plants grown at elevated $[\text{CO}_2]$ reduced foliar $\delta^{15}\text{N}_{\text{lab}}$, $\delta^{13}\text{C}$ and N
382 concentration (CO_2 , $P \leq 0.001$ for $\delta^{15}\text{N}_{\text{lab}}$, $\delta^{13}\text{C}$ and N). On the other hand, plants
383 associated with mycorrhizal fungi and grown at ACO_2 showed higher $\delta^{15}\text{N}_{\text{lab}}$ than
384 their respective non-mycorrhizal controls (AMF, $P \leq 0.001$). In contrast, when plants
385 were subjected to limited irrigation, $\delta^{15}\text{N}_{\text{lab}}$ values were reduced in both -M and +M
386 wheat, especially under ACO_2 conditions (W, $P \leq 0.001$).

387

388 *3.3. Sugars, starch, proteins and proline determinations*

389 The factor that most influenced the sugar profile in flag leaves of wheat plants was
390 water availability (W, $P \leq 0.001$ for glucose, xylose, sorbitol, fructan, fructan DP>3,
391 fructan + S + F + G and total sugars) (Table 5). In fact, wheat plants subjected to
392 drought and grown at ECO_2 contained higher concentrations of glucose and xylose
393 than plants grown under well-watered and ACO_2 conditions (W, $P \leq 0.001$; CO_2 , $P \leq$
394 0.001 ; $\text{CO}_2 \times \text{W}$, $P \leq 0.001$). However, lower concentrations of sorbitol and fructan
395 were detected in wheat grown with limited irrigation (W, $P \leq 0.001$). Mycorrhizal
396 symbiosis never reduced foliar total sugar concentrations under WW conditions
397 (AMF, $P > 0.05$). Nevertheless, the levels of total sugars were the lowest in those +M
398 plants cultivated with water restrictions (W, $P \leq 0.001$; $\text{AMF} \times \text{W}$, $P \leq 0.01$).
399 Moreover, sucrose content was reduced in +M plants grown at ACO_2 (AMF, $P \leq$
400 0.01), while fructan concentrations increased in wheat inoculated with AMF (AMF, P
401 ≤ 0.05).

402 Although amounts of starch declined in flag leaves of wheat plants due to
403 elevated CO_2 concentrations and drought (CO_2 , $P \leq 0.001$; W, $P \leq 0.05$), inoculation
404 with AMF only reduced starch levels in the leaves of WW plants and at ACO_2 (Table

6). The interaction between the three factors in protein concentration resulted in the highest values in droughted -M plants and well-watered +M plants grown at ACO₂ in comparison with their respective controls (CO₂ × AMF × W, $P \leq 0.01$). Levels of proline increased in plants subjected to limited irrigation (W, $P \leq 0.001$), regardless of whether they were inoculated or not with AMF. However, WW plants exposed to ECO₂ showed the lowest concentrations of proline in flag leaves (CO₂, $P \leq 0.01$; CO₂ × W, $P \leq 0.001$).

4. Discussion

4.1. Effects of mycorrhizal association at ACO₂

Under optimal irrigation (WW) the development of vegetative organs (shoot and root) was similar in -M and +M wheat but the number of spikes was slightly higher in +M than in -M plants (Table 1), indicating that phenology may be accelerated in wheat inoculated with AMF. In agreement with this hypothesis, Baslam and others (2012b) also observed that the association of alfalfa with AMF can shorten the vegetative period of this forage legume. However, while the application of water deficit (D) accelerated the production of spikes in -M plants, it did not affect the number of spikes in +M plants, suggesting that water restriction only accelerated life cycle in -M wheat plants. Mycorrhizal symbiosis can delay (Goicoechea and others 1995) or accelerate (Goicoechea and others 2004) the senescence of vegetative organs in host plants subjected to drought depending on the type of plant and its own ecological adaptations to cope with water deficit. Under water restriction (D) root biomass was significantly lower in +M than in -M wheat (Table

429 1) which may be because fungal hyphae associated with roots can uptake water and
430 in the whole plant, soil-to-root or root-to-leaf hydraulic conductance were
431 improved in plants associated with AMF and subjected to drought (Augé 2001).

432 Arbuscular mycorrhizal (AM) symbioses often modify stomatal behaviour
433 although the degree of such an effect is not always apparent and is unpredictable.
434 In several previous studies, AMF have been reported to favour stomatal opening,
435 with the effects being more pronounced under drought than under well watered
436 conditions (Augé and others 2015). In the present study, leaf conductance (Table 2)
437 was lower in the flag leaves from +M wheat plants than in their respective –M
438 controls when cultivated at ACO₂ and optimal irrigation (WW). The reduced leaf
439 conductance (Table 2) together with lower N content (Table 4) in flag leaves of +M
440 plants in comparison with those of –M plants at ACO₂ and WW conditions, also
441 supports the idea that phenology was accelerated in wheat inoculated with AMF
442 under optimal irrigation (Vos and Oyarzún 1987). The above-mentioned decline in
443 stomatal conductance in flag leaves of +M wheat at ACO₂ and optimal irrigation
444 (WW) was not correlated with reduced photosynthetic rates, thus resulting in
445 smaller Ci concentrations than in flag leaves from –M plants (Table 2). Similarly,
446 Aranjuelo and others (2015) found that leaf conductance in durum wheat declined
447 somewhat more with age than photosynthesis. The higher values of the ratio of
448 Fv/Fm in +M wheat at ACO₂ and WW conditions in comparison with those of –M
449 plants (Table 2) indicate that AMF improved photochemistry efficiency of PSII (Zhu
450 and others 2012) and this beneficial effect became more evident after applying
451 water deficit (D) indicating that mycorrhizal symbiosis reduced the degree of stress
452 suffered by wheat plants (Baker 2008).

453 When comparing the carbohydrate profile in flag leaves of –M and +M wheat
454 grown at ACO₂ under WW conditions (Table 5), we found that +M plants
455 accumulated almost three times more fructan than –M plants. Müller and others
456 (1999) observed that mycorrhizal symbiosis exerted systemic effects on assimilate
457 partitioning in shoots so that levels of fructan were lower, similar or higher in leaves
458 of mycorrhizal plants than in those of non-mycorrhizal plants depending on plant
459 phenology, age of leaf and the fertilization applied to plants. Some studies have
460 demonstrated that when plants are transformed with the ability to synthesize
461 fructan, a concomitant increase in drought and/or freezing tolerance occurs (see
462 the review by Livingston and others 2009). In our study, the application of water
463 deficit (D) caused a marked decrease in the levels of fructan in the flag leaves of +M
464 plants (reduction exceeding 80%) at ACO₂ (Table 5). Moreover, the proportion of
465 hexoses (glucose, fructose and sorbitol) in relation to sucrose and fructan increased
466 from 0.09 in WW plants to 0.22 in stressed (D) plants. Such data suggest that some
467 specific enzymes such as invertase, fructan 1-fructosyltransferase and fructan 1-
468 exohydrolase might have contributed to generate the hexose pool (Oliveira and
469 others 2013) and thus, to increase the tolerance of +M wheat cv. Amilcar to drought
470 (Gupta and others 2011). In –M plants cultivated at ACO₂, the application of water
471 deficit (D) did not significantly enhance the proportion of hexoses in relation to
472 sucrose and fructan (0.13 in WW plants and 0.15 in stressed plants)(Table 5).

473 Another relevant benefit of AMF for wheat plants cultivated at ACO₂ was the
474 increased uptake of N (demonstrated by the higher $\delta^{15}\text{N}$) (Table 4) under both well
475 watered (WW) and drought conditions (D) in comparison with their respective –M
476 plants. Moreover, this enhanced uptake of N (Table 4) occurred despite the lower

477 (under WW conditions) or similar (under D conditions) leaf conductance (Table 2)
478 measured in +M plants. Recently, Calvo-Molina and others (2014) found increased
479 root hydraulic conductivity in +M tomato that was not correlated with enhanced
480 stomatal conductance but with higher expression and/or phosphorylation state of
481 plant and fungal aquaporins. The improved uptake of N under drought conditions
482 (D) in +M wheat in comparison with –M plants (Table 4) could benefit the
483 accumulation of proline (Table 6) in flag leaves of +M plants undergoing water
484 restriction (D), which may have contributed to an osmotic adjustment that would
485 allow an adequate water status in order to sustain metabolism in flag leaves to be
486 maintained. Contrary to the idea that proline accumulation seems to occur only
487 when plant growth is already retarded by drought stress (Aspinall 1986), the
488 accumulation of proline in +M wheat at ACO₂ (Table 6) took place before water
489 restriction exerted any deleterious effect on plant growth (Table 1). Moreover, the
490 higher N uptake by roots and accumulation in flag leaves of +M wheat undergoing
491 water deficit (D) in comparison with the –M controls (Table 4), could be related to
492 the high amount of gliadins found in grains of +M wheat subjected to drought
493 (Goicoechea and others 2016).

494

495 *4.2. Effects of mycorrhizal association under ECO₂*

496 In well watered (WW) –M wheat, the greater photosynthetic activity under ECO₂
497 (+55%) (Table 2) resulted in increased content of TSS (+44%) in flag leaves (Table 5)
498 and, in agreement with Aranjuelo and others (2015) working with cv. Sula of durum
499 wheat, enhanced amounts of monosaccharides (mainly glucose and xylose) in
500 comparison with that of –M plants cultivated at ACO₂. However, the increased level

501 of glucose together with an accumulation of fructan (Table 5) suggests that
502 utilization and export of carbohydrates from flag leaves was limited under ECO₂ in –
503 M plants (Long and others 2004). In contrast, enhanced photosynthetic rates in +M
504 plants cultivated under optimal irrigation (WW) under ECO₂ (+84%) (Table 2),
505 coincided with a small increase in the level of TSS in flag leaves (+16%) (Table 5).
506 This may be as a consequence of a sink effect of the mycorrhizal fungus (Baslam and
507 others 2012a) associated with wheat roots. This sink effect by AMF may have
508 limited the translocation of sugars to spikes (Table 1) in +M wheat in comparison to
509 –M plants.

510 The application of water deficit (D) drastically decreased the content of fructan
511 in flag leaves of both –M and +M wheat plants under ECO₂, and such a reduction
512 coincided with significant increases in the concentrations of glucose and xylose in
513 both types of plants and, to a lesser extent, sucrose in –M plants (Table 5). Similarly
514 to findings of Oliveira and others (2013) working with *Viguiera discolor*
515 (Asteraceae), the proportion of hexoses to sucrose plus fructan in stressed wheat
516 (D) under ECO₂ were more than double in –M plants and more than six times higher
517 in +M plants than in their respective well watered (WW) controls. Contrary to
518 findings of Goicoechea and others (2014) working with alfalfa, the significant
519 accumulation of TSS in flag leaves of +M wheat plants subjected to drought (D) (an
520 increase of 74% compared to stressed +M wheat at ACO₂) (Table 5) did not cause
521 photosynthetic acclimation (Table 3) and could result in an osmotic adjustment that
522 may have allowed +M plants to sustain their metabolic activity under drought
523 conditions (Chaves and others 2003). Osmotic adjustment could be reinforced by
524 the increased levels of proline in flag leaves after applying water deficit (D) in both

525 +M and -M plants in comparison with their respective WW controls under ECO₂
526 (Table 6). However, in contrast with results at ACO₂, proline accumulation (Table 6)
527 occurred when the production of shoot biomass (Table 1) was already negatively
528 affected by drought stress. Similar results were obtained by Hudak and others
529 (1999) in spring wheat subjected to ECO₂ and water deficit.

530 It has been reported that plant responses to ECO₂ lead to stomatal closure
531 (Long and others 2004) and this also occurred in the flag leaves of wheat cultivated
532 under ECO₂ independently of mycorrhizal inoculation and water regime (Table 2).
533 However, compared to values measured at ACO₂, decreases of g_w under ECO₂ were
534 higher in -M wheat plants (exceeding 50%) than in +M plants (reductions ranging
535 from 25% in WW plants to 30% after applying drought) (Table 2). Surprisingly, the
536 greatest decreases in concentration of N under ECO₂ (as reported by the recovery of
537 N¹⁵) (Table 4) were found in +M plants (decreases of around 54-56% depending on
538 irrigation regime), which contrasts with results obtained at ACO₂. The lack of
539 relationship between g_w and N¹⁵ recovery indicates that g_w was not the main factor
540 influencing the translocation of N from roots to shoots and its later accumulation in
541 flag leaves at either ACO₂ or ECO₂. Jauregui and others (2015) reported decreased
542 expression of genes encoding PIP (plasma membrane intrinsic proteins) and TIP
543 proteins involved in leaf water conductance in flag leaves of Sula wheat grown
544 under ECO₂ as compared to plants cultivated at ACO₂. Moreover, Jauregui and
545 others (2015) also show that expression of nitrate can be negatively affected by
546 ECO₂. Zhu and others (2016) observed that mycorrhizal inoculation increased N
547 partitioning into the root system of wheat plants and this effect was more evident
548 under ECO₂ than at ACO₂ and, in agreement with our results, Chen and others

549 (2007) reported negative or no effect of mycorrhizal colonization on plant N uptake
550 under ECO_2 (results depending on plant species). In the present study, the
551 decreased Fv/Fm ratio in +M wheat subjected to drought (D) under ECO_2 (Table 2)
552 suggests limited electron transport in chloroplasts and consequently less energy
553 available for nitrate assimilation in flag leaves. In contrast, WW +M wheat under
554 ECO_2 did not show a reduced Fv/Fm ratio.

555

556 **5. Conclusions**

557 Mycorrhizal symbiosis improved the tolerance of wheat against water deficit when
558 plants were cultivated at ACO_2 . In some cases the benefits provided by the AMF
559 (fructan accumulation in the flag leaf) were observed even without imposing
560 drought conditions and could prevent the deleterious effects of water deficit. In
561 other cases, the beneficial effect of AMF was observed under both well-watered
562 and drought conditions but became more evident when plants were undergoing
563 water restriction (higher photochemistry efficiency of PSII in the flag leaf and
564 increased uptake of N). Finally, only wheat associated with AMF displayed some
565 mechanisms (proline accumulation) that can allow water retention in tissues under
566 drought.

567 However, our data also showed that, although mycorrhizal association can
568 modulate the effects of ECO_2 on the physiology of durum wheat (i.e. avoiding or
569 delaying acclimation of photosynthesis and leaf conductance), ECO_2 impaired some
570 of the benefits provided by mycorrhizal association to host plants at ACO_2 (i.e.,
571 improved uptake and transport of N to the flag leaves). The mechanisms underlying
572 the interactions between mycorrhizal association and atmospheric CO_2

573 concentration remain unclear but our results suggest that they can be crucial for
574 the benefits that mycorrhizal symbiosis can provide to wheat plants undergoing
575 water deficit.

576

577 **Acknowledgments**

578 The authors wish to thank Amadeo Urdiáin, Eliseu G. Fabbrin, Marcelle M Bettoni
579 and Rosa Giménez for technical support and Paul William Miller for the language
580 revision. This study has been supported by the Ministerio de Economía y
581 Competitividad (MINECO) and Gobierno de Aragón (Spain) (AGL2011–30386-C02–
582 02, BFU 2011-26989, Group A-44).

583

584 **References**

585

586 Amthor JS (2001) Effects of atmospheric CO₂ concentration on wheat yield: review
587 of results from experiments using various approaches to control CO₂
588 concentration. *Field Crops Res* 73:1–34.

589 Aranjuelo I, Cabrera-Bosquet L, Morcuende R, Avise JC, Nogués S, Araus JL,
590 Martínez-Carrasco R, Pérez P (2011) Does ear C sink strength contribute to
591 overcoming photosynthetic acclimation of wheat plants exposed to elevated
592 CO₂? *J Exp Bot* 62:3957–3969.

593 Aranjuelo I, Sanz-Saez A, Jauregui I, Irigoyen JJ, Araus JL, Sánchez-Díaz M, Erice G
594 (2013) Harvest index, a parameter conditioning responsiveness of wheat plants
595 to elevated CO₂. *J Exp Bot* 64:1879–1892.

596 Arnon DI, Hoagland DR (1939) A comparison of water culture and soil as media for
 597 crop production. Science 89:512-514.

598 Aspinall D (1986) Metabolic effects of water and salinity stress in relation to
 599 expansion of leaf surface. Aust J Plant Physiol 13:59-73.

600 Augé RM (2001) Water relations, drought and vesicular-arbuscular mycorrhizal
 601 symbiosis. Mycorrhiza 11:3-42.

602 Augé RM, Toler HD, Saxton AM (2015) Arbuscular mycorrhizal symbiosis alters
 603 stomatal conductance of host plants more under drought than under amply
 604 watered conditions: a meta-analysis. Mycorrhiza 25:13-24.

605 Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. Ann
 606 Rev Plant Biol 59:89-113.

607 Baslam M, Garmendia I, Goicoechea N (2012a) Elevated CO₂ may impair the
 608 beneficial effect of arbuscular mycorrhizal fungi (AMF) on the mineral and
 609 phytochemical quality of lettuce. Ann Appl Biol 161:180-191.

610 Baslam M, Erice G, Goicoechea N (2012b) Impact of arbuscular mycorrhizal fungi
 611 (AMF) and atmospheric CO₂ concentration on the biomass production and
 612 partitioning in the forage legume alfalfa. Symbiosis 58:171-181.

613 Biswal AK, Kohli A (2013) Cereal flag leaf adaptations for grain yield under drought:
 614 knowledge status and gaps. Mol Breeding 31:749-766.

615 Bradford MM (1976) A rapid and sensitive method for the quantification of
 616 microgram quantities of protein utilizing the principle of protein-dye binding.
 617 Anal Biochem 72:248-254.

618 Bunce JA (2001) Direct and acclamatory responses of stomatal conductance to
 619 elevated carbon dioxide in four herbaceous crop species in the field. *Glob*
 620 *Change Biol* 7:323-331.

621 Calvo-Polanco M, Molina S, Zamarreño AM, García-Mina JM, Aroca R (2014) The
 622 symbiosis with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* drives
 623 root water transport in flooded tomato plants. *Plant Cell Physiol* 55:1017-1029.

624 Chaves MM, Maroco JP, Pereira JS (2003) Understanding plant responses to
 625 drought- from genes to the whole plant. *Funct Plant Biol* 30:239-264.

626 Chen X, Tu C, Burton MG, Watson DM, Burkey KO, Hu S (2007) Plant nitrogen
 627 acquisition and interactions under elevated carbon dioxide: impact of
 628 endophytes and mycorrhizae. *Glob Change Biol* 13:1238-1249.

629 De Luis I, Irigoyen JJ, Sánchez-Díaz M (1999) Elevated CO₂ enhances plant growth in
 630 droughted N₂-fixing alfalfa without improving water status. *Physiol Plantarum*
 631 107:84-89.

632 FAO (2016) Food and Agriculture Organization of the United Nations.
 633 www.fao.org/worldfoodsituation/csdb/en/ (accessed on 30th May 2016).

634 Fitzpatrick EA (1970) The expectancy of deficient winter rainfall and the potential
 635 for severe drought in the southwest of Western Australia. In: *Miscellaneous*
 636 *Publication Vol. 70/1. pp. 37. The University of Western Australia, Institute of*
 637 *Agriculture, Agronomy Dept, Perth, Australia.*

638 Genty B, Briantais JM, Baker NR (1989) The relationship between the quantum yield
 639 of photosynthetic electron transport and quenching of chlorophyll
 640 fluorescence. *Biochim Biophys Acta* 990:87-92.

641 Goicoechea N, Dolézal K, Antolín MC, Strnad M, Sánchez-Díaz M (1995) Influence of
 642 mycorrhizae and *Rhizobium* on cytokinin content in drought-stressed alfalfa. J
 643 Exp Bot 46:1543-1549.

644 Goicoechea N, Merino S, Sánchez-Díaz M (2004) Contribution of arbuscular
 645 mycorrhizal fungi (AMF) to the adaptations exhibited by the deciduous shrub
 646 *Anthyllis cytisoides* under water deficit. Physiol Plantarum 122:453-464.

647 Goicoechea N, Baslam M, Erice G, Irigoyen JJ (2014) Increased photosynthetic
 648 acclimation in alfalfa associated with arbuscular mycorrhizal fungi (AMF) and
 649 cultivated in greenhouse under elevated CO₂. J Plant Physiol 171:1774-1781.

650 Goicoechea N, Bettoni MM, Fuertes-Mendizábal T, González-Murua C, Aranjuelo I
 651 (2016) Durum wheat quality traits affected by mycorrhizal inoculation, water
 652 availability and atmospheric CO₂ concentration. Crop Pasture Sci 67:147-155.

653 Gupta AK, Kaur K, Kaur N (2011) Stem reserve mobilization and sink activity in
 654 wheat under drought conditions. Am J Plant Sci 2:70-77.

655 Hirel B, Gallais A (2006) Rubisco synthesis, turnover and degradation: some new
 656 thoughts on an old problem. New Phytol 169:445-448.

657 Hudak C, Bender J, Weigel H-J, Miller J (1999) Interactive effects of elevated CO₂, O₃,
 658 and soil water deficit on spring wheat (*Triticum aestivum* L. cv. Nandu).
 659 Agronomie 19:677-687.

660 Irigoyen JJ, Emerich DW, Sánchez-Díaz M (1992) Water stress induced changes in
 661 concentrations of proline and total soluble sugars in nodulated alfalfa
 662 (*Medicago sativa*) plants. Physiol Plantarum 84:55-60.

663 Jansa J, Bukovská P, Gryndler M (2013) Mycorrhizal hyphae as ecological niche for
 664 highly specialized hypersymbionts – or just soil free-riders? *Front Plant Sci* 4:
 665 article 134.

666 Jarvis CE, Walker JRL (1993) Simultaneous, rapid, spectrophotometric determination
 667 of total starch, amylose and amylopectin. *J Sci Food Agric* 63:53-57.

668 Jauregui I, Aroca R, Garnica M, Zamarreño AM, García-Mina JM, Serret MD, Parry M,
 669 Irigoyen JJ, Aranjuelo I (2015) Nitrogen assimilation and transpiration: key
 670 processes conditioning responsiveness of wheat to elevated [CO₂] and
 671 temperature. *Physiol Plantarum* 155:338-354.

672 Jiménez S, Ollat N, Deborde C, Maucourt M, Rellán-Álvarez R, Moreno MA,
 673 Gogorcena Y (2011) Metabolic response in roots of *Prunus* rootstocks
 674 submitted to iron chlorosis. *J Plant Physiol* 168:415-423.

675 Krüger M, Krüger C, Walker C, Stockinger H, Schüßle A (2012) Phylogenetic
 676 reference data for systematics and phylotaxonomy of arbuscular mycorrhizal
 677 fungi from phylum to species level. *New Phytol* 193:970–984.

678 Livingston DP III, Hinch DK, Heyer AG (2009) Fructan and its relationship to abiotic
 679 stress tolerance in plants. *Cell Mol Life Sci* 66:2007-2023.

680 Lon SP, Ainsworth EA, Rogers A, Ort DR (2004) Rising atmospheric carbon dioxide:
 681 plants FACE the future. *Ann Rev Plant Biol* 55:591-628.

682 Manderscheid R, Weigel HJ (1995) Do increasing atmospheric CO₂ concentrations
 683 contribute to yield increases of German crops? *J Agro Crop Sci* 175:73–82.

684 Morales F, Pascual I, Sánchez-Díaz M, Aguirreolea J, Irigoyen JJ, Goicoechea N,
 685 Antolín MC, Oyarzun M, Urdiain A (2014) Methodological advances: Using
 686 greenhouses to simulate climate change scenarios. *Plant Sci* 226:30-40

687 Müller J, Mohr U, Sprenger N, Bortlik K, Boller T, Wiemken A (1999) Pool sizes of
688 fructans in roots and leaves of mycorrhizal and non-mycorrhizal barley. New
689 Phytol 142: 551-559.

690 Oliveira VF, Silva EA, Zaidan LBP, Carvalho MAM (2013) Effects of elevated CO₂
691 concentration and water deficit on fructan metabolism in *Viguiera discolor*
692 Baker. Plant Biol 15:471-482.

693 Oury F, Godin C, Mailliard A, Chassin A, Gardet O, Giraud A, Heumez E, Morlais J,
694 Rolland B, Rousset M, Trottet M, Charmet G (2012) A study of genetic progress
695 due to selection reveals a negative effect of climate change on bread wheat
696 yield in France. Eur J Agron 40:28–38.

697 Parry MAJ, Andralojc PJ, Scales JC, Salvucci ME, Carmo-Silva AE, Alonso H, Whitney
698 SM (2013) Rubisco activity and regulation as targets for crop improvement. J
699 Exp Bot 64:717–730.

700 Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining
701 parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of
702 infection. T Brit Mycol Soc 55:158-161.

703 Rawson HM, Gifford RM, Condon BN (1995) Temperature gradient chambers for
704 research on global environment change. I. Portable chambers for research on
705 short-stature vegetation. Plant Cell Environ 18:1048-1054.

706 Reynolds M, Bonnett D, Chapman SC, Furbank RT, Manés Y, Mather DE, Parry MAJ
707 (2011) Raising yield potential of wheat. I. Overview of a consortium approach
708 and breeding strategies. J Exp Bot 62:439–452.

709 Schüßler A, Schwarzott D, Walker C (2001) A new phylum, the Glomeromycota:
710 phylogeny and evolution. Mycol Res 105:1413-1421.

711 Singh AK, Hamel C, DePauw RM, Knox RE (2012) Genetic variability in arbuscular
712 mycorrhizal fungi compatibility supports the selection of durum wheat
713 genotypes for enhancing soil ecological services and cropping systems in
714 Canada. Can J Microbiol 58:293-302.

715 Varga B, Vida G, Varga-László E, Hoffmann B, Veisz O (2016) Combined effect of
716 drought stress and elevated atmospheric CO₂ concentration on the yield
717 parameters and water use properties of winter wheat (*Triticum aestivum* L.)
718 genotypes. J Agro Crop Sci (in press). DOI: 10.1111/jac.12176

719 von Caemmerer S, Farquhar GD (1981) Some relationships between the
720 biochemistry of photosynthesis and the gas exchange of leaves. Planta 153:376-
721 387.

722 Vos J, Oyarzún PJ (1987) Photosynthesis and stomatal conductance of potato
723 leaves- effects of leaf age, irradiance, and leaf water potential. Photosynth Res
724 11:253-264.

725 Zadoks JC, Chang TT, Konzak CF (1974) A decimal code for the growth stages of
726 cereals. Weed Res 14:415-421.

727 Zhu XC, Song FB, Liu SQ, Liu TD, Zhou X (2012) Arbuscular mycorrhiza improves
728 photosynthesis and water status of *Zea mays* L. under drought stress. Plant Soil
729 Environ 58:186-191.

730 Zhu X, Song F, Liu S, Liu F (2016) Arbuscular mycorrhiza improve growth, nitrogen
731 uptake, and nitrogen use efficiency in wheat grown under elevated CO₂.
732 Mycorrhiza 26:133-140.

733

734

735 **Figure legends**

736

737 **Figure 1** Percentage of roots showing mycorrhizal structures (hyphae, arbuscules
738 and/or vesicles) in wheat plants non-inoculated (-M) or inoculated (+M) with
739 arbuscular mycorrhizal fungi (AMF), cultivated either under well-watered conditions
740 (WW, white histograms) or drought (D, black histograms), and grown either at ambient
741 (ACO₂) or under elevated (ECO₂) CO₂. Values are means (n = 8 plants per treatment) ±
742 SE. Data were subjected to arc-sin transformation before applying the χ^2 -test. Test
743 results were considered significant at $P \leq 0.05$. Similar letters indicate that values were
744 not significantly different. ND = not detected.

Figure 1

Table 1 Growth parameters of wheat plants non-inoculated (-M) or inoculated (+M) with arbuscular mycorrhizal fungi (AMF), cultivated under either well-watered (WW) or limited irrigation (D), and grown at either ambient (ACO₂) or elevated (ECO₂) CO₂. For each mycorrhizal and irrigation treatment, values in parenthesis are percentages of increases (+) or decreases (-) of values under ECO₂ compared to those at ACO₂.

Treatments			Shoot DM (g plant ⁻¹)	Root DM (g plant ⁻¹)	Spike DM (g plant ⁻¹)	No. of spikes per plant
ACO ₂	-M	WW	6.1 ± 0.2 c	4.1 ± 0.2	3.6 ± 0.2 ab	1.6 ± 0.1 a
		D	6.7 ± 0.2 bc	4.7 ± 0.0	3.3 ± 0.4 b	2.2 ± 0.0 abc
	+M	WW	6.2 ± 0.1 c	3.9 ± 0.2	3.5 ± 0.0 ab	1.9 ± 0.2 bc
		D	6.2 ± 0.5 c	3.7 ± 0.4	3.9 ± 0.1 ab	1.8 ± 0.2 c
ECO ₂	-M	WW	8.7 ± 0.3 a (+43%)	4.7 ± 0.3 (+15%)	4.3 ± 0.3 a (+20%)	2.8 ± 0.1 a (+75%)
		D	7.4 ± 0.2 b (+10%)	4.3 ± 0.1 (-9%)	3.6 ± 0.1 ab (+9%)	2.5 ± 0.3 ab (+14%)
	+M	WW	8.6 ± 0.2 a (+39%)	4.4 ± 0.2 (+13%)	3.6 ± 0.4 ab (+3%)	2.5 ± 0.0 ab (+32%)
		D	7.4 ± 0.4 b (+19%)	3.8 ± 0.2 (+3%)	4.0 ± 0.3 ab (+3%)	2.0 ± 0.1 bc (+11%)
ANOVA						
CO ₂			***	ns	ns	**
AMF			ns	*	ns	ns
Water (W)			*	ns	ns	ns
CO ₂ × AMF			ns	ns	ns	ns
CO ₂ × W			**	ns	ns	*
AMF × W			ns	ns	*	ns
CO ₂ × AMF × W			ns	ns	ns	ns

Values are means (n = 8) ± S.E. separated by Duncan's multiple range test ($P \leq 0.05$). Within each parameter means followed by the same letter are not significantly different. ANOVA: ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Table 2 CO₂ exchange rate (CER), total leaf conductance to water vapour (g_w), intercellular CO₂ concentration (C_i) and maximum quantum yield of PSII (Fv/Fm) of wheat plants non-inoculated (-M) and inoculated (+M) with arbuscular mycorrhizal fungi (AMF), cultivated under either well-watered (WW) or limited irrigation (D), and grown at either ambient (ACO₂) or elevated (ECO₂) CO₂. Photosynthetic rates were measured at 400 µmol mol⁻¹ CO₂ for plants grown at ACO₂ and at 700 µmol mol⁻¹ CO₂ for plants grown under ECO₂. For each mycorrhizal and irrigation treatment, values in parenthesis are percentages of increases (+) or decreases (-) of values under ECO₂ compared to those at ACO₂.

Treatments			CER ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	g_w ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)	C_i ($\mu\text{mol mol}^{-1} \text{CO}_2$)	Fv/Fm
ACO ₂	-M	WW	15.2 ± 0.5 c	0.20 ± 0.02 a	248 ± 15 abc	0.72 ± 0.02 a
		D	8.5 ± 0.9 de	0.11 ± 0.01 bc	248 ± 13 abc	0.57 ± 0.07 b
	+M	WW	14.7 ± 1.1 c	0.12 ± 0.01 b	142 ± 25 d	0.79 ± 0.02 a
		D	7.3 ± 0.5 e	0.10 ± 0.01 bc	230 ± 17 bc	0.75 ± 0.01 a
ECO ₂	-M	WW	23.5 ± 1.6 b (+55%)	0.09 ± 0.01 bc (-55%)	210 ± 6 bcd (-15%)	0.76 ± 0.03 a (+5%)
		D	10.7 ± 1.8 d (+26%)	0.05 ± 0.01 d (-55%)	278 ± 40 ab (+12%)	0.71 ± 0.02 a (+24%)
	+M	WW	27.0 ± 1.8 a (+84%)	0.09 ± 0.01 bc (-25%)	178 ± 19 cd (+25%)	0.77 ± 0.03 a (-2.5%)
		D	14.7 ± 1.1 c (+100%)	0.07 ± 0.01 cd (-30%)	311 ± 34 a (+35%)	0.55 ± 0.02 b (-27%)
ANOVA						
CO ₂			***	***	ns	ns
AMF			ns	ns	ns	ns
Water (W)			***	***	***	***
CO ₂ × AMF			**	**	ns	***
CO ₂ × W			**	ns	ns	ns
AMF × W			ns	*	*	ns
CO ₂ × AMF × W			ns	ns	ns	**

Values are means (n = 8) ± S.E. separated by Duncan's multiple range test ($P \leq 0.05$). Within treatments, means for each parameter followed by the same letter are not significantly different. ANOVA: ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Table 3 Acclimatory responses of stomatal conductance and photosynthesis rate to elevated CO₂ of wheat plants non-inoculated (-M) and inoculated (+M) with arbuscular mycorrhizal fungi (AMF), cultivated under either well-watered (WW) or limited irrigation (D), and grown at either ambient (ACO₂) or elevated (ECO₂) CO₂. The acclimatory responses of stomatal conductance and photosynthesis rate are expressed as the values for plants grown under ECO₂ relative to those of plants cultivated at ACO₂ when both measured at 700 μmol mol⁻¹.

	-M		+M	
	WW	D	WW	D
Acclimation of conductance	0.60 ± 0.04*	0.54 ± 0.11*	1.43 ± 0.15	1.00 ± 0.12
Acclimation of photosynthesis	0.95 ± 0.07	0.67 ± 0.07*	1.10 ± 0.03	1.05 ± 0.08

Values are means (n = 8) ± S.E. analyzed by single-group *t*-tests. *indicates a ratio significantly different from 1.0 at *P* ≤ 0.05

Table 4 Nitrogen isotopic composition ($\delta^{15}\text{N}$) corresponding to K^{15}NO_3 -non-labeled ($\delta^{15}\text{N}_{\text{non-lab}}$) or K^{15}NO_3 -labeled ($\delta^{15}\text{N}_{\text{lab}}$) wheat plants, carbon isotopic composition ($\delta^{13}\text{C}$) and C and N concentration in flag leaves corresponding to wheat plants non-inoculated (-M) and inoculated (+M) with arbuscular mycorrhizal fungi (AMF), cultivated under either well- watered (WW) or limited irrigation (D), and grown at either ambient (ACO_2) or elevated (ECO_2) CO_2 . For each mycorrhizal and irrigation treatment, values in parenthesis are percentages of increases (+) or decreases (-) of values under ECO_2 compared to those at ACO_2 .

Treatments			$\delta^{15}\text{N}$	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$	N	C
			(‰)	(‰)	(‰)	(%)	(%)
			($\delta^{15}\text{N}_{\text{non-lab}}$)	($\delta^{15}\text{N}_{\text{lab}}$)			
ACO ₂	-M	WW	12.2 ± 0.3 cd	145.0 ± 5.2 b	-30.7 ± 0.3 b	2.53 ± 0.04 a	46.2 ± 0.3 abc
		D	12.8 ± 0.3 bc	95.4 ± 7.5 c	-29.4 ± 0.3 a	1.71 ± 0.08 b	47.2 ± 0.6 ab
	+M	WW	11.9 ± 0.2 cd	209.5 ± 12.0 a	-28.9 ± 0.1 a	1.71 ± 0.03 b	44.9 ± 0.6 bc
		D	13.9 ± 0.1 b	130.6 ± 6.3 b	-29.0 ± 0.3 a	2.79 ± 0.09 a	47.4 ± 1.2 a
ECO ₂	-M	WW	15.5 ± 0.3 a (+27%)	79.1 ± 6.3 cde (-45%)	-42.9 ± 0.6 cd (-40%)	1.79 ± 0.05 b (-29%)	47.6 ± 0.8 a (+3%)
		D	11.6 ± 0.7 cd (-9%)	71.4 ± 4.4 de (-25%)	-43.7 ± 0.3 de (-49%)	1.41 ± 0.07 c (-18%)	45.4 ± 0.6 abc (-4%)
	+M	WW	11.3 ± 0.3 d (-5%)	91.5 ± 5.3 cd (-56%)	-44.3 ± 0.5 e (-53%)	1.39 ± 0.02 c (-19%)	44.4 ± 0.6 c (-1%)
		D	11.7 ± 0.5 cd (-16%)	60.4 ± 5.9 e (-54%)	-42.6 ± 0.3 c (-47%)	1.38 ± 0.20 c (-51%)	45.7 ± 0.7 abc -4%)
ANOVA							
CO ₂		ns	***	***	***	ns	
AMF		**	***	ns	ns	ns	
Water (W)		ns	***	*	ns	ns	
CO ₂ × AMF		***	***	*	*	ns	
CO ₂ × W		***	***	ns	*	*	
AMF × W		***	*	ns	***	*	
CO ₂ × AMF × W		*	ns	**	***	ns	

Values are means ($n = 8$) ± S.E. separated by Duncan's multiple range test ($P \leq 0.05$). Within each column means followed by the same letter are not significantly different. ANOVA: ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Table 5 Sugar profile in flag leaves of wheat plants non-inoculated (-M) and inoculated (+M) with arbuscular mycorrhizal fungi (AMF), cultivated under either well- watered (WW) or limited irrigation (D), and grown at either ambient (ACO₂) or elevated (ECO₂) CO₂. Total sugars: for each mycorrhizal and irrigation treatment, values in parenthesis are percentages of increases (+) or decreases (-) of values under ECO₂ compared to those at ACO₂.

Treatments			Glucose (G)	Xilose	Fructose (F)	Sorbitol	Sucrose(S)	Fructans	Fructans DP>3	Fructans DP=3	S+G+F	Fructans+S+F+G	Total sugars	
									(mg g ⁻¹ DM)					
ACO ₂	-M	WW	6.6 ± 0.5 c	5.0 ± 1.2 d	3.8 ± 0.7 a	0.5 ± 0.1 bc	50.4 ± 8.6 a	36.5 ± 6.9 bc	12.3 ± 2.8 bc	11.6 ± 0.8 bcd	67.4 ±13.4	103.9 ± 1.0 cd	109.7 ± 2.3 bc	
		D	8.8 ± 0.6 bc	8.5 ± 0.3 cd	3.4 ± 0.9 a	0.5 ± 0.1 abc	43.5 ± 6.4 ab	42.0 ± 8.4 b	26.3 ± 5.6 b	15.7 ± 2.8 abc	58.5 ± 9.1	103.2 ± 12.7 cd	112.2 ± 12.5 bc	
	+M	WW	8.5 ± 1.9 bc	8.2 ± 2.0 cd	4.0 ± 0.4 a	0.5 ± 0.1 abc	33.7 ± 5.0 bc	105.9 ± 8.3 a	85.5 ± 7.1 a	19.0 ± 2.8 a	47.3 ± 5.0	150.6 ± 23.2 ab	160.0 ± 12.4 ab	
		D	6.2 ± 0.8 c	4.8 ± 0.9 d	2.2 ± 0.2 a	0.4 ± 0.1 cd	25.6 ± 4.5 c	14.4 ± 0.8 c	8.1 ± 0.7 c	6.3 ± 0.7 d	42.1 ± 8.6	56.1 ± 7.7 e	62.7 ± 8.0 c	
ECO ₂	-M	WW	13.2 ± 0.6 b	10.5 ± 0.9 c	3.7 ± 0.6 a	0.6 ± 0.1 a	35.6 ± 0.5 abc	83.6 ± 7.2 a	71.7 ± 7.2 a	13.2 ± 2.2 abc	58.2 ± 9.2	137.9 ± 7.2 abc	157.5 ± 23.1 ab (+44%)	
		D	23.4 ± 1.8 a	16.6 ± 1.0 b	4.2 ± 0.8 a	0.3 ± 0.1 cd	48.8 ± 5.5 ab	28.1 ± 4.5 bc	18.6 ± 3.5 bc	9.8 ± 1.7 cd	87.0 ± 8.4	115.2 ± 13.0 bcd	134.7 ± 13.8 b (+20%)	
	+M	WW	11.0 ± 0.9 bc	10.6 ± 1.1 c	3.0 ± 0.3 a	0.6 ± 0.1 ab	46.9 ± 3.6 ab	103.9 ± 9.8 a	87.8 ± 8.6 a	17.2 ± 2.9 ab	66.1 ± 7.2	175.3 ± 23.7 a	185.1 ± 24.5 a (+16%)	
		D	25.3 ± 3.5 a	20.8 ± 1.6 a	4.0 ± 0.2 a	0.3 ± 0.0 d	26.5 ± 2.2 c	18.1 ± 0.6 c	10.8 ± 1.4 bc	6.5 ± 0.8 d	66.4 ± 3.2	80.1 ± 4.2 de	108.9 ± 10.4 bc (+74%)	
ANOVA														
CO ₂			***	***	ns	ns	ns	ns	**	ns	*	*	**	
AMF			ns	ns	ns	ns	**	*	***	ns	*	ns	ns	
Water (W)			***	***	ns	***	ns	***	***	**	ns	***	***	
CO ₂ × AMF			ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	
CO ₂ × W			***	***	*	**	ns	*	***	ns	ns	ns	ns	
AMF × W			ns	ns	ns	ns	*	***	***	***	ns	**	**	
CO ₂ × AMF × W			ns	**	ns	ns	*	**	***	ns	ns	ns	ns	

Values are means (n = 8) ± S.E. separated by Duncan's multiple range test ($P \leq 0.05$). Within each column means followed by the same letter are not significantly different. ANOVA: ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. DM: dry matter; DP: degree of fructans polymerization.

Table 6 Starch, total soluble proteins and proline concentration in flag leaves of wheat plants non-inoculated (-M) and inoculated (+M) with arbuscular mycorrhizal fungi (AMF), cultivated under either well- watered (WW) or limited irrigation (D), and grown at either ambient (ACO₂) or elevated (ECO₂) CO₂. For each mycorrhizal and irrigation treatment, values in parenthesis are percentages of increases (+) or decreases (-) of values under ECO₂ compared to those at ACO₂.

Treatments			Starch (mg g ⁻¹ DM)	Proteins (mg g ⁻¹ DM)	Proline (nmol g ⁻¹ DM)
ACO ₂	-M	WW	4.8 ± 0.1 a	13.1 ± 0.3 bc	25.0 ± 3.1 ab
		D	2.6 ± 0.3 bc	17.6 ± 1.4 a	19.5 ± 3.7 b
	+M	WW	3.4 ± 0.2 b	15.0 ± 0.9 ab	19.7 ± 3.2 b
		D	3.3 ± 0.0 b	12.4 ± 1.9 bcd	30.7 ± 3.1 a
ECO ₂	-M	WW	2.0 ± 0.2 c (-58%)	10.6 ± 0.2 cd (-19%)	5.4 ± 0.8 c (-78%)
		D	1.9 ± 0.0 c (-27%)	9.8 ± 1.4 cd (-44%)	30.1 ± 2.5 a (+54%)
	+M	WW	2.9 ± 0.3 bc (-15%)	8.3 ± 0.5 d (-47%)	3.8 ± 0.2 c (-81%)
		D	2.9 ± 0.4 bc (-12%)	12.3 ± 0.3 bcd (-1%)	27.6 ± 3.1 ab (-10%)
ANOVA					
CO ₂			***	***	**
AMF			ns	ns	ns
Water (W)			*	ns	***
CO ₂ × AMF			**	ns	ns
CO ₂ × W			*	ns	***
AMF × W			*	ns	ns
CO ₂ × AMF × W			ns	**	*

Values are means (n = 8) ± S.E. separated by Duncan's multiple range test ($P \leq 0.05$). Within each column means followed by the same letter are not significantly different. ANOVA: ns, not significant; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$. DM: dry matter.